ADIPOSE TISSUE REMODELLING BY POLYPHENOLS IN OBESE RATS

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DOCTORAL THESIS

Directed by Prof. Maria Cinta Bladé

and

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Department of Biochemistry and Biotechnology

UNIVERSITAT ROVIRA i VIRGILI

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FEM CONSTAR que aquest treball, titulat "**Adipose tissue remodelling by polyphenols in obese rats**", que presenta Aïda Pascual Serrano per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que compleix els requeriments per poder optar a Menció Internacional.

HACEMOS CONSTAR que el presente trabajo, titulado "**Adipose tissue remodelling by polyphenols in obese rats**", que presenta Aïda Pascual Serrano para la obtención del título de Doctor, ha sido realizado bajo nuestra dirección en el Departamento de Bioquímica y Biotecnología de esta universidad y que cumple los requisitos para poder optar a la Mención Internacional.

WE STATE that the present study, entitled "**Adipose tissue remodelling by polyphenols in obese rats**", presented by Aïda Pascual Serrano for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of this university and that it fulfills the requirements to be eligible for the International Mention.

Tarragona, 29 de juny de 2017 / Tarragona, 29 de junio de 20017 / Tarragona, 29th June 2017

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Bé, ja som aquí, hem arribat al cim tot i que semblava llunyà i impossible. De fet, sense l'ajuda de tots els que m'heu acompanyat en aquests quasi bé quatre anys no hagués estat possible. I és per aquest motiu que us mereixeu un espai dins aquest llibret que tanta suor ha costat.

Primerament m'agradaria donar gràcies al Grup de Recerca en Nutrigenòmica, i en particular al Lluis Arola per la gran oportunitat d'iniciar els estudis de doctorat i seguir endavant amb la recerca.
En especial voldria agrair a les meves supervisores, directores de tesi i companyes de patiments, il·lusions i desil·lusions, Cinta i Anna. He après moltíssim amb vosaltres: nous coneixements, esforç, perseverança, escriptura i fins i tot, crec que he madurat una miqueta gràcies a la vostra tenacitat. Heu estat una part molt i molt important d'aquesta tesi i un ànim constant a seguir endavant. Us estaré eternament agraïda. Sou un exemple a seguir. Gràcies.

A la parte indispensable de cualquier tesis en el grupo de Nutrigenòmica: nuestras queridas técnicos. Niurka, Rosa i Yaiza. Sin vuestra tarea puedo asegurar que ninguna tesis de este grupo llegaría muy lejos. Siempre disponibles y nunca tenéis un no por respuesta y además con una sonrisa en la cara. Gracias, de verdad. Ayuda saber que siempre se puede contar con vosotras. Estoy muy contenta de haber convivido estos casi cinco años trabajando en el grupo durante los cuales hemos compartido crisis, lloros, risas y locuras varias. A parte de buenas profesionales sois bellísimas personas. ¡Espero que sigáis teniendo la misma paciencia y energía durante muchos años! ¡Gracias! Y a nuestro técnico de prácticas Santiago, por ayudarnos con la docencia y resolvernos todo. Haces que la tarea de dar prácticas sea mucho más sencilla. Gracias.

A tots els professors dels grups de Nutrigenòmica, MoBioFood i Quimioinformàtica, perquè en un moment o altre sempre heu tingut un suggeriment, heu resolt algun dubte i heu estat companys en aquest procés.

I would like to say thank you to the Animal Sciences group and all the people from the Human and Animal Physiology department (the HAPpy people for me). I want to thank the opportunity you gave me for my PhD stay in the Netherlands and for the lovely treatment I had during my time in Wageningen. Everybody was nice and gentle to me. I learned a lot and got big help from you all. I specially want to thank you, Jaap and Evert for your kindness and the trust you put in me since the first moment I arrived. And also thanks to all the people in Wageningen, inside and outside the uni. It was so nice to meet you all. I arrived back home with some new friends. Hope to see you all again soon!

Als meus companys, amics i estimats! No es pot passar pel grup de Nutrigenòmica i no sortir amb bons records i un somriure a la cara. Hem conviscut durant quatre anys, passant més temps junts que amb la família i, per això, sou la part indispensable d'aquesta

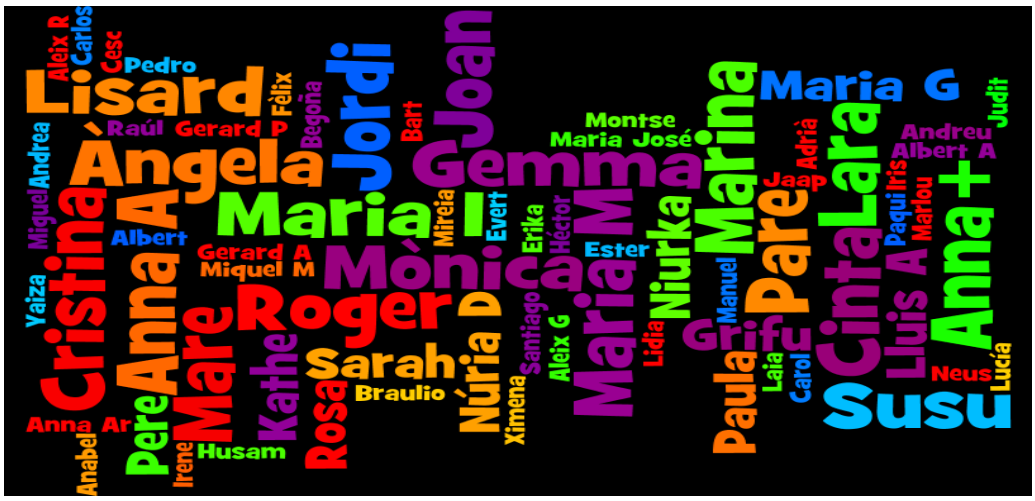
tesi. No em vull posar a donar mencions especials, ja que acabaríem tots plorant i no cal, oi? Tots i cadascun de vosaltres sabeu el que signifiqueu per mi, crec que us ho he deixat ben clar quan ha fet falta. La tesi no té el mateix sentit sense els nostres moments de ploreres, rialles, sopars, festes, bogeria al laboratori, experiments d'animals interminables, dies dolents, dies millors, calor, fred, desastres als resultats, alegries quan les PCRs sortien bé, celebracions, comiats d'alguns i arribades d'altres i algun que altre "bailoteo" desestressant pel despatx, laboratori o passadís. No es pot explicar amb paraules, s'ha de viure! Sou tots molt especials i tant els que ja hi éreu quan vaig arribar (dels quals vaig aprendre moltíssim) com els que us quedeu quan marxo heu deixat una miqueta de vosaltres en mi i en la meva tesi. Gràcies a tots!

Als amics externs, que sempre heu estat recolzant, animant i tenint una estona per a fer-me desconnectar de l'estrès diari. Sou molt macos i molt importants i necessaris per a mi!

Als meus pares i la meva estimada germana, família incondicional que m'ha recolzat. Sense vosaltres no hagués arribat mai tan lluny, ni pensar-hi. Sou els meus tres pilars, m'heu acompanyat de la mà tota la vida, fins que vau veure que ja podia caminar sola i em vau fer la darrera empenta per no tirar mai enrere. Gràcies per la confiança posada en mi, l'ajuda per a que mai em faltés de res, la dedicació i assistència SEMPRE que ho he necessitat. No hi ha paraules, sé que sóc aquí gràcies al vostre esforç continu.

I com no, al meu estimat Jordi, que m'has suportat durant tot aquest procés, m'has animat en totes les circumstàncies, m'has acompanyat quan ha calgut, m'has recolzat quan estava enfonsada, m'has cuidat i t'has encarregat de tot per a que jo pogués centrar-me en la tesi. Has esperat i esperat a que aquest dia arribés i has confiat en tot moment en mi. No cal que et digui res, ho saps. Una casa no s'aguanta amb tres pilars i tu n'ets el quart.

Gràcies a tots!



ALS MEUS PARES, LA
GEMMA i EL JORDI

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"It is not the mountain we conquer but ourselves"

Edmund Hillary

Sir Edmund Percival "Ed" Hillary KG ONZ KBE (1919–2008) was a New Zealand mountaineer, explorer, and philanthropist. On 29 May 1953, Hillary and Nepalese Sherpa mountaineer Tenzing Norgay became the first climbers confirmed to have reached the summit of Mount Everest. Following his ascent of Everest, Hillary devoted most of his life to helping the Sherpa people of Nepal through the Himalayan Trust, which he founded. Through his efforts, many schools and hospitals were built in Nepal.

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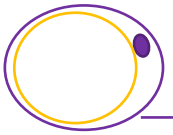
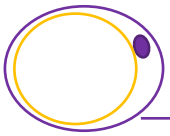


INDEX

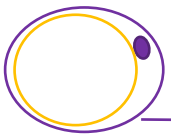


I. ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
ADD-1	Adipocyte determination and differentiation factor 1
AKT	serine/threonine kinase
APC	Adenomatous polyposis coli
APOA1	Apolipoprotein A-1
BAT	Brown adipose tissue
BMI	Body Mass Index
BMP	Bone morphogenetic protein
C/EBP	CCAAT/enhancer-binding protein
CAF	Cafeteria Diet
cAMP	Cyclic adenosine monophosphate
COL6A2	Type VI collagen alpha chain 2
COMT	Catechol-O-methyltransferase
CPT1	Carnitine palmitoyltransferase 1
CVD	Cardiovascular disease
DACT1	Dapper 1
DKK-1	Dickkopf homolog-1
DNA	Deoxyribonucleic acid
EC	Epicatechin
ECG	Epicatechin Gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ERK 1/2	Extracellular signal-regulated kinases 1/2
ER	Estrogen receptor
FA	Fatty acids
FABP4	Fatty acid-binding protein 4
FAS	Fatty acid synthase
FFA	Free fatty acids
FXR	Farnesoid X receptor
GA	Gallic Acid
GH	Growth hormone
GLUT4	Facilitated glucose transporter member 4
GSPE	Grape Seed Proanthocyanidin Extract
HDAC	Histone deacetylase
HDL-C	High density lipoprotein cholesterol
HFD	High-fat diet
HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase

GA	Gallic Acid
IGF	Insulin-like growth factor
IL-6	Interleukin 6
IRF	Interferon-Regulatory Factor
IRS	Insulin Responsive Substrate
Kg	Kilograms
KLF	Kruppel-like factor
LDL	Low density lipoprotein
m	Meters
MAPK	mitogen-activated protein kinase
MHO	Metabolically healthy obese
miRNA	microRNA
mRNA	Messenger RNA
MS	Metabolic syndrome
MSC	Mesenchymal stem cell
NAFLD	Non-alcoholic fatty liver disease
NELL-1	NEL-like molecule-1
NF-κB	Nuclear factor kappa B
PA	Proanthocyanidin
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PKA	Protein kinase A
PLIN1	Perilipin 1
PPAR	Peroxisome proliferator-activated receptor alpha
PRDM16	PR domain containing 16
PREF-1	Preadipocyte factor 1
pre-miRNA	precursor microRNA
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Resveratrol
RXRα	Retinoid X receptor alpha
sFRP	Frizzled-related protein
SHP	Small heterodimer partner
SIRT	Sirtuin
SREBP	Sterol response element binding protein 1
STAT	Signal transducer and activator of transcription
SULT	Cytosolic sulfotransferase

SUMO	Small ubiquitin-like modifier
sWAT	Subcutaneous adipose tissue
T3	Triiodothyronine
T4	Thyroxine
TAG	Triglycerides
TNFα	Tumour necrosis factor alpha
TR	Thyroid hormone receptor
TREM2	Triggering receptor expressed on myeloid cells 2
UCP1	Uncoupling protein 1
UGTs	Glucuronosyltransferase
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
vWAT	Visceral white adipose tissue
WAT	White adipose tissue
WHO	World Health Organisation
WISP2	Wnt-inducible protein 2
XBP1	X-box binding protein 1



II. INTRODUCTION

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1. Obesity

1.1. Obesity definition

Obesity is defined as a body mass index (BMI) of 30 or higher. BMI is the most extensively and simple method used for measuring and determining obesity, it is calculated by dividing the person's weight in kilograms (kg) by the square of the height in meters (m). Other indexes for measuring and predicting obesity have been also suggested because they can give more accurate information about the body fat distribution state, such as waist circumference, waist-hip ratio and waist-height ratio [1].

Obesity develops from a positive energy balance due to excessive food intake and physical inactivity exerting an enlargement of fat depots. Importantly, nowadays one of each ten individuals in the world suffers from obesity and it has been described as one of the major chronic diseases according to the World Health Organisation (WHO)[2]. This health problem is a concern because obesity's prevalence is annually increasing in most developed countries and what makes the situation even more alarming is the fact that an increasingly young population is becoming obese [3].

1.2. Obesity-related diseases

Obesity is one of the main central disorders in metabolic syndrome (MS) together with insulin resistance, dyslipidaemia and hypertension among others [4,5]. MS is a grouping of interrelated metabolic risk factors that appear to directly promote the development of cardiovascular disease (CVD) and type II diabetes mellitus [6]. There is no consensus in defining the presence or absence of MS, but some criteria have been proposed to identify MS. In this sense, it has been postulated by scientific and the medical community, based the Adult Treatment

Panel III in 2001 criteria with some minor modifications, that individuals can be diagnosed of MS when they meet 3 of the 5 criteria indicated in Table 1, which include elevated waist circumference, elevated triglycerides (TAGs), reduced high density lipoprotein cholesterol (HDL-C), elevated blood pressure and elevated fasted glucose [7].

Table 1. Criteria for Clinical Diagnosis of Metabolic Syndrome. Grundy SM *et al*, 2004 [7].

Measure (any 3 of 5 constitute diagnosis of metabolic syndrome)	**Categorical Cutpoints**
Elevated waist circumference	≥102 cm (≥40 inches) in men
	≥88 cm (≥35 inches) in women
Elevated triglycerides	≥150 mg/dL (1.7 mmol/L)
	or
	On drug treatment for elevated triglycerides
Reduced HDL-C	<40 mg/dL (1.03 mmol/L) in men
	<50 mg/dL (1.3 mmol/L) in women
	or
	On drug treatment for reduced HDL-C
Elevated blood pressure	≥130 mm Hg systolic blood pressure
	or
	≥85 mm Hg diastolic blood pressure
	or
	On antihypertensive drug treatment in a patient with a history of hypertension
Elevated fasting glucose	≥100 mg/dL
	or
	On drug treatment for elevated glucose

Energy homeostasis maintain body fat stores in balance. An imbalance in this stability towards an excess of energy accumulation leads to obesity [8]. Energy homeostasis involves different processes, signals and tissues that together gather information around the body about the status of body energy stores. In this sense, there are specialized neurons in different brain areas that sense several factors and control appetite and energy expenditure to protect body against extreme changes in fat stores. However, obesity is not only generated from a passive increased body weight but also involves the activation of multiple processes to fight against all changes during obesity and fat accumulation [3,8]. This response includes many cell types, which trigger a multiple adverse cellular response to energy excess. For instance, when catabolism increases to oxidize the surplus energy accumulation, reactive oxygen species (ROS) are generated in higher amounts increasing the damage of some cellular structures and leading to an inflammatory response at systemic level [9]. The chronic inflammation characterized by irregular cytokine production, increased ROS and activated inflammatory signalling pathways can be the link between obesity, insulin resistance and type II diabetes [10].

Although the inflammatory response that comes with obesity is generated at systemic level, adipose tissue seems to be its host and the predominant promoter [9,10]. As adipose tissue expands, due to the increased need of fat storage, the immune cell population increases, especially macrophages, which tend to infiltrate within white adipose tissues (WAT) [11]. Therefore, macrophages inside WAT release cytokines, which alter the adipocytokine secretion by adipocytes. In fact, different studies have demonstrated the existence of this communication between the adipose tissue and the immune system that, together with morphological alterations of adipocytes, enhances adipose tissue inflammation which ends up with an impaired adipose tissue function, typical in obesity [12,13].

Obesity highly increases the risk of developing CVD [14]. Despite all components of the MS are risk factors for CVD, obesity for itself has been considered as a risk factor for CVD. In this sense, it has been described that a high BMI is sufficient to significantly increase CVD symptoms [15]. Moreover, all grades of obesity are associated to increased risk factors for CVD and higher grades are directly related to an increased mortality risk [15].

However, the location and type of fat accumulated in the body play an important role determining CVD and MS risks [16]. In this sense, for the same BMI, an imbalance towards an increased quantity of visceral adipose tissue is associated with insulin resistance, increased cholesterol, TAG and low-density-lipoprotein levels (LDL), all connected with atherogenic profile [16]. On the other hand, subcutaneous obesity is related with lower metabolic disease risk profile [16]. Therefore, BMI is not the best index to evaluate CVD risk associated to obesity.

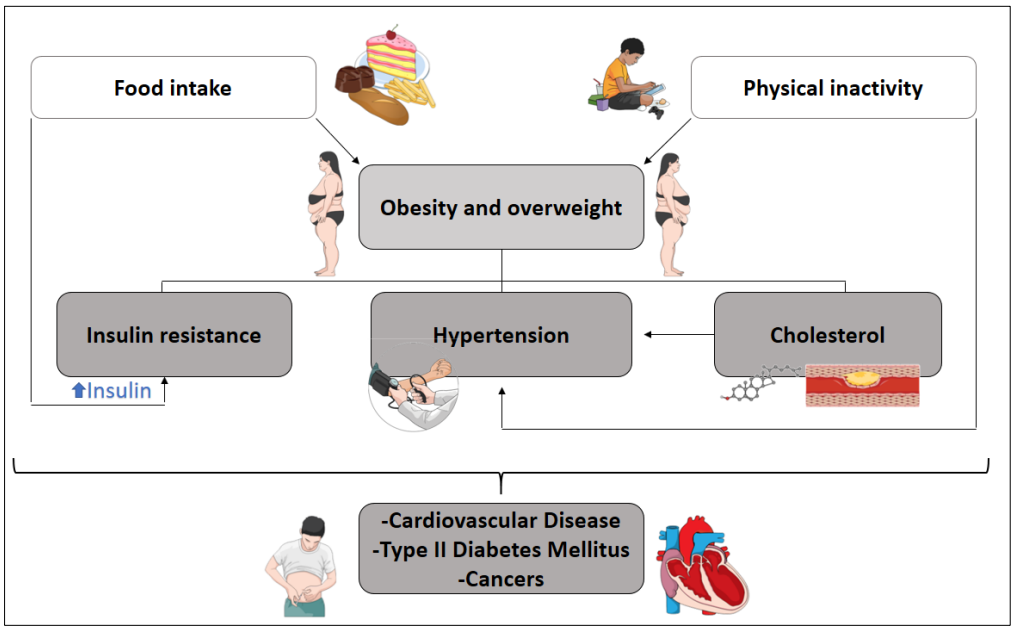


Figure 1. Interplay between the risk factors for metabolic syndrome. *Adapted from S. O'Neill and L. O'Driscoll, 2015* [17]

Ultimately, obesity seems to be the trigger for the development of other risk factors present in metabolic syndrome as shown in Figure 1 [18]. Taking this information into account, obesity and adipose tissue should be main targets to deal with metabolic syndrome.

1.3. Metabolically healthy obese (MHO)

Although obesity is one of the most spread diseases and is associated to increased CVD and diabetes risks, some obese people are identified as metabolically healthy. In fact, numerous scientific works support this new concept, the metabolically healthy obese (MHO) individuals [19–22].

Different works have investigated the factors that characterise this MHO phenotype [20,21,23,24]. These individuals have benign obesity, which has been characterized by a higher insulin sensitivity, lower ectopic fat accumulation in the liver and muscle, increased circulating adiponectin levels and lower estimated CVD risk [25]. Some studies have reported that MHO individuals do not present defects in adipocyte morphology, showing lower adipocyte size than obese individuals that develop metabolic syndrome [19]. This fact is important because small adipocytes are more sensible to insulin than large adipocytes [26,27]. Despite these improvements, it has been postulated that MHO individuals are not protected from typical obesity consequences but they are slower than the typical obese individuals in developing adverse metabolic complications [28].

2. Adipose tissue

Adipose tissue is a multilocated organ. In mammals, it is distributed in different fat depots which contribute to many basic needs in survival such as fuel for metabolism, thermogenesis, lactation and immune responses among others [29]. In humans, adipose tissue is one of the most extensive organs, representing around a 10-30% of the body mass in healthy subjects, being a little higher in women. In contrast, adipose tissue can reach up to the 70% of the body weight in obese individuals [30].

The first role given to adipose tissue, and the one that has been attributed to it for many years, is the fat storage function. Firstly, adipose tissue was only considered as an energy repository organ that provided thermal insulation to the body. However, it was not until 1994 that the importance of the adipose tissue in the whole body functionality was evidenced with the discover of the first adipokine, leptin [31]. From then, adipose tissue and specially leptin became more important in obesity studies and afterwards, other molecules and hormones started to arise [32,33]. Moreover, this organ was evidenced to be able to respond to different stimuli from the organism and to send many signals, which made scientist link it to an important role as an endocrine organ [34]. Nowadays, it is considered one of the most important organs for its implication in obesity, inflammation and its role as one of the mediators of energy metabolism. It has been demonstrated that altered adipocyte function and thus, also adipose tissue function, can lead to alterations in systemic energy balance or deregulate several physiologic processes [31,35].

2.1. Adipose tissue types

Adipose tissue is classified in brown adipose tissue (BAT), WAT and a new described form of adipose tissue, the brite or beige adipose tissue. Specifically, BAT is predominant in humans neonates and in all rodents but it can also be found in adult human when some specific signals activate it [18,36].

All types of fat contain adipocyte as the common central unit, a cell which its basic function is TAG storage. Nevertheless, adipocytes have shown to regulate multiple processes by secreting numerous signalling molecules [37]. However, the composition of the adipose organ can differ depending on the anatomical location and it can also be changed under different physiological or pathological conditions such as cold exposure or obesity state [29]. In this sense, the major form of adipose tissue, which is the WAT, includes pre-adipocytes, adipocytes, fibroblasts and also other cell types as endothelial cells and immune system cells [18].

2.1.1. White adipose tissue

It has been defined that white adipocytes arise from mesenchymal stem cells (MSCs). MSCs can differentiate into many different types of cells including brown and white adipocytes. These stem cells are found in stromal vascular fraction of WAT and in the bone narrow and, under appropriate stimuli, they become restricted to adipocyte lineage. Recruitment to this lineage gives rise to preadipocytes [38]. The transition from preadipocyte to adipocyte, adipogenesis, involves different stages which end in terminal differentiation in mature adipocytes. These stages are controlled by signalling cascades involving the important roles of Peroxisome proliferator-activated receptor γ (PPARγ) together with the CCAAT/enhancer-binding protein (C/EBP) family members among others, which will be widely described further, in the adipogenesis regulation

section. PPARγ has been shown to be necessary for adipocyte differentiation and also for the maintenance of the final differentiated state of these cells. The early expression of C/EBPβ and C/EBPδ is one of the first steps of adipocyte differentiation and it is really involved in the induction of C/EBPα and PPARγ expression, which are central transcriptional regulators of adipogenesis [39–41].

White adipocytes are a unique type of cell which is characterized by the typical presence of large lipid droplets composed by TAG molecules. These particular cells have the ability to enlarge when more energy storage is necessary. In fact, white adipocytes can increase from 30µm to 230µm in they diameter [42]. Thus, usually WAT adipocytes consist in unilocular lipid droplets and they take up to the 90% of the cytoplasm space, forcing the nucleus of the cell to move to the periphery of adipocyte. In WAT adipocytes energy is stored in form of TAG inside lipid droplets which are surrounded by phospholipids and some proteins; one of the most important and characteristic proteins around the lipid droplets is perilipin1 (Plin1). Plin1 is a key regulator of TAG mobilization and, when body increases its energy demand, Plin1 is phosphorylated by protein kinase A (PKA) and there is an activation of lipolysis [43]. Moreover, mature adipocytes are also characterized by having terminal differentiation markers such as fatty acid synthase (FAS) and leptin, among other specific proteins. Otherwise, preadipocytes are difficult to discriminate from other undifferentiated cell types with the same precursor morphology. Specifically, there is only one well-studied marker for preadipocytes, preadipocyte factor 1 (Pref-1), which will be profoundly described in future sections. It is expressed in preadipocytes and its expression is reduced while differentiation progresses. Otherwise, its overexpression leads to a decreased fat mass, which puts Pref-1 in a role of adipogenesis inhibition [44–46]. There are other presumed preadipocyte markers such as type VI collagen alpha 2 chain (COL6A2) which is also more expressed in

preadipocytes than in mature adipocytes. However, this marker is not adipose tissue specific either [47].

WAT major cells are adipocytes, but on its whole organ different types of cells are found such as the already mentioned preadipocytes, immune cells, as macrophages and leukocytes and blood and endothelial cells. All this other non-adipose cell types form is what is called stromal cell fraction of the adipose tissue [48,49].

Given the endocrine functions of adipose tissue and its high secretion of adipokines, it is logical to believe that adipose tissue has also a high level of vascularization and innervation [50,51].

In humans and rodents the WAT distribution is quite similar [37]. Specifically, WAT depots can be found in between visceral tissues called visceral WAT (vWAT) and also inside subcutaneous compartment (sWAT). Even though both types of adipose tissue are called WAT but, differ in functions and numerous aspects. For example, in vWAT the production of pro-inflammatory molecules related to insulin resistance, type II diabetes mellitus and CVD in obesity state is really well-defined [52–54]. Also, in vWAT the lipolysis is highly active in response to catecholamines and the repression of this process by insulin has a lower effect compared to sWAT [55,56]. These differences between fat depots and the higher risk for metabolic diseases associated to vWAT could be explained by the adipose tissue anatomical location; vWAT drains into the portal vein and thus, the liver is the first organ to sense the secreted vWAT signals. This secreted vWAT signals are part of the factors that contribute to the attributed adverse consequences of visceral obesity [57–59]. In this sense, some studies have shown a correlation of circulating molecules related to lipid metabolism, insulin sensitivity and inflammation with visceral fat mass [60–62]. Conversely, sWAT has plasticity on its phenotype and can be modified to a BAT or vWAT characteristics depending on the conditions [63,64]. Although lipolysis is not very active in sWAT adipocytes, their

insulin sensitivity is quite good and it has been demonstrated that abdominal sWAT is as sensible to insulin, when talking about glucose metabolism, as it is vWAT [65,66].

As commented before, one of the main functions of WAT is to mobilize energy substrates when negative energy balance is present and to store energy excess when this balance is positive. Besides, WAT has other diverse important functions from structural to endocrine ones. WAT is able to protect organs and body parts from physical injuries (such as eyes, gut, heels), from extreme changes in temperature, with its insulation properties, but the function that is being importantly attributed to WAT is that it works as a relevant endocrine organ [51,67].

2.1.2. Brown adipose tissue

Unlike WAT, BAT is characterized for burning energy to produce heat. Brown adipocytes are smaller than white adipocytes being their diameters from 20µm to 60µm [42]. Brown adipocytes are filled with multiple lipid droplets and are characterised by having a rounded nucleus and a large number of mitochondria with high respiration rates. The Having these multiple small lipid droplets makes them easier to be used for BAT to burn fatty acid and produce heat instead in the called non-shivering thermogenesis [29,68–70].

As indicated before, both white and brown adipocytes arise from MSCs, but some signals and markers differ to lead to different types of adipocytes. In this sense, the myf5 expressing progenitor cells lead to the characteristic brown preformed adipocytes, found in interscapular and perirenal regions [71]. However, not all brown adipocytes come from this myf5+ precursor cells. Recently, a huge number of studies have reported the presence of brown adipocytes located in unexpected sites, such as within WAT and skeletal muscle, and those cells are derived from myf5- precursors. These different brown adipocytes are receiving

names such as beige and brite for “brown-in-white” adipocytes because of their inducible brownish phenotype and will be further explained in next section [72–74].

Brown adipocytes morphology is different from white adipocytes and they also present specific markers. Specifically, their specialized function comes from their high mitochondrial content and the presence of uncoupling protein 1 (UCP1). UCP1 is a transmembrane protein which is able to uncouple protons from the respiratory chain and dissipating heat [71]. This process is called non-shivering thermogenesis and it is the more distinctive function of BAT. Due to this active process, BAT is highly innervated with sympathetic nervous system and well vascularised for covering the oxygen and lipid demand in the process [75–77].

There are different factors participating in the non-shivering thermogenesis process. The activation starts at sympathetic level, when norepinephrine is released and activates β_3 adrenergic receptors in BAT. This activation induces the production of cyclic Adenosine Monophosphate (cAMP) which activates PKA, that in turn increases the lipolysis rate through phosphorylation of Plin1. When free fatty acids (FFA) are released after lipolysis, they get inside mitochondria via carnitine palmitoyltransferase 1 (CPT1), located in the external mitochondrial membrane, and UCP1 is activated causing the early mentioned uncoupling of protons.

Cold exposure has shown to be an important stimulus in increasing fatty acid (FA) uptake in BAT leading to heat production through the dissipation of protomotive energy from respiratory chain [78,79]. Besides, BAT has also been studied for increasing the expression of genes involved in glucose metabolism, lipogenesis and uptake and catabolism of FA, activating UCP1 and regulating fuel utilization as part of cold adaptation [78].

In rodents, BAT is majorly present in interscapular depot. In humans, however, it was believed that active BAT was widely distributed during first decade of life, but then it disappeared gradually. Nowadays, many studies have

demonstrated that adults still have some brown adipocytes depots located around cervical regions of the body [36,80]. A part from typical brown adipocytes, there have been found within WAT, adipocytes with brown characteristics. These new findings have found a definition for this novel class of adipocytes called brite or beige because they are not exactly like typical brown fat cells [81,82].

2.1.3. Brite/beige adipose tissue

Beige or brite "brown-in-white" fat cells are a novel defined class of adipocytes which can exhibit brown adipocyte characteristics in response to specific stimuli, like beta-adrenergic signalling or cold exposure, but are usually found within WAT. These adipocytes are capable of expressing UCP1 and thus, showing thermogenic capabilities comparable to those exerted by typical brown adipocytes [83].

Brite cells, like BAT adipocytes, are characterised by presenting multilocular lipid droplets, high number of mitochondria, and by the expression of specific markers such as the mentioned UCP1, Cidea and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α). The presence of these brite cells inside fat depots provides a brownish coloration to the adipose tissue due to the increased mitochondria content (Figure 2).

Most of the authors refuse the primary idea that brite and brown adipocytes are the same due to their different developmental origin [79,84]. Moreover, the origin of brite cells has been a much-debated issue in the last decade. The most approved theory is the transdifferentiation one. This theory is based on adipose tissue plasticity and it postulates that with cold exposure or other pharmacological stimuli, the signals that come with the need of increasing body temperature lead to conversion from white to brown-like adipocytes (Figure 2) [69,85–87].

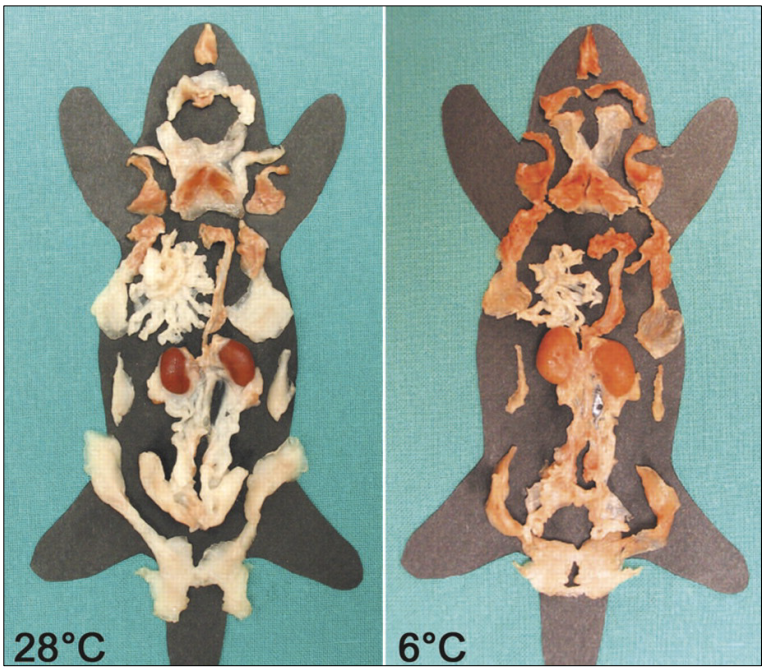


Figure 2. Representative image of changes in adipose tissue of female mice after cold exposure for 10 days. Cold exposure is one of the signals that induce the conversion from white to brown-like adipocytes. *Adapted from Cinti S, 2009* [88].

However, this plasticity also works the other way around, when body is exposed to a continuous surplus of energy storage needs, BAT adipocytes are able to transform into WAT-like cells to increase their storage capacity [86,89]. Both processes (cold exposure and pharmacological stimuli) are meant to lead to brite adipocytes, which makes authors hypothesise that these new cells as an intermediate type cell between both brown and white cells. For the mentioned development of brite cells, some studies have shown that the expression of PR domain containing 16 (PRDM16), which is a transcription factor, is necessary for the commitment of embryonic progenitors towards brown adipocyte lineage and also for the browning of white adipocytes within WAT depots. PRMD16 acts increasing the activity of the transcription factors of C/EBP family, concretely the C/EBPβ factor, PPARγ and PGC1α [90]. The transcriptional pathways towards brite

cells is really close to the one needed for the brown adipocytes development, although these two types of cells seem to arise from different origins [73].

Both brown and beige adipocytes are metabolically important due to their roles in energy expenditure through thermogenesis and are considered important targets to correct body energy imbalances. It has been demonstrated that the activity of these cells is inversely correlated with diet-induced obesity in animal models and thus, its relevance as targets in obesity treatment is increasing [79].

2.2. Adipogenesis and its regulation

Adipogenesis is the process of adipocyte generation, from adipocyte precursors to mature adipocytes. It is a complex biological machinery in which lots of different signals and transcription factors are involved to orchestrate all modifications that adipocytes go through for the formation or modification of adipose tissue depots [19]. Therefore, adipogenesis is regulated by a huge number of factors, including hormones, cytokines, transcription factors, other proteins and microRNAs (miRNAs). Next, this introduction focus on the most relevant factors regulating adipogenesis [37,91–96].

2.2.1. Transcription factors and other proteins that govern adipogenesis

Notably PPARγ, several members of the C/EBP, STAT, KLF, IRF and SREBP families have been described for having key roles in regulating adipogenesis. Moreover, GATA and β-catenin, acting through wnt-pathways, also participate on adipogenesis regulation.

PPARγ

Inside nuclear receptors superfamily there is PPARγ, a ligand-dependent transcriptional factor that has been really relevant in the study of adipogenesis transcriptional regulation [97]. PPARγ dimerize with retinoid X receptor alpha (RXRα) forming an heterodimer and then modulates transcription of nearby genes [98]. The importance of PPARγ in adipogenesis regulation is over the rest of transcription factors. In fact, a lot of different transcription factors involved in adipogenesis regulation do their functions through the induction or reduction of PPARγ expression [99–102]. PPARγ is not only important in adipogenesis regulation but also in other processes such as glucose homeostasis and insulin sensitivity [103,104]. In adipose tissue, PPARγ acts over the so-called non-precursor cells which include fibroblasts and myocyte precursors [100,105].

PPARγ exists in two different isoforms PPARγ1 and PPARγ2. These two isoforms differ in their amino-terminal region in which PPARγ1 has an extension of 30 amino acids [106]. It has been described that the PPARγ2 isoform is more important for a proper adipogenesis. However, some knock-out studies have shown that both isoforms are basic and critical for the adipocyte development [101,107,108]. PPARγ1 is less expressed in multiple tissues while PPARγ2 is predominantly and highly expressed in adipose tissue.

PPARγ directly binds numerous ligands. For example, PPARγ binds to long chain FA, arachidonic acid derivatives or oxidized FA and LDLs [109–111]. Moreover, it is assumed that a high number of different PPARγ ligands are yet described. Thus, this transcription factor differs from others nuclear receptors that bind a single specific ligand.

The expression and activity of PPARγ, and thus adipogenesis, can be modulated through various processes. In this sense, the posttranslational regulation of PPARγ has been studied extensively. PPARγ can be phosphorylated

by mitogen-activated protein kinases (MAPKs) which results in transcriptional inactivation of PPARγ [112]. Moreover, other kinases can phosphorylate PPARγ, attenuating PPARγ action over important adipocyte gene expression, like adiponectin; Choi *et al.* reported that PPARγ phosphorylation by Cdk5 was able to change adiponectin expression [113]. Also, PPARγ is target of small ubiquitin-like modifier (SUMO) and SUMOylation of PPARγ is a relevant regulator of its function [114]. Like SUMOylation, acetylation is also a reversible covalent modification of PPARγ. Thus, PPARγ can be acetylated and deacetylated by different types of molecules, being the most important histone deacetylases (HDACs) and nicotinamide adenine dinucleotide-dependent deacetylases (Sirtuins). However, despite the importance of acetylation in modulating PPARγ transcriptional activity [115,116], more work on this field is needed to confirm if these posttransductional modification of PPARγ are relevant in the regulation of adipocyte development. PPARγ activity is also regulated by the ubiquitin-proteasome pathway, the ligand binding domains of PPARγ is targeted by ubiquitin and directed to the proteasome for degradation [97,117].

Summing up, PPARγ activity can be regulated by many mechanism, pointing out the huge importance of modulating PPARγ expression and activity for the adipogenesis regulation [91].

C/EBPs

A further substantial family of transcription factors involved in adipocyte differentiation and proliferation is C/EBPs. This family is expressed in numerous mammalian species. Six members of the family have been described; C/EBP α, β, γ, δ, ε, and ζ which can form homodimers and heterodimers. These dimers bind to the promoter region of genes with a complementary CCAAT sequence in order to regulate their expression. The most important C/EBPs involved in adipogenesis

regulation are α, β and δ. They work together in different stages of differentiation and the deletion of any of these results in the impairment of adipocyte development [118–120]. C/EBPβ and C/EBPδ are expressed at the beginning of adipocyte differentiation and are reported to modulate C/EBPα production. The importance of C/EBPβ in regulation is not only for its location upstream of C/EBPα and PPARγ [121], but also because it has been shown to play roles in the activation of unfolded protein response (UPR), a pathway related to endoplasmic reticulum which, in turn, is relevant in adipogenesis [122]. Moreover, during adipocyte formation, C/EBPβ can suppress Wnt/β-Catenin signalling, a negative regulator of adipogenesis [123,124].

The C/EBP family can be regulated in transcription steps but they can also be controlled through translational and post-translational mechanisms. C/EBPα can be translated into two different isoforms (whole and truncated) [125] while C/EBPβ does it in three different isoforms (full length, one shorter and a truncation). It has been shown that these different translations of the isoforms are regulated by some translation initiation factors [126].

Numerous messenger RNA (mRNA)-binding proteins have also been reported to regulate C/EBPα and β translation through interactions with special motifs and/or secondary structures of their mRNAs [118,127]. Although the modulation of C/EBPδ translation has not been widely studied, some recent works have reported that it can also be repressed by a phosphorylation cascade [127]. In relation to post-translational control, C/EBPs hold a highly-conserved sequence which is target for SUMOylation, as PPARγ, decreasing their transcriptional activity [125,128]. In the concrete process of adipogenesis, the post-translational modifications have only been well reported for C/EBPβ which, besides SUMOylation, can be phosphorylated and acetylated [129,130].

Glucocorticoids promote adipocyte differentiation by upregulating several genes, including C/EBPβ and δ and have also been described to promote

adipogenesis by affecting C/EBPα and PPARγ promoters. Zinc finger containing transcription factors have also been shown to modulate adipogenesis by affecting C/EBPβ function [19].

In summary, C/EBP family have an important role in adipocyte differentiation and all the information that is already known about their regulation could help in making them good targets for adipogenesis modulation.

STATs

The signal transducer and activator of transcription (STAT) family of transcription factors is another family significant for adipogenesis and for the regulation of diverse pathways.

STAT proteins are majorly present in the cytosol and are recruited by specific cell receptors where they are phosphorylated by the Janus kinases (JAKs). This phosphorylation activates the STAT proteins and after that they undergo dimerization and are translocated to the nucleus, where they can regulate transcription by binding to their target gene sequences. As their name infers, STATs regulate transcription by transducing cell surface signals to the nucleus in a rapid manner [19]. They are able to regulate metabolisms transducing signals from hormones, to which STATs are really responsive [131].

In the context of adipose tissue and adipocyte development, only STAT5 have been extensively demonstrated, by both *in vitro* and *in vivo* studies, to have a role in adipogenesis being positive regulators of the process [132–136]. Nevertheless, the specific target genes that mediate the proadipogenic functions are not clear yet. Some studies suggest that STAT5 can induce PPARγ expression [137] but it seems not to be strictly required for it during adipocyte development, at least not in a direct manner [138,139].

The role of STAT3 in adipogenesis has also been studied, both *in vitro* and *in vivo*. The results suggest a possible role of STAT3 in adipogenesis. However, because some STAT3 activators enhance adipogenesis while other have no effects it is not clear whether STAT3 is strictly necessary for adipogenesis (reviewed by White *et al.* 2011 [140]).

KLFs

The Kruppel-like factor (KLF) protein family consists of a large number of transcription factors that bind specific rich GC DNA regions and act as both positive and negative regulators [141].

This family have been related to impairment or promotion of adipogenesis with different expression patterns [142,143]. Inside this big family, the most important proteins that have been related to adipogenesis modulation include KLF2, KLF3, KLF4, KLF5, KLF6 and KLF15. KLF2 inhibits adipogenesis *in vitro* and repress PPARγ promoter activity [144,145]. KLF3 has anti-adipogenic effects through modulation of C/EBPα [143]. KLF4 is considered an initiator of the adipogenic program through its transactivation of C/EBPβ and also is considered an adipogenic effector working together with Krox20 for an optimal adipocyte differentiation[146]. KLF5 acts as a positive regulator of adipogenesis taking actions together with C/EBPβ and C/EBPδ in early adipocyte development stages [147]. KLF6 works impairing Dlk1 expression which is a known inhibitor of adipogenesis [148]. KLF15 may operate together with C/EBPα in later stages of adipocyte development by activating PPARγ transcription [142].

IRFs

The Interferon-Regulatory factors (IRFs) family of transcription factors were discovered in relation with immune system but they have also been shown

to be involved in adipocyte development. The family consists in nine members and although all of them have been related to adipocyte development, the most important are IRF3 and IRF4. IRFs repress adipogenesis and some of their actions have been compared to those of Nuclear factor kappa B (NF-kB), a transcription factor that also activates immune system genes and exerts inhibition over some adipocyte-specific genes [149,150].

SREBPs

Sterol Response Element Binding Protein-1 (SREBP-1) was identified as a homologous of the rat Adipocyte Determination and Differentiation factor-1 (ADD-1) which was reported to have a protein structural motif, helix-loop-helix, that gives the transcription factors the ability to bind the E-box DNA domain [151]. The name of SREBP-1 arose when the transcription factor was reported to have the ability to bind sterol response elements in the promoter region of LDL-receptor gene [152]. SREBP family includes different isoforms SREBP-1a, SREBP1-c and SREBP-2. The SREBP1 isoforms are hypothesised to be originated from the same gene, being generated by an alternative splicing [153].

SREBP1-c is the predominant isoform in WAT and its function is related to regulation of lipogenesis [154]. However, *in vitro* studies have shown that SREBP-1a is the most expressed isoform in adipose cells [154]. Although different *in vitro* experimentation showed an important role for SREBP-1 in adipocyte development, numerous *in vivo* studies failed to demonstrate the direct involvement of SREBP-1 in adipogenesis. For instance, SREBP-1-null mice have normal WAT growth and whole-body knocked out of SREBP-1 mice present a normal gene expression pattern in adipocyte. However, both animal models showed a compensatory increase in SREBP-2 expression, thus SREBP-1 should not be discarded yet as regulator of adipocyte development [155,156].

SREBP-1 and 2 are known to participate in cholesterol biosynthesis regulation [157] and SREBP-1 gene contains intronic miRNA miR-33b (in humans), while SREBP-2 gene includes miR-33a, which have been both reported to be important lipid metabolism regulators [158].

GATAs

The GATA family of transcription factors consists of six described members that have structural zinc finger DNA binding domains in common and their name is due to their ability to bind the DNA sequence GATA and then regulate cellular differentiation and proliferation. From all the factors in the family, only GATAs 2 and 3 have been shown to be expressed in preadipocytes of WAT. These two transcription factors are repressed during adipocyte formation, suggesting that they have an antiadipogenic function on adipocyte precursors. Knockdown and overexpression studies have shown that GATA2 reduction enhanced adipogenesis while its ectopic expression reduced fat cell development by repressing PPARγ activity by binding to its promoter [159]. GATAs 2 and 3 can also attenuate adipogenesis by association with C/EBPs alpha and beta, disrupting their transcriptional activity.

GATA proteins can be regulated by posttranscriptional modifications and serine/threonine kinase (Akt) phosphorylation of GATA proteins results in their nuclear translocation inhibition and thus, also an inhibition of their functions [160–162].

Altogether, research works have shown that GATA proteins can intervene in adipocyte development through different pathways.

β-Catenin and Wnt signalling pathways

Another important transcription factor in adipogenesis regulation is β-catenin. It has been profoundly studied for being the major mediator of the Wnt signalling pathway. This pathway is highly conserved in evolution and function as a negative adipogenesis regulator. β-catenin is translocated into the nucleus, where it coactivates other transcription factors. This translocation is only possible when Wnt protein activates the signalling pathway and β-catenin can be released from other ligands to the cytosol. This contributes to adipogenesis inhibition as it can be schematically observable in Figure 3 [163].

β-catenin is regulated by numerous elements that affect its stability, structure and localization [164]. For example, β-catenin is modulated by PPARγ which makes it susceptible to be ubiquitinated and thus degraded [165].

β-catenin is basic in adipocyte development. For instance, conditional deletion of β-catenin induces a switch towards adipogenesis in the mesenchyme of mouse in development [166] and ectopic exposure of preadipocytes to an active form of β-catenin inhibits adipogenesis by preventing induction of PPARγ and C/EBPα [167,168]. Moreover, a deficiency in β-catenin phosphorylation reduces the adipogenic activity through the activation of Wnt signalling pathway downstream of PPARγ, blocking a select subset of adipogenic genes [165].

As commented before, β-catenin modulates adipogenesis through Wnt signalling pathways. There are three Wnt signalling pathways: the canonical signalling pathway, which involves the transcription factor β-catenin, the non-canonical planar cell polarity pathway and the non-canonical Wnt/calcium pathway [169].

Wnt act in all three signalling pathways as ligands by binding to frizzled receptors and LDL receptor-related proteins (LRP) [157]. Wnt is a family (19 described members) of extracellular effectors secreted by different cell types

that play a key role during early development and adipogenesis [39,170,171]. The β-catenin dependent pathway (canonical pathway) is the rout directly related to adipogenesis and many proteins relevant for adipocyte development are regulated through this pathway [169]. It is assumed that Wnt activators inhibit adipocyte development through β-catenin repression function while Wnt antagonists lead to degradation of β-catenin. This results in an increase in adipocyte development [172–174].

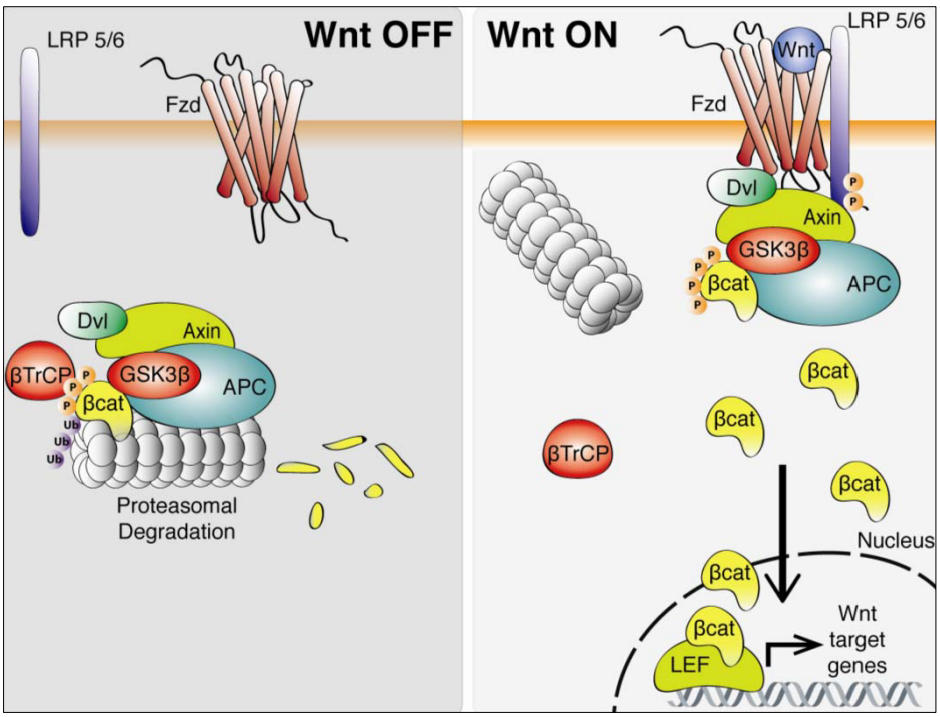


Figure 3. Canonical Wnt/β-catenin signalling pathway. (Left panel) In the absence of a Wnt protein, GSK-3β phosphorylates β-catenin which targets it for ubiquitination and degradation in the proteasome. (Right panel) Activation of the signalling pathway by the binding of a Wnt ligand to frizzled receptor. In this condition, the ubiquitination is blocked and β-catenin is stabilized in the cytoplasm and enters the nucleus to regulate the transcription of Wnt target genes. *Adapted from Nibaldo C. et al, 2013* [175].

In contrast to the canonical pathways, non-canonical pathways are less investigated. However, some studies show that non-canonical pathways could act against the canonical ones through Wnt5a family member [41].

Also, some positive regulators of Wnt signalling pathway are Wnt proteins, such as Wnt10b and Wnt6, some other effectors, like NELL-1, Gremlin 2 and WISP2, and some miRNAs, such as miR-135a-5p [168,176,177].

Wnt10b is the most well-described agonist of the Wnt signalling pathway in adipocyte development. Some studies showed that Wnt10b works through stabilization of β-catenin when it is still in the cytosol, supressing adipocyte differentiation. In this sense, anti-sera addition of Wnt10b to 3T3-L1 adipocyte media increases adipogenesis [168,172] and free expression of Wnt10b strongly reduces body fat in a mice model of diet-induced obesity [178]. All these studies reaffirm the important role of Wnt10b in activating Wnt canonical signalling pathway and, eventually, in the repression of WAT expansion by inhibiting preadipocyte differentiation.

Wnt6 also activates the Wnt/β-catenin signaling pathway. Wnt6 is down-regulated during adipogenesis, together with Wnt10b, and the ectopic expression of these two Wnt members suppress adipogenesis [171].

The differentiation factor NEL-like molecule-1 (NELL-1) and the bone morphogenetic protein (BMP) antagonist Gremlin 2 are also agonist of the Wnt signaling pathway, thus repressing adipogenesis [176]. Moreover, Wnt-inducible protein 2 (WISP2) activates the canonical Wnt signaling pathway in adipogenic precursor cells by forming complex with the ZFP423 factor, thus preventing ZFP423 translocation into the nucleus and the activation of PPARγ [179]. In contrast, WISP2 unbinds ZFP423 in presence of BMP4, a protein secreted by differentiated adipose cells, leading ZFP423 translocation to the nucleus and its interaction to PPARγ promoter, activating adipocyte differentiation [179].

Many negative regulators of the Wnt canonical pathway have been characterized, the best well-described include Dickkopf homolog-1 (Dkk-1) and some of the Frizzled-related proteins (sFRP) [173,174]. Among all of the sFRP proteins identified, sFRP1 and sFRP4 promoted adipogenesis by targeting Wnt signaling pathway. In this sense, sFRP1 is upregulated in mice fed with a high-fat diet and the treatment with recombinant sFRP4 results in lipid droplet accumulation and expression of adipocyte characteristic genes in non-adipocyte precursor cells [174,180].

Nucleorodoxin, a protein involved in redox homeostasis during cell differentiation, also act as negative regulator of Wnt-pathway, upregulating the expression of adipogenic genes through the modulation of β-catenin activity [181]. In addition, the triggering receptor expressed on myeloid cells 2 (TREM2), which is related to the anti-inflammatory response, is an activator of adipogenesis *in vitro* and *in vivo.* Park M. *et al.* showed that TREM2 could activate adipogenesis through reducing Wnt10b expression [182]. Moreover, the X-box binding protein 1 (XBP1) [183] and Dapper 1 (DACT1) [184] also suppress Wnt10b expression and thereby inhibit the canonical Wnt pathway, inducing adipocyte differentiation.

Furthermore, numerous works have focused on miRNAs as regulators of adipogenesis, which will be discussed further in the miRNA section. Specifically, several studies have shown that some miRNAs target Wnt signaling factors. For instance, miR-135a-p inhibits adipogenesis by targeting adenomatous polyposis coli (APC). APC contributes to the translocation of β-catenin from cytoplasm to nucleus, and then activates the expressions of other genes inducing the canonical Wnt/β-catenin signaling pathway [177]. Also, miR-204-5p promotes the differentiation into mature adipocytes through directly targeting Wnt/β-catenin pathway agonists [185].

Overall, there are many agonists and antagonists of the canonical Wnt signaling pathway and Wnt/β-catenin pathway plays a critical role in adipocyte development and cell fate [41].

Pref-1

Pref-1 is a transmembrane protein that belongs to a family of EGT-repeat-containing proteins [186]. It is highly expressed in preadipocytes but its levels are reduced during adipocyte development process [44,187]. Pref-1 is activated by proteolytic cleavage to regulate cell-fate specification [188]. In this sense, constitutive expression of Pref-1 reduces PPARγ and C/EBPα expressions and, consequently, inhibits adipocyte differentiation [189]. In contrast, mice deficient or knockout for Pref-1 showed growth retardation, accelerated adiposity and accelerated body weight gain due to the increase of adipose tissue mass [190]. Histological analyses of WAT showed bigger adipocytes in Pref-1 null-mice compared to the wildtype and Pref1-knockout mice overexpress genes related to adipogenesis and have altered plasma metabolite, exhibiting a pattern typically associated with obesity [191]. Moreover, transgenic mice with an induced ectopic expression of Pref-1 in adipose tissue, showed hypertriglyceridemia and lipoatrophy [192]. These data demonstrated that ablation of Pref-1 expression enhances adipogenesis and that Pref-1 can be considered a powerful negative regulator of the adipogenic process.

2.2.2. Sirtuin 1

Sirtuin 1 (SIRT1) is a NAD^+-dependent lysine deacetylase involved in numerous cellular processes, such as apoptosis, stress responses, glucose homeostasis, insulin secretion, cell proliferation, adipocyte differentiation and adipogenesis [193,194]. Focusing on adipogenesis, SIRT1 activation inhibits adipocyte

development while SIRT1 inhibition increases adipocyte number and promotes adipocyte marker expression [195]. The paper of SIRT1 inhibiting adipogenesis is also demonstrated in experiments with SIRT1 null mice where the adipocyte size are smaller and the expression levels of leptin is decreased by 60% of wildtype [196]. SIRT1 can also interact with Wnt signalling pathway because Sirt1 promotes constitutive Wnt signalling in several cancer cells [197] and stabilizes and stimulates nuclear accumulation of β-catenin in osteoblastic ST2 cells [198].

2.2.3. Hormones

Many hormones regulate the adipogenic process. Between them stand out steroids, thyroid hormones, growth hormone and insulin.

Steroid Hormones

Steroids are hydrophobic hormones that regulate numerous biological processes, including adipose tissue development and distribution. Consequently, adipose cells express a high number of steroid hormone receptors. The action of steroid hormones goes through dimerization and binding to specific receptors to induce their response [91]. The most important steroid hormones modulating the adipogenesis process are estrogens, androgens and glucocorticoids.

Estrogens mediate their effects through receptors estrogen receptors (ERs), ERα and ERβ. *In vitro* studies are controversial about the positive or negative influence of estrogens in adipogenesis [199–203] but *in vivo* studies are more consistent, showing that estrogens have an inhibitory effect on adipocyte development [204,205].

Androgens can be found in adipocytes and androgen receptors are expressed in both rats and human preadipocytes and in mature adipocytes [206–

[208]. Androgen effect in humans can be different in men and women. In this sense, low circulating androgen levels in men are associated with increased abdominal adiposity and when these levels are restored at its physiological levels, abdominal fat is reduced [209]. In women, the association between androgens and abdominal obesity is far more complex. Women with hyperandrogenism show higher abdominal obesity [210,211]. However, discrepancies appear when testing testosterone due to sensitivity problems in women [212]. Moreover, in both *in vivo* and *in vitro* [213,214] models there is still a lot of controversy about the role of androgens controlling adipogenesis because different and contradictory results have been found , as it was reviewed by Blouin *et al.* [215]. Thus, more research is needed to clarify exact effects of androgens over adipocyte development. However, it can be stated that androgens exert some kind of regulation over adipogenesis and the majority of animal experiments suggest that these steroid hormones have inhibitory effects [216,217].

Glucocorticoids are another class of steroid hormones that regulate adipocyte differentiation. It is well stablished that glucocorticoids promote adipogenesis. In fact, Dexamethasone is a synthetic glucocorticoid used as a standard component to induce differentiation in preadipocyte cell line 3T3-L1 [218]. Studies focused in their mechanisms of action showed that Dexamethasone induces C/EBPδ which, in turn, activates the transcription factor PPARγ, which has been widely described above for being the master regulator of adipogenesis [219,220]. Glucocorticoids have been also shown to inhibit Pref-1 and Wnt10b, which would result in increased adipogenesis because these two elements are known for being negative regulators of adipogenesis as commented in previous sections [221].

Thyroid hormones

Thyroid hormones triiodothyronine (T3) and thyroxine (T4) are regulated by a well-defined negative-feedback loop. T3 is the biological active form. Thyroid hormones act by regulating genes related to differentiation in various tissues such as brain, muscle, heart, liver and adipose tissue among other by controlling lipid and carbohydrate metabolism, the basal metabolic rate as well as the transcription of numerous proteins [222]. The action of T3 takes part through nuclear receptors named thyroid hormone receptor alpha and beta (TRα and TRβ) which bind to thyroid response elements present in the promoters of target genes. T3 actions are modulated by corepressors and coactivators like deiodinases 1, 2 and 3 that control the amount of T3 that should be present in each tissue [223]. In adipogenesis, T3 regulates many enzymes involved in the process either directly, by targeting its own nuclear receptors, or by interacting with others nuclear receptors, like PPARs and coactivators [224]. Deiodinases play crucial role in adipogenesis control; Deiodinase 2 helps in producing or limiting T3 levels and plays crucial role in adipogenesis, thermogenesis and lipid metabolism while Deiodinase 3 increases during proliferation. Numerous studies suggest that thyroid hormones promote adipogenesis in the majority of models [225–227]. There is evidence that SUMOylation of thyroid receptors is important to control adipocyte development [228] and it is clear that thyroid hormones can regulate adipogenesis and modulate expression of genes required for lipogenesis [229].

Growth hormone

Growth hormone (GH), a 22-kDa polypeptide, is secreted by the anterior pituitary gland and exerts important biological and physiologic effects [230,231]. Both preadipocytes and adipocytes are responsive to GH. As mentioned before, STAT5

protein is able to induce adipogenesis and GH is a potent activator of STAT5. Thus, GH is an adipogenic promoter. The action of GH-STAT5 pathway upregulates C/EBPβ, C/EBPδ and PPARγ expression [137]. Although it has been established that GH promotes adipogenesis in clonal cell lines, some studies have shown that GH can inhibit differentiation in primary preadipocytes [232,233]. Also, newer studies have shown that GH modulates in a negative way the maturation and accumulation of lipids in adipocytes [234]. Furthermore, some trials in humans try to use GH as an adjuvant of diet to reduce obesity [235,236]. Thus, GH is known for modifying adipocyte development but there is still controversy about whether it activates or inhibits the process.

Insulin

Insulin is a polypeptide hormone formed by two peptide chains, A and B [237]. It is secreted by the beta cells located in pancreatic islets and it is known for regulating carbohydrate, lipid and protein metabolism as well as promoting cell growth and division [238]. It mediates its effects by binding to insulin receptors which, in turn activate a cascade that ends with phosphorylation of substrate proteins known as Insulin Responsive Substrates (IRSs). IRS bind then to other signalling molecules which mediate cellular actions of insulin [239]. Molecular events associated with preadipocyte differentiation have been mostly analysed in 3T3-L1 cells [240–242]. Differentiation of 3T3-L1 preadipocytes into adipocytes requires insulin or insulin-like growth factor 1 (IGF1). IGF1 was first thought to be the most potent inducer of adipogenesis, but recent studies have shown that insulin is more physiologically important in terms of adipogenesis regulation. Concentrations of insulin inside physiological range showed to induce adipocyte differentiation [243,244]. It is understood that insulin is an activator of fat cell development and studies show that insulin signalling cascade can regulate

expression of adipogenic genes. Mice lacking insulin receptor in adipose tissue have reduced fat mass and lower expression of SREBP1 and C/EBPα [245]. This data suggest that insulin is capable of activating adipogenesis.

2.2.4. MicroRNAs

mi(cro)RNAs constitute a novel class of 20–25 nucleotide-long noncoding RNAs that regulate the expression of many different genes [246]. miRNAs undergo their functions by inhibition of protein translation or by the induction of cleavage of mRNAs of their target genes.

miRNAs have a different and unusual biogenesis, they are first transcribed as part of a longer precursors in the form called pri-miRNA, then this form fold on itself forming the hairpin structure. Pri-miRNA is then processed inside the nucleus by the microprocessor complex with endonuclease activity formed by Drosha and DGCR8, which generates a 70-nucleotide loop known as precursor miRNA (pre-miRNA) which is then exported to the cytoplasm by exportin 5. In the cytoplasm, pre-miRNAs undergo final processing by ribonuclease Dicer. Mature miRNAs are incorporated inside the RNA-induced silencing complex (RISC) by associating with the protein Argonaut. Inside RISC, miRNA is unwound in single-strand form and is directed for target mRNA selection [247–250].

Obese patients show an altered pattern of miRNA expression in different locations, which seem to be strongly related to the manifestation of metabolic disorders in these individuals [251]. In fact, altered circulating miRNA levels [252–254] and altered miRNAs levels in tissues involved in energy metabolism, such as liver or adipose tissue [255,256], are proposed as potential biomarkers of obesity .
For instance, miR-142-3p, which is related to acute and chronic inflammation, is increased in plasma of obese subjects while it is down-regulation after a weight-

loss intervention [257]. Moreover, the increased expression of miR-802 in the liver [255], miR-335 in the liver and WAT [258] and miR-143 in adipose tissue [259] have also been related to obesity.

Several miRNAs have been described to regulate the expression of adipogenesis-related genes, acting as activators or repressors of adipogenesis. Bork *et al* [260] showed that miR-369-5p was able to reduce adipogenic differentiation of mesenchymal stromal cells through inhibition of adiponectin and fatty acid-binding protein 4 (FABP4) mRNA expressions. Also, miR-302a inhibits adipocyte differentiation by directly targeting 3'UTR region of PPARγ mRNA, the master regulator of adipogenesis [261]. In addition, miR-27a and miR-130 also suppress PPARγ expression and the downregulation of these two miRNAs is related to adipose tissue dysfunction observed in obese subjects [262,263].

Other miRNAs act as activators of adipogenesis. Specifically, miR-148 inhibits Wnt1 mRNA [264] and miR-146b downregulates Sirt1 [265], thus promoting adipogenesis. In addition, miR-375 stimulates adipocyte differentiation, this miRNA is up-regulated during adipocyte differentiation in obesity, because it seems to act through stimulation of C/EBPα and PPARγ. The proposed mechanism for miR-375 includes the repression of extracellular signal-regulated kinases 1/2 (ERK1/2) which in turn could up-regulate PPARγ expression [266]. miR-146b is also overexpressed in both subcutaneous and visceral adipose tissue of obese individuals, resulting in the repression of KLF7 transcription factor mRNA, increasing the expression of adipogenic transcription factors [265].

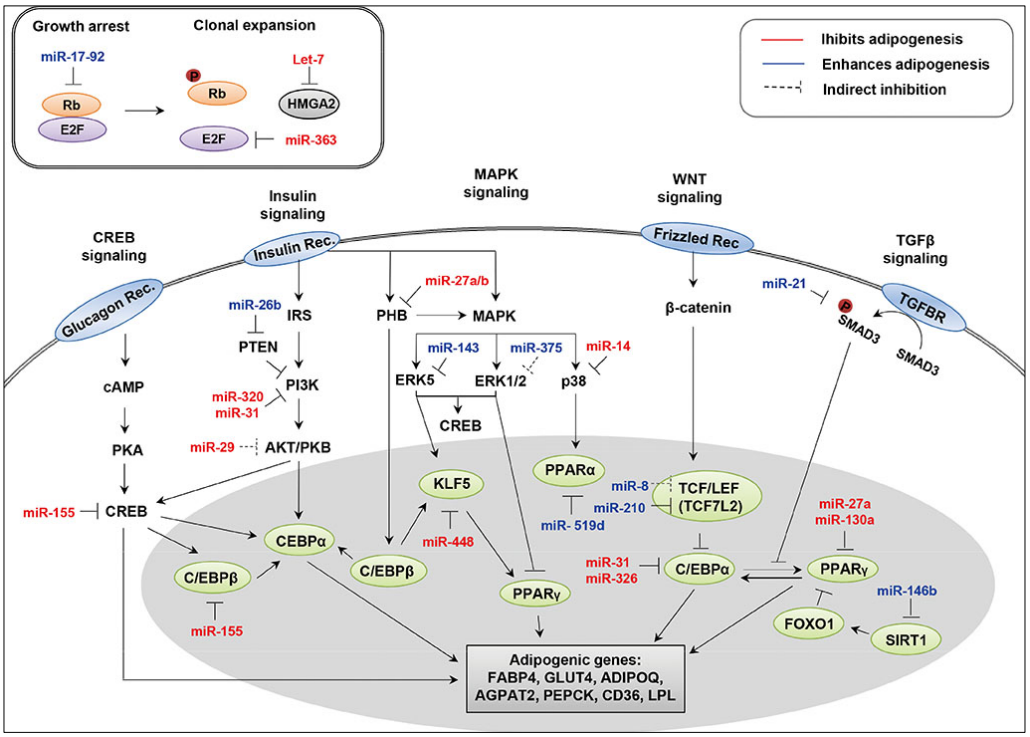


Figure 4. Signals and microRNAs involved in adipogenesis. Abbreviations: *HMGA2, high mobility group AT-hook2; CREB, cAMP response element binding; MAPK, mitogen-activated protein kinase; WNT, wingless and INT-1; TGF-β, transforming growth factor β; TGFBR, TGF-β receptor; IRS, insulin receptor substrate; PHB, prohibitin; SMAD3, Sma and Mad related protein 3; cAMP, cyclic adenosine monophosphate; PTEN, phosphatase and tensin homolog gene; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; PKA, protein kinase A; PKB, protein kinase B; KLF, Kruppel-like factor; PPAR, peroxisome proliferator-activated receptor; TCF, T-cell-specific transcription factor; LEF, lymphoid-enhancer-binding factor; C/EBP, CCAAT/enhancer-binding protein; FOXO, forkhead box protein O; SIRT1, sirtuin 1; FABP4, fatty acid binding protein 4; GLUT4, glucose transporter type 4; ADIPOQ, adiponectin; AGPAT2, 1-acyl-sn-gylcerol-3-phosphate acyltransferase beta; PEPCK, phosphoenolpyruvate carboxykinase; LPL, lipoprotein lipase*. *Son Y et al., 2014* [267].

As reviewed by Son Y *et al.* [267], many different miRNAs have been described to regulate adipocyte differentiation and every day new miRNA candidates are proposed to take part on it. To summarize, Figure 4 shows a schematic overview of some of the relevant miRNAs involved in WAT adipogenesis regulation.

With respect to brown and brite adipocyte adipogenesis, miRNAs are differently expressed in brown adipocytes compared to white adipocytes and brown adipocytes show a pattern of expression closer to muscle cells. For example, miR-1, miR-206 and miR-133a are highly expressed in brown pre-adipocytes [268]. Concretely, miR-133a has been reported to modulate brown adipocyte differentiation in both brown and white fat depots. miR-133a targets PRDM16 mRNA sequence, inhibiting its expression and thus, the modulation of miR-133a is a proposed strategy to induce brown-in-white adipocyte differentiation. An increase in PRDM16 would lead to an increase in energy expenditure inside white fat depots being a good point in counteracting excessive energy storage in WAT in obesity state [269,270].

Regarding lipid metabolism regulation in obese individuals, miR-33a and miR-33b have shown to target the genes involved in the regulation of cholesterol metabolism, FA β-Oxidation, and Insulin signalling [271]. miR-33a and miR-33b are encoded inside their host genes Srebp2 and Srebp1 respectively although in rodents, only miR-33a isoform is present. These miRNAs have been demonstrated to act through modulating mRNA expression of ATP-binding cassette transporter A1 (ABCA1) and SREBP1 [272] among many other target genes. SREBP1 activates FAS enzyme while ABCA1 regulates cholesterol efflux to apolipoprotein A-1 (ApoA1), being essential for the reverse transport of cholesterol [272,273]. Thus, miR-33a and miR-33b should be considered potential targets for treatment and prevention of obesity-related diseases. Also, miR-122, which is highly expressed in liver, has been strongly correlated to fat metabolism modulation. Selective inhibition of miR-122 has shown multiple beneficial health effects such as HMGCR decreased levels, reduced cholesterol levels, decreased Fas levels and consequent decreased fatty acid synthesis, increased FA oxidation, reduced TAG and cholesterol serum levels among many others [274–277]. Together these findings suggest that miR-122 is a plausible target for obesity treatment.

Together with lipid metabolism and adipogenesis, miRNAs have also been reported to regulate glucose and insulin homeostasis, for example miR-375, miR-124a and miR-let-7b [278]; diabetes through miR-375 and miR-9 which have shown to regulate insulin release [279–281]; energy balance in the hypothalamus via miR-30a-5p and miR-195 [282], and many other disorders making them relevant for the whole MS approach.

Summing up, hundreds of factors are currently known to play a role in adipocyte development. Despite all known factors, identifying novel regulators of fat cell development is still an important goal to get further into the mechanisms and new molecules through which adipogenesis could be regulated.

2.3. Adipose tissue expansion and remodelling in obesity

When obesity develops, adipose tissue is the main organ to store the energy excess because of its unique properties for this function, which include an almost unlimited capacity to expand to fulfil the required energy-storage demands [87].

WAT expansion in healthy states is defined as the enlargement of the fat pad mass through recruitment of adipocyte precursor cells that will be differentiated into adipocytes, together with recruiting other stromal cells and the associated vascularization [283]. However, when energy intake overpasses the limits of the fat pad storage, pathological expansion can occur. Pathological expansion can be described as the fast growth of the adipose tissue through enlargement of the existing adipocytes. In this scenario, the demand for energy storage is much higher than the rate of adipose tissue function inducing a

dysfunctional adipose tissue that increases the risk of developing metabolic and/or cardiovascular obesity-related diseases [284]. Adipose tissue function impairment arises together with other factors such as visceral fat accumulation, hypoxia and oxidative stress [284]. Such pathological expansion is also associated with chronic inflammation, which may result in developing insulin resistant adipocytes and also insulin resistance at systemic level.

Although all obese patients hold a positive energy balance, this does not ensure they will have the same risk for obesity-related diseases. As indicated before, vWAT mass is positively correlated with obesity-related diseases. Thus, individuals that are unable to expand their sWAT in a healthy manner in response to a continuous energy income in the body, are the ones in high risk of vWAT lipid accumulation, giving the first step that leads to adipose tissue dysfunction [284–287]. Moreover, in cases of unfunctioning or insulin resistant sWAT, the excess of TAG may accumulate in other organs, such as liver, muscle and heart inducing ectopic fat deposition [288]. Given the mentioned features, which determine the development of MS, there are two simply proposed criteria for diagnosing MS: enlarged waist perimeter and increased plasma TAG levels in fasted state. This conditions appearing together have been defined as “hypertriglyceridaemic waist”[289].

WAT tissue suffers from contraction and expansion along all the individual’s life. It can account from 5% [290] until 65% of the body depending on the human in particular [291]. Some athletes have shown to be under the average when they are extremely lean while other highly obese individuals can touch the upper limit of body fat percentage [290,291]. This plasticity makes the adipose tissue unique among other organs in mammals.

At cellular level, in all fat depots, the pool of adipocytes is renewed every day to balance with the adipocyte death. This refilling of adipocytes accounts about 10% of adipocytes annually in humans while in mice is around 2% every

day [292,293]. This renewal of adipocytes is postulated to arise from adipocyte precursors (preadipocytes) already placed inside WAT that undergo *de novo* adipocyte differentiation process. Adipocyte turnover has a stable rate if no different stimulus is given, but it has been shown that at advanced ages the dead adipocyte replacement becomes slower [294]. Adipocytes contribute to WAT expansion through hypertrophy and hyperplasia mechanisms [295]. Adipocyte hypertrophy is defined as the response of adipose tissue to positive energy balance by increasing adipocyte volume in order to increase adipocyte's TAG storage capacity. On the other hand, hyperplasia is the way of adipose tissue growth by increasing adipocyte number [296].

2.3.1. Adipocyte hypertrophy and related disorders

The remodelling of adipose tissue in response to energy surplus is not driven by hypertrophy or hyperplasia aleatory. It has been described that, against TAG excess, the first activated process is hypertrophy because it seems that the adipocyte number in adults is set during childhood and is almost constant trough all adult individuals' life independently of their leanness or obesity degree [293]. Thus, during WAT growth by energy oversupply, the most important activated mechanism is hypertrophy, so this process is one of the major determinants of obesity development [297,298].

A persistent hypertrophy state in adipose tissue has been extremely linked to a reduced insulin sensitivity of adipocytes [284,299], increased circulating levels of inflammatory molecules and increased infiltration of macrophage inside adipose tissue [300]. This occurs due to the fact that after overpassing a certain adipocyte volume threshold, adipocytes are not well-functioning anymore and they are not able to perform their physiological role in a correct manner [301]. In

addition, hypertrophy come together with alterations in lipid metabolism and local lipotoxicity [301,302].

Moreover, hypertrophic adipocytes have an increased oxygen demand that can lead local hypoxia in WAT that further contributes to a pro-inflammatory adipokine secretion pattern [303–308]. To counteract the hypoxia caused by hypertrophic cells, adipose tissue requires new angiogenesis to meet the oxygen demand. Vascular endothelial growth factors (VEGFs) promote angiogenesis and VEGFA expression is higher in the obese subjects that showed lower insulin resistance [309]. Obese animals with increased VEGFA expression have an increased number of blood vessels within adipose tissue, which could be a protective mechanism to counteract hypoxia and insulin resistance in hypertrophic adipocytes [310,311]. Thus, angiogenesis could be a desired activated process when pronounced hypertrophy is being developed in WAT adipocytes (Figure 5).

Numerous studies evidence that obesity can be thought as an inflammatory disease because of infiltration of immune cells in WAT [312], concretely in rodents and humans the most infiltrated immune cells are macrophages [313]. It has been shown that macrophages are recruited in response to the dead of hypertrophied cells by aggregating in crown-like structures that surround adipocytes [314] [314]. Some studies support the idea that macrophage infiltration into visceral fat depots is increased by hypertrophic and visceral obesity and plays a role in the link between abdominal adiposity and associated conditions such as diabetes and coronary heart disease [315,316]. Thus, the whole inflammatory process in adipose tissue is one of the symptoms of adipose tissue dysfunction that contributes to worsening obesity.

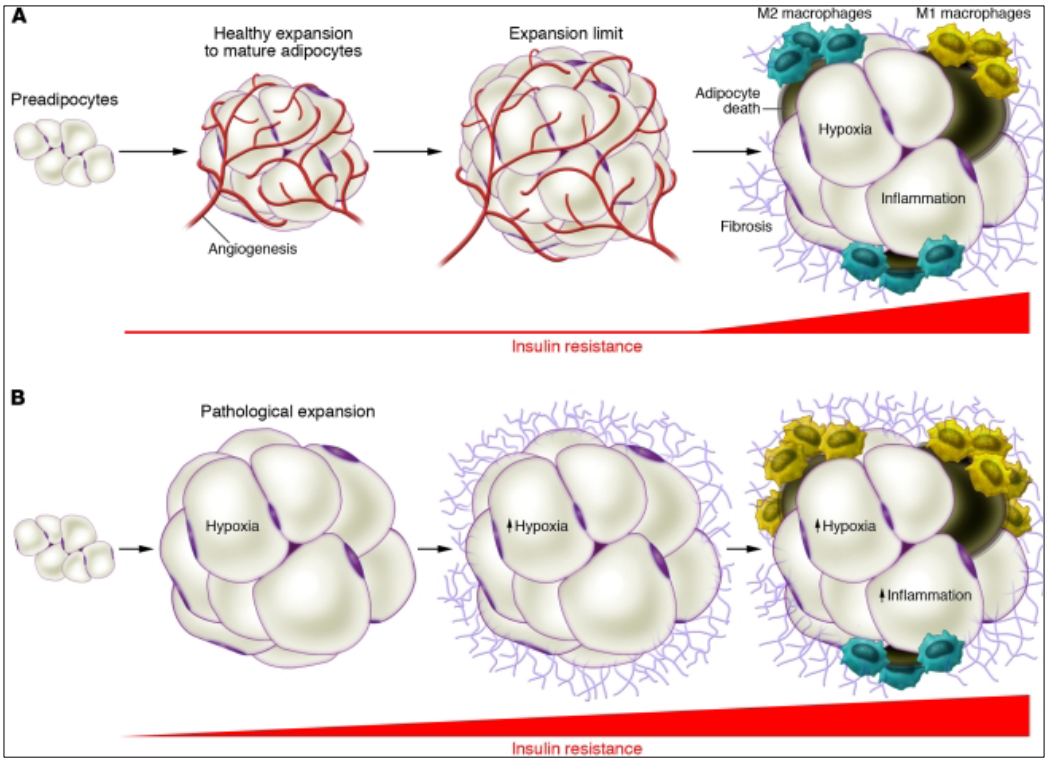


Figure 5. Healthy and unhealthy expansion in obesity. A. Progressive growth of adipose tissue with the proper corresponding angiogenesis. B. Pathological expansion of adipose tissue, hypoxia and insufficient angiogenesis and early development of insulin resistance. *Sun K et al., 2011* [283].

Leptin mediates food intake, energy expenditure and metabolism [317], while adiponectin is known to be protective in diabetes and atherosclerosis for its anti-inflammatory effects [318]. Both hyperleptinemia and hypoadiponectinemia have been correlated with insulin resistance and increased risk of CVD in animal and human obesity models [317,318]. Interestingly, some studies have shown that adipokine secretion dysregulation could be, in a high percentage, due to adipocyte size and not only to immune cell infiltration. Concretely, adipocyte size was shown to be directly related to leptin [319–322] and/or adiponectin [306] mRNA expression and protein secretion by adipocytes. A study from Skurk *et al.* [306] showed a direct correlation between adipocyte size and the ability of adipocytes

to secrete different cytokines. Specifically, adipocyte size is positively correlated with leptin, interleukin-6 (IL-6) and TNF-α [306].

Figure 6 shows that an unhealthy expansion leads to severe disorders in WAT that are related to diseases that imply the whole organism. These changes have been reflected in clinical studies where obese patients with a hypertrophic adipose tissue matched the mentioned increased inflammatory adipokine levels compared to those patients with smaller adipocytes in their fat depots. These patients are more likely to develop obesity-related diseases including low grade inflammation that can become chronic [323]. In contrast, when the excessive lipid storage is reduced (by caloric restriction or by decreasing glucose uptake into adipocytes, through the disruption of IRS in adipose tissue) adipocytes are smaller and the individual has improved whole body insulin sensitivity and even increased longevity [245,324]. Thus, the approach of reducing fat cell volume is a promising strategy to reverse the adipose tissue dysfunction. This should be seen as an anti-obesity intervention that could also ameliorate obesity-related disorders.

Adipose tissue dysfunction due to initial hypertrophy, as commented above, belongs to the primary effects in obesity and is the start of obesity-related health problems. Obesity and inflammation are processes which are really interrelated in pathogenesis of insulin resistance, diabetes, atherosclerosis and non-alcoholic fatty liver disease (NAFLD) [313,325,326].

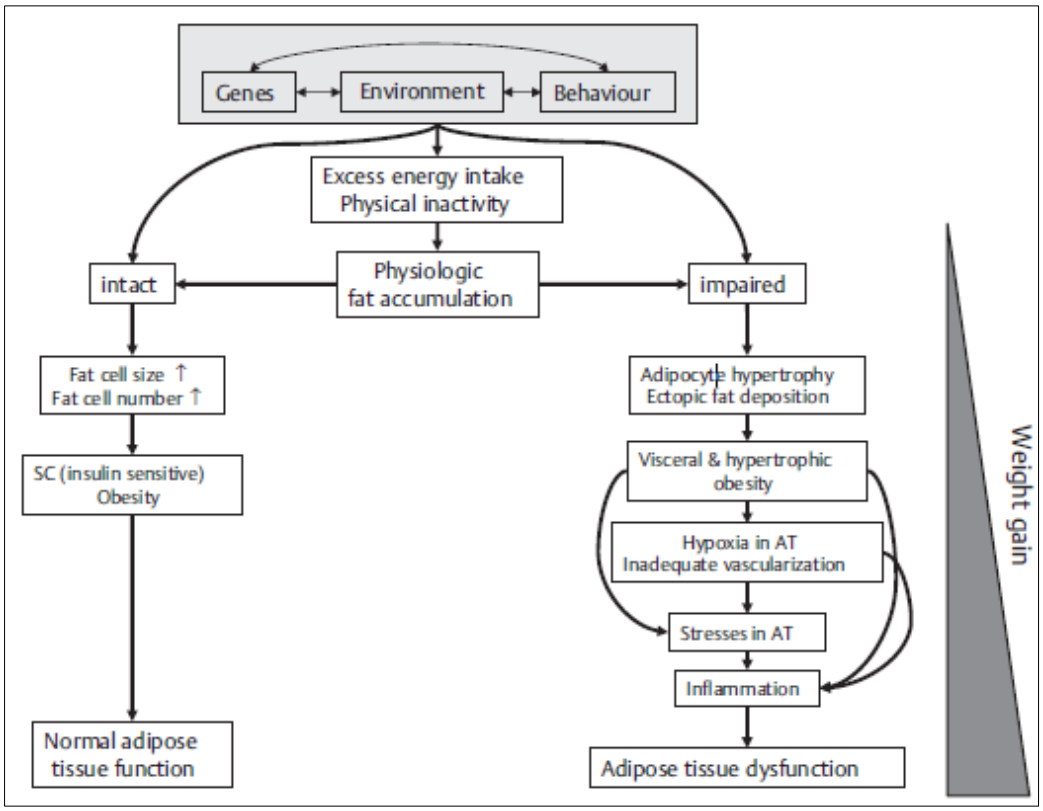


Figure 6. Adipose tissue changes with weight gain. Possible consequences of intact or impaired adipose tissue growth due to fat accumulation. *Blüher, 2009* [284].

2.3.2. Adipocyte hyperplasia and adipose tissue remodelling

As mentioned above, adipose tissue hypertrophy has been linked to the risk of developing obesity-related metabolic diseases. On the other hand, adipocyte hyperplasia has been related to a protective effect against obesity-related diseases [285,327]. Hyperplasia is produced by differentiation of pre-adipocytes; this process is regulated by a high number of transcription factors and other elements which have been already widely explained in the previous section.

New formed adipocytes derive from pre-existing pool of adipose stem cells. The adipose precursor cells are the MSCs. The induction of MSCs to enter

the commitment pathway that leads to hyperplasia is triggered by unknown signals that start with the obese metabolic state. This state is generated by an excessive energy intake together with an elevated glucose during an extended time period [328].

Several factors have been identified that commit or inhibit the conversion of pluripotent stem cells to the adipocyte lineage. These include the BMP family members BMP4 and BMP2, Wnt and many others [168,329–331] already described in the previous section of adipogenesis. All these factors work together in a balance of signals that determines the developmental pathway by inhibiting some pathways and activating others. For example, BMP4 promotes adipogenesis while inhibits myogenesis [332] and Wnt10b inhibits adipogenesis but promotes osteogenesis and may be also activating myogenesis [333].

In severe obesity states, individuals are predisposed to metabolic syndrome development while some studies show that MSCs contribute in a relevant manner to adipocyte generation increasing adipocyte number in fat depots [38,334]. This role is significant in maintaining the function of adult adipose tissue because it provides new cells, preventing the existing adipose cells from evolving towards hypertrophic adipocytes. However, this MSCs contribution to adipocyte hyperplasia is reduced in obesity state. Obese adipose tissue shows reduced levels of adipogenic differentiation-specific genes suggesting that differentiation is constrained by endogenous adipose factors in obesity state, contributing even more to the dysfunction of WAT [335]. For this, some works have focused in the research of agents that promote adipogenesis, mainly targeting PPARγ. The upregulation of PPARγ induces adipogenic differentiation of new small adipocytes, which are insulin sensitive and thus produce less inflammatory adipokines [336,337]. In diabetic obese subjects, adipocyte PPARγ is downregulated and adipocytes are less insulin sensitive. Hence, PPARγ is a potential target to be induced for ameliorating adipocyte hypertrophy by inducing hyperplasia which is

why PPARγ agonists are some of the drugs designed for diabetes treatment. However, PPARγ activation has secondary effects, such as weight gain [338].

3. Polyphenols

Phytochemicals are part of secondary metabolites from plants. Thus, phytochemicals are not required for the plant survival, as they are not essential for plant development. However, these phytochemicals can protect plants against microbial infections, UV radiation or herbivores [339].

Polyphenols are one of the major groups of phytochemicals and are some of the most important plant secondary metabolites. They are not essential for animal survival, like vitamins, but polyphenols can contribute to human health by giving protection against diseases such CVD, cancer and obesity-related diseases, like type II diabetes [339–341].

Phenol family is composed of a huge number of molecules which include in their chemical structure at least one aromatic ring that, in turn, contain one or more hydroxyl groups as substituents. Numerous phenolic structures have been characterized and these are widely dispersed inside the whole vegetal kingdom [342]. Most of them are present in food such as fruits, vegetables, cereals, olives, chocolate and beverages, including tea, coffee and wine [343].

3.1. Structural classification of polyphenols

Dietary polyphenols can be classified by different criteria like by their chemical structure [343,344].

In this sense, polyphenols are divided in two big groups: flavonoid and non-flavonoid forms.

Flavonoids compose the largest group of phenolic compounds, with more than 4,000 different identified structures [340]. The main skeleton of flavonoids is C_6-C_3-C_6 characterized by 2 aromatic rings (named A and B), which are connected by a 3-carbon bridge (named C ring) (Figure 7) [345]. Flavonoids are mainly sub-classified in: in flavones, isoflavones, flavanones, anthocyanidins, flavonols and flavanols (monomeric and oligomeric forms). Figure 4 shows these classes of flavonoids with their basic chemical structure [339].

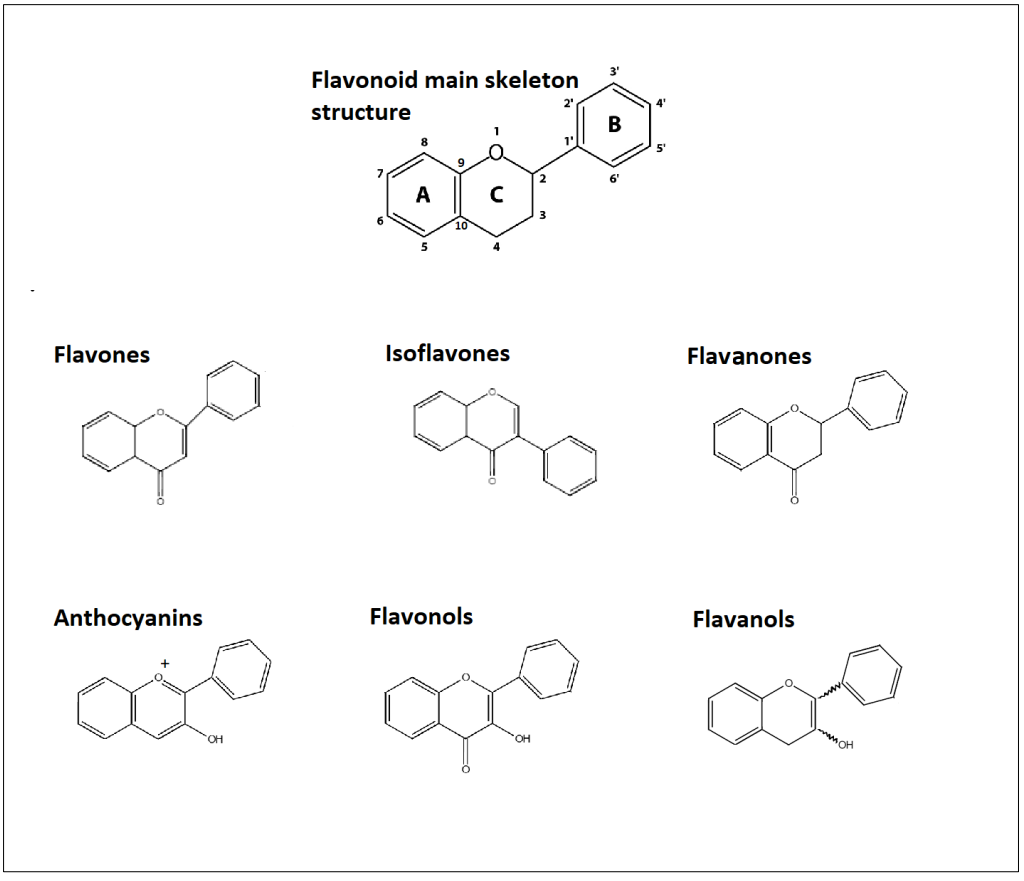


Figure 7. Flavonoids basic chemical structure.

The presence of the different flavonoids is spread in several foods. Flavones are found in some herbs such as celery or parsley [346]. Isoflavones occur mainly in leguminous plants including the soybean [347]. Flavanones, such as naringenin, are mostly present in citrus fruits [348]. Anthocyanidins are easily detectable in flowers and fruits for their colours, which range from orange to purple [349]. Flavonols are found spread around all plant kingdom [350]. Flavanols, also named flavan-3-ols, are the most complex structure of flavonoids and are mainly found in tea, cocoa, apples, grapes and thus in red wine [339,346,351,352]. Flavanols range from simple monomers to complex structures that include oligomeric and polymeric proanthocyanidins (PAs), also known as condensed tannins (Figure 8) [353]. Inside flavanol monomers, the most common structures include (+)-catechin, and (-)-epicatechin (EC) but (-)-catechin, and (+)-epicatechin are also relevant even though they are less frequent. Flavanols are also found as gallated forms (+)-gallocatechin (GC), (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG) depending on the bond position [339,354].

PAs, are the most complex subclass of flavonoids [355]. They are composed of monomeric units that are linked between them mainly through C4-C6 or C4-C8 bonds, and these PA are B-type. However, less frequent monomeric units have an additional link through C2-C7, forming PA of A-type [356–358].

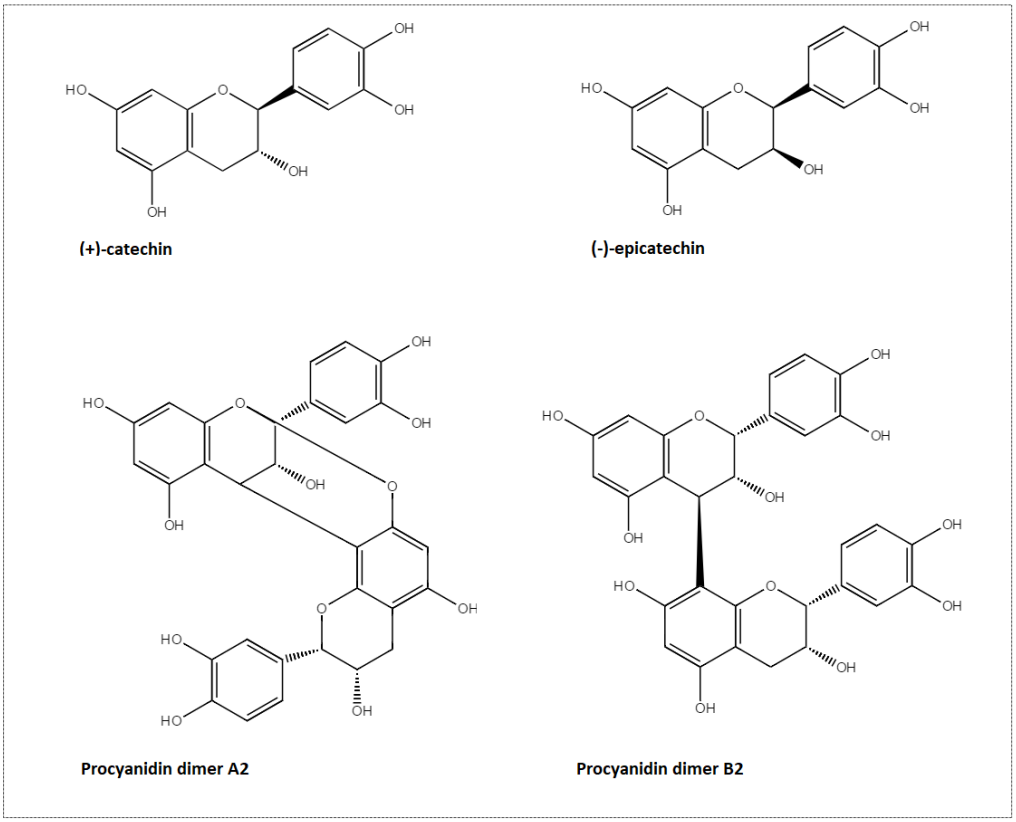
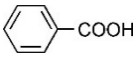
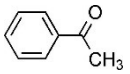
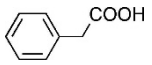
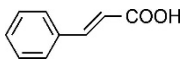
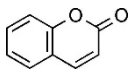
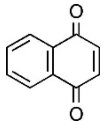
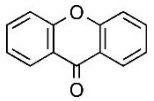
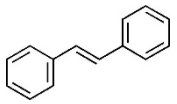
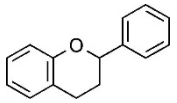


Figure 8. Flavanol monomeric ((+)-catechin and (-)-epicatechin monomers) and type A2 and B2 procyanidin dimer structures.

Among the non-flavonoid polyphenols, phenolic acids, hydroxycinnamic acids and stilbenes are the principal dietary non-flavonoids (Table 2) [339]. Phenolic acids are usually found in free forms inside fruits and vegetables while inside grains and seed there are often in the bound forms [359,360]. Hydroxycinnamic acids have been shown to have health protective benefits against oxidative stress and inflammatory damage. Curcumin is a well-known hydroxycinnamic acid derivative exerting these mentioned properties, however its bioavailability is poor and thus its clinical use is lower than other phenolic compounds[361]. The stilbenes have a C6-C2-C6 structure and are produced by plants in response to injury, stress and diseases [340]. Red wines contain high diversity of stilbene derivates but they are present in lower concentrations than other phenolic compounds [362] The most studied from inside Stilbenes is Resveratrol (RSV). There

are also several non-flavonoid polyphenols found in foods that are considered important for human health. A part from RSV, ellagic acid, which consists of a dimer of gallic acid (GA), and its derivates are found un betty fruits and in tree nuts skin but their effects on health have been related to anti-nutrient outcome [344].

Table 2. Structural classification and skeletons of the different phenolic compounds. Adapted from [339].

Skeleton	Classification	Basic structure
C_6-C_1	Phenolic acids	
C_6-C_2	Acetophenones	
C_6-C_2	Phenylacetic acid	
C_6-C_3	Hydroxycinnamic acids	
C_6-C_3	Coumarins	
C_6-C_4	Naphthoquinones	
C_6-C_1-C_6	Xanthones	
C_6-C_2-C_6	Stilbenes	
C_6-C_3-C_6	Flavonoids	

3.2. Polyphenols bioavailability and beneficial effects on health

3.2.1 Bioavailability of polyphenols

Bioavailability of polyphenols, the ability of these compounds to be absorbed and exert subsequent effects in the organism they are absorbed, includes the liberation of polyphenols from the food matrix, their digestion, their transport through intestinal barrier towards bloodstream, their distribution to the different tissues, their metabolisation and their final excretion [363].

Polyphenol bioavailability depends on many different factors such as their chemical structure, the food matrix in which are contained, the dose and the intestinal transit among many other aspects [363]. For example, several studies in animal models have demonstrated that flavanol bioavailability is affected by sex, age or by a pathological state [364–366]. The structural complexity as the grade of polymerisation also plays an important role in determining the polyphenol absorption. For example, quercetin glycosides, isoflavones, flavanones, flavanols and GA are the most easily absorbed polyphenols in humans, whereas proanthocyanidins and anthocyanins are absorbed in less proportion [363,367].

Polyphenols are actively metabolised inside small intestine enterocytes and in the liver. They are mainly conjugated by glucuronidation, sulphation and/or methylation, reactions by the enzymatic activity of uridine 5'-diphosphate glucuronosyltransferases (UGTs), cytosolic sulfotransferases (SULTs) and catechol-O-methyltransferases (COMTs), and respectively [340,368,369]. Moreover, large polymers are difficult to absorb in small intestine and reach the colon, where they can undergo microbial transformation in which they will be

hydrolysed in smaller molecular weight metabolites that can be then absorbed and still reach different tissues through systemic circulation [369–372].

Polyphenol appears to be transported in the bloodstream mainly attached to carriers such as albumin. The higher plasma concentrations of polyphenol and polyphenol metabolites goes from 1 to even 24 hours, depending on the absorption site and the structure [363,367,373,374]. Studies in animals have shown that, after the ingestion, polyphenols and their metabolites can reach target tissues such as liver, adipose tissue, heart, spleen and even inside brain tissue, pointing to a capacity to cross the bloodbrain barrier [367,375–377].

Polyphenol are eliminated through different pathways, depending on their structure and molecular weight. Some polyphenols and their metabolites are known to be excreted mainly via renal through urine [378] and the ones more conjugated, can be eliminated in the bile through intestine.

3.2.2 Beneficial effects of polyphenol intake

Oxidative stress is regulated by polyphenols. Many metabolic reactions that take place in the body produce ROS [379]. Some polyphenols described for delaying ageing for oxidative stress include EGCG, quercetin, hydroxytyrosol or RSV [380].

CVDs are the main cause of mortality worldwide. CVDs include coronary heart diseases, hypertension, myocardial infarction and stroke [380,381] . Some risk factors are unchangeable but others depend on lifestyle [382], and the prevention of CVDs is more effective than treating it once it has been developed. A large number of epidemiological studies have related polyphenol consumption with a decreased risk of CVDs development. Specifically, tea, wine, flavan-3-ols, PAs, anthocyanins, flavanones and flavonols consumption have a protective effect on CVDs in an extent particular for each food or polyphenol class [383–386]. Polyphenols could have this CVD protective effect by improving endothelial function,

modulating nitric oxide and/or reducing serum LDL, cholesterol, and some inflammation markers [387–390].

Anticarcinogenic effects of polyphenols have been extensively studied in animal models. Administration of polyphenols to rodents with tumours, or under the effects of carcinogenic agents, protected them against worsening their conditions [391]. Moreover, specific polyphenols even reduced the number and size of tumours or inhibited their growth [391].

Interestingly, several studies have demonstrated that polyphenols can act at the different stages of cancer development: initiation, promotion and progression [387,392]. Even though, the anticarcinogenic activity of polyphenols is much evident in rodents than in humans, possibly because the difficulties in extrapolating results and doses form animal models to humans [393]. However, despite the small number of human clinical studies, there is enough evidence to describe some polyphenols as anticarcinogenic molecules. Specifically, EGCG, a flavonoid found in many plant sources and specially in tea, has been related to chemopreventive effects. In addition, green tea consumption has been linked to lowered risk of different cancers including breast, mouth and prostate. Many studies performed *in vitro*, *in vivo* and in humans have shown that polyphenols contained in grapes, berries, cocoa and olive oil have anticarcinogenic effects [394–397].

Firstly, it was thought that the anticarcinogenic effect of polyphenols was due to their antioxidant properties. However, newer studies have proposed novel mechanisms by which polyphenols can prevent or inhibit cancer. One mechanism is that polyphenols block initial stages of cancer through the modulation of cytochrome P450 enzymes [398], which are involved in activation of carcinogens, and/or through the stimulation of DNA repair in initiated cells. Another proposed mechanism is by the induction of apoptosis of cancer cells by polyphenols, slowing or stopping tumour growth [399].

Polyphenol consumption also have beneficial effects on MS and obesity-related diseases. Most of these studies, both in human and animals, have been performed with tea and grapes polyphenol extracts. The main group of polyphenol in tea is catechins, mostly EC, epicatechin gallate (ECG), EGC and EGCG [400]. Tea extracts have pronounced hypolipidemic effects [401] and suppress some transcription factors related to adipogenesis [402]. Other polyphenols, such as flavan-3-ols, tannins and chalcones inhibit the activity of lipoprotein lipase [403]. In addition, PAs, highly present in grapes, improve dyslipidaemia and hyperglycaemia associated to MS [404–407].

To sum up, dietary polyphenols have been widely demonstrated to improve several diseases and even revert some of them. Anyway, some controversy may occur when translating results from animal models to human subjects. In any case, numerous clinical studies have shown that polyphenol consumption may protect against many diseases and that these compounds are a good strategy to target several diseases with a nutraceutical treatment approach.

Next sections explain in detail the characteristics and beneficial effects of polyphenols studied in this doctoral thesis, namely grape seed proanthocyanidin extract, GA and RSV. Moreover, because the core of this doctoral thesis is adipose tissue remodelling, this introduction focus on the effects of these polyphenols on adipose tissue and lipid metabolism.

3.3. Grape Seed Proanthocyanidin Extract

The Grape Seed Proanthocyanidin Extract (GSPE) is an extract rich in PAs with numerous healthy properties, such as hypolipidemic, anti-hyperglucemic, anti-hypertensive, and anti-carcinogenic [389,408–411].

The GSPE used in this thesis was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). GSPE composition is presented in Table 3. GSPE contains different phenolic compounds including GA, monomeric flavan-3-ols; C and EC, their hydroxylated EGC form and their forms esterified with GA (ECG and EGCG), dimers and a considerable amount of oligomeric and polymeric forms. Therefore, GSPE consists mainly of PAs[365].

Table 3. Main polyphenols (flavanols and phenolic acids) of the grape seed proanthocyanidin extract (GSPE). Adapted from [365]. The results are expressed as mg of phenolic compound/g of GSPE on a wet basis as the mean ± SD (n=3).

Compound	Concentration (mg/g)
Catechin	121.32 ± 3.41
Epicatechin	93.44 ± 4.27
Proanthocyanidin dimer B1	88.80 ± 3.46
Proanthocyanidin dimer B3	46.09 ± 2.07
Proanthocyanidin dimer B2	33.24 ± 1.39
Gallic acid	31.07 ± 0.08
Epicatechin gallate	21.24 ± 1.08
Dimer gallate	8.86 ± 0.14
Proanthocyanidin trimer	4.90 ± 0.47
Protocatechuic acid	1.34 ± 0.02
Vanillic acid	0.77 ± 0.04
Epigallocatechin	0.27 ± 0.03
Epigallocatechin gallate	0.03 ± 0.00
Proanthocyanidin tetramer	0.05 ± 0.01

The relevance of this polyphenols is because PAs are the most abundant polyphenols in human diets [412]. PAs are considered bioactive compounds for their influence in cellular and physiological processes that result in beneficial health effects. PAs ameliorate oxidative stress, microbial infections, genotoxicity, cancer and inflammation and have cardioprotective properties [390,413–417].

Two major mechanisms have been proposed to explain the biological actions of PAs: basic biochemical mechanisms, which focus on PAs' ability to bind strongly to proteins, and epigenetic mechanisms, which include histone modifications, DNA methylation and modulation of miRNAs. Both mechanisms allow PAs to modulate enzymatic activities, cell signalling cascades and gene expression, ultimately resulting in the modulation of cell functionality [412].

One of the best described activity of PAs is its capacity to ameliorate hyperlipidaemia [355]. *In vivo* studies in rodents show that PAs reduce apoB-TAG-rich lipoproteins, LDL-C and increase HDL-C [355]. However, the effects on lipid metabolism in humans is confusing and there is controversy about whether PAs exert positive, negative or no effect in TAG levels [418–420], but the dose and the specific PA composition of extracts should be taken into account to make accurate comparisons between human studies.

GSPE modulates lipid metabolism by inducing the transactivation of the farnesoid X receptor (FXR) [421] and overexpression of the nuclear receptor Small Heterodimer partner (SHP) [408] that results in the repression of SREBP1 and lipogenic genes in the liver. Furthermore, recent studies from our group have demonstrated that GSPE modulate cholesterol homeostasis through the repression of miR-33 which, in turn, activates ABCA1 gene expression [422], thus increasing HDL formation and the reverse transport of cholesterol for its elimination in the liver [423,424]. In addition, these same studies also demonstrated that GSPE reduces lipogenesis by repressing miR-122 [423,424], a miRNA that

regulates the expression of FAS, SREBP1-C AND CPT1A. Interestingly, GSPE is also effective repressing miR-33 and miR-122 levels in obesity rodent model [158].

GSPE targets adipose cells [425] and modifies adipose tissue gene expression [426]. Specifically, GSPE-treated rats presented higher expression of facilitated glucose transporter member 4 (GLUT4) and IRS1 in mesenteric adipose tissue, suggesting an amelioration of peripheral insulin resistance [427].

As stated before, remodelling of adipose tissue in obesity is produced by hypertrophy and hyperplasia. Adipocyte hypertrophy is related to TAG mass, which is determined by lipid synthesis and/or degradation and, importantly, both processes are modulated by PAs. However, hyperplasia is managed by preadipocyte maturation by adipogenesis, preadipocyte replication and adipocyte cell death [425]. *In vitro* studies have revealed that GSPE inhibits 3T3-L1 differentiation to mature adipocytes, but only when GSPE is added at the onset of differentiation (between days 0 and 2) [428]. Furthermore, GSPE treated 3T3-L1 cells showed a modified expression profile of cell cycle genes, that suggested an interference of GSPE with the progression of mitotic clonal expansion and thus, partially inhibiting differentiation [428]. However, these results were shown in an immortal cell line and were not tested in *in vivo* models. On the contrary, *in vivo* studies show that GSPE increases adiposity in pregnant rats fed with a high-fat diet, and even in their offspring. However, the offspring possessed a more favourable adipose tissue development as a result of an increased angiogenesis and decreased macrophage infiltration [429]. A comparative study of Montagut *et al.* [430] suggest that the capacity of GSPE to modulate adipocyte differentiation depends on the physiological conditions and the fat depot [430]. In summary, not many specific studies have been performed in order to obtain reliable results regarding adipose tissue remodelling with GSPE or PAs treatments.

3.4. Gallic acid

The most important source of polyphenols in diet is the flavonoid family. However, non-flavonoid compounds are also important in human diet[340].

Gallic Acid (3,4,5-trihydroxybenzoic acid; GA) is one of the most common and studied phenolic acids. It is a C_6-C_1 phenolic acid (Figure 9) which is abundant in vegetables, like broccoli and aubergine, in tea leaves, in fruits, such as strawberries, pineapples, bananas, apples and lemons red and white wines and extracts of grape seed , like GSPE [431–435]. GA in foods exists as its free form or as a conjugate of flavanols such as EGCG or PA dimer B2 gallate [436–438].

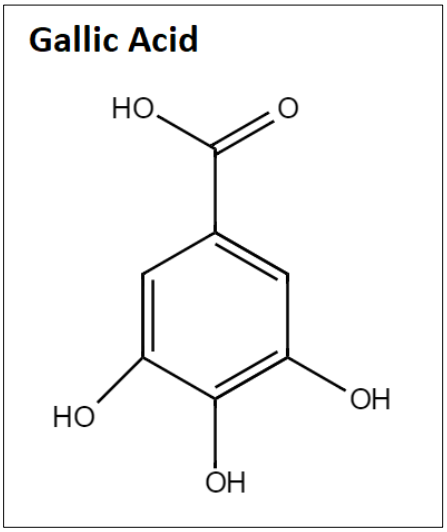


Figure 9. Chemical structure of Gallic Acid (3,4,5-trihydroxybenzoic acid).

GA has been reported for its antimutagenic, anticarcinogenic, antiallergenic and anti-inflammatory activity [439–441]. It is thought that a daily intake of GA is the way of obtaining health benefits and its action is performed through interfering with several biochemical pathways [434,439].

GA has been reported to reduce body weight , TAG and LDL-C in high fat diet (HFD) induced obese rodent models [442,443]. Furthermore, GA reduced leptin and insulin levels in rats fed a HFD [444]. Some studies tested GA effects using an

extract of the *Terminalia Bellirica* fruit, a rich source of GA. Interestingly, this extract prevents vWAT fat accumulation and improves glucose intolerance in TSOD mice, a model of spontaneous metabolic syndrome [445,446].

In vivo studies have demonstrated that oral administration of GA reduces lipid droplets and adipocyte size in high-fat diet obese mice [443]. GA administered intraperitoneally is also able to reduce adipocyte size in subcutaneous and epididymal adipose tissue of obese animals. Importantly, this effect is concomitant with the enhancement of PPARγ expression, in both subcutaneous and visceral fat depots, suggesting that GA induces adipogenesis [447]. Furthermore, *in vitro* studies confirm the adipogenic activity of GA. In this sense, an extract of *Terminalia Bellirica* enhances 3T3-L1 cell differentiation at the same extent than the troglitazone (one of the thiazolidinediones known for activating PPARγ) [448] and increases adiponectin expression and secretion in adipocytes [449], suggesting that GA could be used as a potential drug for enhancing differentiation and adiponectin secretion.

Taken together all these studies, GA could be a potential agent for ameliorating adipose tissue dysfunction through the increase of WAT storage capacity and the reduction of adipocyte size. However, the published results focusing on hypertrophic and hypoxic adipocytes with oral GA administration have not been quantified and are obtained from qualitative measures.

3.5. Resveratrol

RSV (3, 5,4'-trihydroxystilbene) [340] is a phenolic compound included in the non-flavonoid family, specifically in the stilbenes subfamily [340,450,451]. RSV exists in *cis*- and *trans*- isomeric forms [340,450,451] (Figure 10). RSV is very relevant because of its reported health benefits.

RSV occurs in numerous plant species that synthesise it in response to pathogens [450] and is a characteristic polyphenol present in red wine [452]. The existing studies about RSV have focused on grapes, peanuts and their products for its high RSV content [453]. However the cis- isomeric form has never been identified in grape extract and the trans-isomeric form is the most well-studied for its beneficial effects [450].

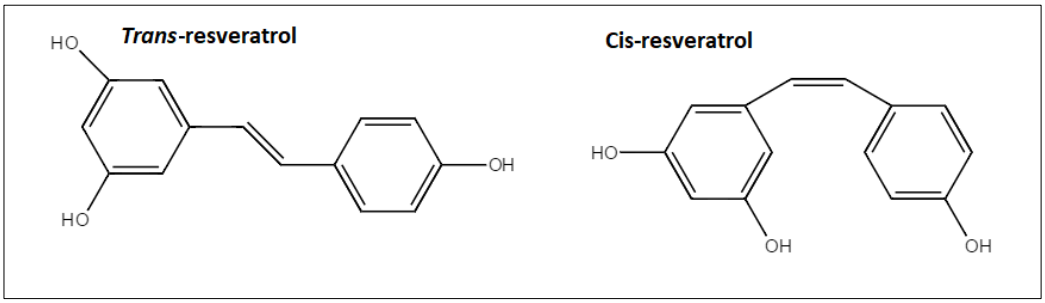


Figure 10. Chemical structure of Resveratrol isomeric forms.

RSV is mainly absorbed in the small intestine, in the duodenum fraction, and it is absorbed in approximately a 20% [454]. The glucuronide form is the most abundant when compared to sulphate forms [454]. RSV is distributed in all organs, being detectable in the liver and kidneys after 1.5 h of its administration and in lung, spleen, heart and other tissues after 3h [455]. The free form of RSV is rarely found in plasma and it is short lived compared to all the metabolised forms [456].

Numerous studies have reported the potential health benefits of dietary RSV on MS [457–459] and the French paradox is one of the most popular topic among researchers when talking about RSV [452,456,460]. In both *in vitro* and *in vivo* models,

RSV has shown many properties: antioxidant effects, such as inhibiting LDL oxidation; cardioprotective functions, by preventing platelet aggregation inside arteries; chemopreventive action, by acting over cell cycle; and anti-inflammatory properties among many others [461–466]. Furthermore, RSV modifies the metabolism through miRNA modulation. The most widely studied field regarding RSV and miRNAs is cancer [467,468]. However, it has also been demonstrated that RSV can modulate lipid metabolism regulators such as miR-122 and miR-33a in an *in vitro* hepatic cell model and that RSV can directly bind to these miRNAs by ^{1}H-MNR spectroscopy [469].

There are diverse rodent studies that suggest RSV as an effective antidiabetic treatment because RSV reduces plasma glucose levels [470–473], decreases plasma insulin levels [457,470–472,474] and improves insulin sensitivity or glucose tolerance [457,475–477]. Furthermore, clinical trials in type II diabetic subjects support this anti-diabetic effects of RSV [478–480]. However, studies with non-diabetic patients are less consistent regarding glucose and insulin homeostasis regulation [481–484].

Despite all the beneficial effects of RSV indicated above can ameliorate obesity-related complications or reduce the risk of developing them, RSV can also improve obesity by modulating metabolic pathways that are directly related to the development or worsening of this disease. In this sense, several studies with rodent fed a high-fat diet have shown that oral high doses of RSV (around 400mg/kg) have multiple beneficial effects on obesity. Specifically, RSV reduces body weight gain, visceral weight and adipocyte size in epididymal adipose tissue [457,471]. Remarkably, RSV increases energy expenditure in rodent [457] and non-human primate models [485], indicating that this is the mechanism through which RSV reduces body weight gain. Interestingly, other studies have involved the browning process in the increase of energy expenditure and fat lowering actions of RSV. In this sense, RSV increases PGC1α and mitochondrial DNA content in

WAT depots of rodents fed a high-fat diet [457] and mice fed with high or low doses of RSV show an overexpression of UCP1 and SIRT1 and activation of PGC1α in adipose tissue [486,487].

In contrast, RSV human trials have not been able to show consistent results concerning body weight changes. It is also important to note that human studies were performed with lower doses of RSV than rodent studies (from 75 to 2000mg/day) and humans were not under HFD conditions [479,482,488,489]. In this sense, rodent experiments with low doses of RSV (around 22 mg/kg) show that RSV is not effective reducing body weight [470]. Therefore, it is important to stablish the dose, time of the study and the health state of the cohort to provide definitive answers of RSV effects on energy expenditure and body composition in humans. Even though, data from animal studies is encouraging and clinical trials are limited and the conditions are not always the best in terms of control groups, doses or time exposure to RSV. Thus, further research should be performed through human intervention studies to assure health claims of RSV are solid.

RSV is very active in remodelling white to a brown-like adipose tissue, such it has been indicated before. Furthermore, several studies, all performed *in vitro*, demonstrate that RSV acts as an anti-adipogenic effector over murine, porcine and human pre-adipocytes in concentrations ranging from 20 to 100μM, reducing the expression of PPARγ and C/EBPα and decreasing lipid accumulation in most of the cases [490–496]. However, lower doses of RSV, which are equivalent to those found in plasma after ingestion of RSV rich food sources, do not affect lipid content in 3T3-L1 adipocytes [497]. In contrast, an analysis of the effects of different doses of RSV, from 1 to 100μM, during seven days on 3T3-L1 cell line shows that RSV enhances adipocyte differentiation in a dose dependent manner by up-regulating PPARγ and C/EBPα [498].

Only few studies have focused on the effect of RSV on adipocyte size. Nevertheless, obese mice [499] following a HFD supplemented with 400mg/kg of

RSV showed a reduction in adipocyte size from epididymal WAT. Furthermore, a human study carried out in healthy obese man shows a significant reduction in adipocyte size after 30 days of RSV supplementation [500]. This reduction of adipocyte size is also reflected in the gene expression profile, which shows increased adipogenesis pattern even though adipose tissue functionality was not evaluated to confirm whether RSV improves adipose tissue activity [500].

In summary, RSV seems to attenuate adipocyte differentiation in *in vitro* models although some controversy is still present regarding doses and contradictory results. As indicated by Chang C. *et al*, [501] low doses of RSV can exert both inhibition of adipogenesis and lipolysis while high concentrations of RSV can decrease cell survival rate in both preadipocytes and adipocytes. Thus, the anti-obesity effects of RSV could be through adipogenesis inhibition as well as through cytotoxic instigation of adipocytes. Therefore, RSV concentrations must be taken into account for experimental designs in order to obtain comparable results.

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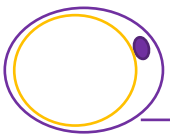


III. OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
ADIPOSE TISSUE REMODELLING BY POLYPHENOLS IN OBESE RATS
Aïda Pascual Serrano

The research work of the present Ph. D. thesis has been developed in the Nutrigenomics Research Group of Universitat Rovira i Virgili. This thesis is included in the research project AGL2013-40707-R "Development of an integrated food to maintain body weight and to prevent the risk of obesity related pathologies" which aims to formulate a MIX, containing several bioactive compounds, useful for the prevention of a set of pathologies that encompass the metabolic syndrome. The final idea is to develop a 2.0 functional food, a functional food able to prevent simultaneously all the risk factors associated to a multifactor situation, such as metabolic syndrome. This MIX is based on proanthocyanidins complemented with other natural bioactive compounds that are able to improve pathologies associated to obesity and metabolic syndrome. Proanthocyanidins have been previously described to ameliorate many components of metabolic syndrome and obesity-related disorders without reducing body weight. More specifically, grape seed proanthocyanidins have been described to exert hypolipidemic effects which improve lipid metabolism and fat accumulation.

Within the framework of this general project, this thesis has focused on the capacity of some polyphenols to remodel adipose tissue thereby counteracting adipose tissue dysfunction associated to obesity and metabolic syndrome.

The specific objectives of the present thesis were:

1. To determine whether proanthocyanidins are able to prevent adipose tissue dysfunction induced by an obesogenic diet by remodelling adipocytes (Manuscript 1).

To accomplish this objective, rats were fed with a cafeteria diet to induce obesity during twelve weeks. Along all experiment, rats were daily administered a grape seed proanthocyanidin extract at dietary (25 mg/Kg body weight) or pharmacological doses (100 or 200 mg/Kg body weight). The specific aims were to determine:

1.1. The effectiveness of proanthocyanidins to improve visceral adipose functionality by measuring both plasma metabolites related to lipid and glucose homeostasis and the expression of leptin and inflammatory markers in visceral adipose tissue.

1.2. The capacity of dietary and pharmacological doses of proanthocyanidins to avoid adipocyte hypertrophy linked to obesity and /or to induce hyperplasia by performing microscopic analyses of visceral adipocytes.

1.3. The molecular mechanism by which a pharmacological dose of proanthocyanidins prevent adipose tissue dysfunctionality, mainly focusing on adipogenesis and browning at the expression level.

2. To evaluate the capacity of a dietary dose of proanthocyanidins or gallic acid to remodel adipose tissue and counteract its impairment in obesity (Manuscript 2).

To achieve this objective, rats were fed a cafeteria diet and once obesity was established they were administered for three weeks with a dietary dose of a grape seed proanthocyanidin extract (25 mg/kg body weight) keeping the obesogenic diet. Grape seed extract contains gallic acid in addition to proanthocyanidins, thus this objective also aims to determine the contribution of

gallic acid on the effectiveness of the grape seed proanthocyanidin extract. Therefore, an additional group of rats was administered with an equivalent dose of gallic acid (7 mg/Kg body weight) present in the grape seed extract at the same conditions. The specific aims were to determine:

2.1. The effectiveness of proanthocyanidins and gallic acid to counteract adipose dysfunction by measuring both plasma metabolites related to lipid and glucose homeostasis and the expression profile of several markers of visceral and subcutaneous adipose tissue functionality such as markers of inflammation and angiogenesis.

2.2. The capacity of proanthocyanidins and gallic acid to correct adipocyte hypertrophy linked to obesity and /or to induce hyperplasia by performing microscopic analyses of visceral and subcutaneous adipocytes.

2.3. The molecular mechanism by which proanthocyanidins and gallic acid prevent adipose tissue dysfunctionality, mainly focusing on adipogenesis and fatty acid oxidation at the expression level.

2.4. The sensitivity of visceral and subcutaneous adipose tissues to be modulated by proanthocyanidins or gallic acid.

2.5. The contribution of gallic acid and proanthocyanidins on the activity of the grape seed extract at remodelling adipose tissue.

3. To test the capacity of pharmacological doses of resveratrol to correct adipose tissue dysfunctionality related to obesity by remodelling adipocytes (Manuscript 3).

To fulfil this objective, rats were fed a cafeteria diet and once obesity was established, they were administered for three weeks with pharmacological doses of resveratrol (50, 100, 200 mg/kg body weight) maintaining the obesogenic diet. The specific aims were to determine:

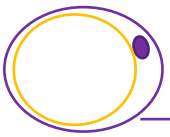
3.1. The effectiveness of pharmacological doses of resveratrol to counteract visceral adipose dysfunction by measuring both plasma metabolites related to lipid and glucose homeostasis and the expression of leptin in visceral adipose tissue.

3.2. The capacity of pharmacological doses of resveratrol to counteract adipocyte hypertrophy linked to obesity and /or to induce hyperplasia by performing microscopic analyses of visceral adipocytes.

3.3. The molecular mechanism by which a pharmacological dose of resveratrol improves adipose tissue functionality studding adipogenesis and browning at the expression level.

3.4. The effect of resveratrol on the expression of miRNAs, and their target genes, involved in the lipid metabolism in visceral adipose tissue and liver.

The research work carried out in this thesis has been supported by the grant AGL 2013-40707-R (AEI/FEDER, UE) from the Spanish Ministry of Economy and Competitiveness. The research work has been performed mainly in the Nutrigenomics Research Group laboratory of Universitat Rovira i Virgili with a personal grant FI-DGR 2014-16 from the Catalan Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR). An international research stay has been done in the Human and Animal Physiology group from Animal Sciences Department in Wageningen University (Netherlands) under the supervision of Prof. Dr. Ir. Jaap Keijer in order to get the International doctorate mention.



IV. RESULTS and DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
ADIPOSE TISSUE REMODELLING BY POLYPHENOLS IN OBESE RATS
Aïda Pascual Serrano

1. Preventive effect of proanthocyanidins in adipose tissue dysfunction in Obesity

1.1. Grape seed proanthocyanidins improve white adipose tissue expansion during diet induced obesity development in rats (Manuscript 1)

UNIVERSITAT ROVIRA I VIRGILI
ADIPOSE TISSUE REMODELLING BY POLYPHENOLS IN OBESE RATS
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Grape seed proanthocyanidins improve white adipose tissue expansion during diet-induced obesity development in rats

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Submitted

Abstract

Scope: The development of metabolic complication associated with obesity has been correlated with a failure of white adipose tissue (WAT) to expand. Our group has previously reported that a 12-week administration of grape seed proanthocyanidin extract (GSPE) together with an obesogenic diet mitigated the development of cardiometabolic complications in rats (Pons Z. et al., Br J Nutr 2017;117:200–8). Using the same cohort of animals, we aim to elucidate whether the prevention of cardiometabolic complications by proanthocyanidins is produced by a healthier expansion of visceral WAT and/or an induction of the browning of WAT.

Methods and results: adipocyte size and number in retroperitoneal WAT (rWAT) were determined by histological analyses, and the gene expression levels of markers of adipogenesis, browning and WAT functionality were quantified by qPCR. Long-term administration of GSPE together with an obesogenic diet expanded rWAT via an increase in the adipocyte number and via a preventive decrease in the adipocyte size in a dose-dependent manner. At the molecular level, GSPE seems to induce WAT adipogenesis through the upregulation of Pparγ in a Sirt1-dependent manner.

Conclusion: the healthier visceral WAT expansion induced by proanthocyanidins supplementation may explain the improvement of cardiometabolic risks associated with obesogenic diets.

***Abbreviations**: Adgre1, Adhesion G protein-coupled receptor E1; C/ebpβ, CCAAT/enhancer binding protein beta; Cpt1, carnitine palmitoyltransferase 1A; Fasn, fatty acid synthase; Gpdh, glycerol-3-phosphate dehydrogenase; GSPE, grape seed proanthocyanidin extract; Lpl, Lipoprotein lipase; Pgc1α, Pparγ coactivator 1 alpha; PPARγ, peroxisome proliferator activated receptor gamma; Prdm16, PR domain containing 16; rWAT, retroperitoneal white adipose tissue; Sirt1, Sirtuin 1; UCP1, Uncoupling protein 1.*

1. Introduction

In obesity, fat is excessively accumulated in white adipose tissue (WAT) under conditions of energy surplus or reduced energy expenditure. The prevalence of obesity is increasing, and this is dangerous since the risk for cardiovascular complications and type 2 diabetes among other diseases increases with obesity [1]. However, it seems that the total fat mass may not be the direct link between obesity and its associated diseases, since some obese individuals are relatively metabolically healthy[2]. In fact, the capacity of the WAT to expand to store the excess energy may prevent the development of metabolic complications in response to the surplus energy since it may prevent the lipids from targeting other organs[3]. However, WAT can only expand up to a certain threshold beyond which their capacity to store more fat is diminished, and this is also associated with metabolic complications [4]. In this regard, several of the obesity-related metabolic complications might be explained by WAT dysfunction as a consequence of an impaired capacity of the fat depots to store more energy [5].

With a continuous energy surplus, the expansion of WAT occurs first via the increase in adipocyte size (hypertrophy) and then via the increase in adipocyte number (hyperplasia) and/or hypertrophy[4]. Thus, adipocyte hypertrophy has been linked to an increased risk of developing metabolic diseases related to obesity, whereas a protective effect has been attributed to adipocyte hyperplasia against obesity-related metabolic alterations[5]. Hyperplasia is produced by the differentiation of pre-adipocytes (i.e., adipogenesis), and a cascade of transcription factors controls this process. Among these transcription factors, peroxisome proliferator–activated receptor (Pparγ) is considered the master regulator of adipogenesis, and coregulators such as the Pparγ-coactivator-1 alpha (Pgc1α) can in turn modulate its activity[6]. In fact, Pparγ controls the functionality of WAT by regulating not only WAT lipogenesis but also the browning of WAT[7].

Brown adipose tissue (BAT) is characterized by a large number of mitochondria and thermogenic capacity, and thus the remodeling from WAT to BAT, known as the browning process, also has beneficial effects on obesity-related disorders[8]. BAT uses the uncoupling protein 1 (UCP1) for its thermogenic function, and UCP1 is the main marker of brown adipocytes. Among others, the expression of Ucp1 gene is controlled by Pparγ, Pgc1α and PR domain containing 16 (Prdm16).

Proanthocyanidins are a class of flavonoid compounds that are present in many food and drinks such as fruits, vegetables and red wine, and thus they are very abundant in the human diet[9]. In particular, grape seeds are rich in this class of flavonoids. In fact, various studies using a grape seed proanthocyanidin extract (GSPE) have demonstrated its capacity to improve metabolic complications associated with obesity[9], including its ability to reduce blood pressure[10], act as a hypolipidemic agent[11] and improve inflammation[12] and insulin resistance. Specifically, previous studies from our research group provided evidence showing that the daily administration of GSPE (at doses of 25, 100 and 200 mg/kg per day) for 12 weeks together with a diet high in carbohydrates and fat (i.e., cafeteria diet or CAF diet) in rats can mitigate the increase in blood pressure and plasma lipids levels in response to the obesogenic diet. However, only with the dose of 200 mg GSPE /kg body weight (BW), a slight attenuation of the CAF diet-induced increase in BW was observed[13]. Therefore, by using the same cohort of animals, the objective of this study was to elucidate whether the GSPE-mediated attenuation of the development of obesity-associated cardiometabolic risk factors due to the prolonged feeding of the CAF diet is due to a healthier expansion of visceral WAT and/or the induction of the browning process of the visceral WAT.

2. Materials and Methods

2.1. Grape Seed Proanthocyanidin Extract

The GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The composition of the GSPE has been previously characterized by Margalef et al.[14] and is described in Supplementary Table 1.

2.2. Animal experimental procedure

In this study, we used the same cohort of animals as the previous study reported by Pons et al.[13] (License number 8867 from Generalitat de Catalunya). Briefly, six-week-old male Wistar rats (Charles River Laboratories, Barcelona, Spain) were randomly divided in five groups. The standard group (STD, n=10) fed a standard chow diet (Panlab A04, Panlab, Barcelona, Spain), the cafeteria control group fed a CAF diet (CAF, n=10) and three CAF groups supplemented with three

different doses of GSPE: 25 mg/kg BW (GSPE 25, n=10); 100 mg/kg BW (GSPE 100, n=10) or 200 mg/kg BW (GSPE 200, n=10). After 12 weeks animals were fasted for 6 hours and sacrificed. Blood from the saphenous vein was collected using heparin (Deltalab, Barcelona, Spain) as the anticoagulant. Plasma was obtained by centrifugation (1500 g, 20 min, 4°C), and the WATs were excised and immediately frozen in liquid nitrogen. Both plasma and tissues were stored at -80°C until further use.

Adiposity index was computed as the sum of the weights of mesenteric WAT (mWAT), retroperitoneal WAT (rWAT) and epididymal WAT (eWAT) depots.

2.3. Quantification of plasma parameters

The level of insulin and leptin in the plasma was measured by ELISA (ELISA kit EZRMI-13K and ELISA kit EZRL-83K, Millipore Ibérica, Madrid, Spain). Plasma triglycerides (TGs), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and glucose (GLU) were measured with enzymatic colorimetric kits (QCA, Barcelona, Spain). Non-HDL-C was calculated by subtracting HDL-C from the level of TC. Moreover, HDL-C/non-HDL-C and TC/HDL-C ratios were calculated using the individual values of each animal. HOMA-IR and QUICKI indexes were calculated from insulin and GLU plasma levels.

2.4. Adipose tissue morphology

Adipocyte size and number were determined by microscopic analyses in rWAT after histological staining with hematoxylin-eosin. The procedure and formulas used are described in supplementary material.

2.5. RNA extraction

Total RNA, including small RNAs, was extracted from the frozen rWATs by using TRIzol® reagent (Ambion, USA) according to the manufacturer's protocol. To isolate both total RNA and miRNA, isopropanol precipitation was performed overnight at -20°C instead of the 10 min at room temperature as recommended for the isolation of only mRNA. The quality of total RNA was checked with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.6. mRNA quantification by real-time qRT-PCR

Relative mRNA levels of Adhesion G protein-coupled receptor E1 (Adgre1), CCAAT/enhancer binding protein (C/EBP) beta (C/ebpβ), carnitine palmitoyltransferase 1A (Cpt1), fatty acid synthase (Fasn), glycerol-3-phosphate dehydrogenase (Gpdh), Leptin (Lep), Lipoprotein lipase (Lpl), Ucp1, Pgc1α, Pparγ, Prdm16 and Sirtuin1 (Sirt1) were analyzed by real-time PCR in the rWATs by using cyclophilin (Ppia) as the endogenous control. Total RNA was retrotranscribed using TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Madrid, Spain) and gene expression was evaluated with a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Barcelona, Spain) using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Barcelona, Spain) and gene-specific SYBR primers designed for each gene using FastPCR software (Supplementary Table 2). The results were normalized to Ppia. The amplification was performed according to the temperature steps of 95°C for 30 seconds followed by 40 cycles at 95°C for 5 seconds and 60°C for 20 seconds. The fold change in the level of mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method, where ΔCt=Ct mRNA−Ct Ppia and ΔΔCt=ΔCt treated samples−ΔCt untreated controls.

2.7. miRNA quantification by real-time qRT-PCR

A specific TaqMan probe (5'-UUUGGUCCCCUUCAACCAGCUG-3') was used for the analysis of microRNA-133a (002246, Applied Biosystems, Madrid, Spain). U87 small nuclear RNA (001712, Applied Biosystems, Madrid, Spain) was used as the endogenous control. Single-stranded cDNAs were synthesized by using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain). The reaction was performed at the following temperature cycles: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. Quantitative polymerase chain reaction (qPCR) was performed using the TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain) The amplification reaction was performed on an ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems, Madrid, Spain) at 95°C for 10 min followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min. Fold change in the miRNA level was calculated according to the equation $2^{-\Delta\Delta Ct}$, where ΔCt=Ct miR-133a−Ct U87 and ΔΔCt=ΔCt treated samples−ΔCt untreated controls.

2.8. Western blotting

Briefly, 200 mg of rWAT were homogenized in 200 µl of RIPA lysis buffer (15 mM Tris-HCl, 165 mM NaCl, 0.5% Na-deoxycholate, 1% Triton X-100 and 0.1% SDS) containing protease inhibitor cocktail (1:1000, Sigma-Aldrich, Madrid, Spain) and 1 mM of phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich 93482, Madrid, Spain). Ucp1 was quantified after Western blotting using Ucp1 (ab23841, Abcam, Cambridge, UK) and β-actin (A2066, Sigma, Madrid, Spain) primary monoclonal antibodies. The detailed procedure is described in supplementary material.

2.9. Statistical analyses

The results are reported as the means±SEM of 3 animal per group for histological analysis. For all other analyses 10 animals per group were used. Group means were compared using one-way analysis of variance (ANOVA) with IBM SPSS statistics 20.0 software (SPSS, Inc, Chicago, IL, USA). The comparisons were considered significant at $p \leq 0.05$. Pearson correlations were calculated using all the values obtained for all animals for each variable. The correlations were judged as significant when the two-tailed p-values were ≤0.05.

3. Results

3.1. Daily administration of GSPE with a CAF diet reduces plasma TG levels without affecting adiposity.

In this study, we used the same cohort of animals as the previous study reported by Pons et al.[13]. The study reported that the daily administration of GSPE with the CAF diet did not prevented the BW gain during the 12 weeks of experiment, although with 200 mg GSPE /kg BW, a tendency to avoid the obesogenic diet-induced increase in BW was observed by the end of the experiment[13]. Similarly, the daily administration of GSPE with the CAF diet did not prevent the alteration of the adiposity index of the animals compared to the CAF diet-fed rats without GSPE (Table 1). However, the CAF diet-induced rWAT accretion was slightly attenuated by the administration of 200 mg GSPE /kg BW

per day. Moreover, a similar effect was seen in the weight of subcutaneous WAT (sWAT) with 100 mg GSPE /kg BW.

Table 1. **Body weight and deposition of white adipose tissue in rats after 12 weeks of feeding a standard (STD) diet, a cafeteria (CAF) diet or a cafeteria diet with the daily administration of different doses of GSPE (CAF+GSPE)**

	STD	*CAF*	*CAF + GSPE 25 mg/kg BW*	*CAF + GSPE 100 mg/kg BW*	*CAF + GSPE 200 mg/kg BW*
Body Weight (g)	406.1 ± 12.5^{a}	514.0 ± 15.1^{b}	513.5 ± 15.3^{b}	501.3 ± 22.6^{b}	487.4 ± 24.3^{ab}
rWAT Weight (g)	11.94 ± 2.95^{a}	23.70 ± 2.31^{b}	26.02 ± 1.60^{b}	23.49 ± 2.70^{b}	22.71 ± 2.40^{ab}
eWAT Weight(g)	7.81 ± 0.90^{a}	22.60 ± 2.06^{b}	22.22 ± 1.88^{b}	21.92 ± 2.91^{b}	18.53 ± 1.98^{b}
mWAT Weight(g)	4.39 ± 0.29^{a}	10.83 ± 1.74^{b}	10.17 ± 1.13^{b}	9.68 ± 1.30^{b}	10.26 ± 1.37^{b}
sWAT Weight(g)	5.35 ± 0.49^{a}	14.25 ± 2.41^{b}	13.48 ± 1.85^{b}	12.20 ± 1.77^{ab}	13.14 ± 1.64^{b}
Adiposity index	5.64 ±0.74^{a}	10.97 ± 0.77^{b}	11.31 ± 0.57^{b}	10.22 ± 0.95^{b}	10.86 ± 0.71^{b}

The values are the means±SEM (n=10). Statistical analyses were performed using one-way ANOVA. Different letters indicate significant differences between groups of at least $p<0.05$.
Abbreviations: BW: Body weight; rWAT: retroperitoneal white adipose tissue; eWAT: epididymal white adipose tissue; mWAT: mesenteric white adipose tissue and sWAT: subcutaneous white adipose tissue.

Although the daily administration of GSPE together with the CAF diet for 12 weeks did not improve the adiposity index, it improved the plasmatic lipid profile as previously reported by Pons et al.[13] in the same cohort of animals. Specifically, the plasmatic TG levels of the rats fed a CAF diet for 12 weeks were

higher compared to the animals fed the STD diet, and when the obesogenic diet was supplemented with the daily administration of 25 or 200 mg GSPE /kg BW, the plasma TG levels decreased by 28% or 25%, respectively (Table 2). Moreover, the plasmatic GLU and leptin levels were also increased in the animals after 12 weeks of CAF diet compared to the STD diet-fed rats, but the daily administration of GSPE with the CAF diet did not change these parameters (Table 2).

Table 2. Plasma parameters in rats after 12 weeks of feeding a standard (STD) diet, a cafeteria (CAF) diet or a cafeteria diet with the daily administration of different doses of GSPE (CAF+GSPE).

Plasma parameters	*STD*	*CAF*	*CAF + GSPE 25 mg/kg BW*	*CAF + GSPE 100 mg/kg BW*	*CAF + GSPE 200 mg/kg BW*
GLU (mg/dL)	112.27 ± 3.76^{a}	137.19 ± 5.92^{b}	150.07 ±3.11^{b}	146.41 ± 8.19^{b}	139.12 ± 4.84^{b}
Insulin (ng/mL)	2.23 ± 0.28	3.98 ± 0.78	4.03± 0.58	3.86 ± 0.86	3.91 ± 0.54
HOMA-IR	15.52 ± 1.54	32.49 ± 8.15	32.98 ± 7.19	32.65 ± 7.69	29.06 ± 4.40
QUICKI	0.265 ± 0.003	0.251 ± 0.008	0.246 ± 0.005	0.250 ± 0.006	0.249 ± 0.004
Leptin (ng/mL)	11.49 ± 0.55^{a}	37.52 ± 3.54^{b}	40.28 ± 4.61^{b}	40.07 ± 4.38^{b}	42.62 ± 6.69^{b}
TG (mg/dL)	39.50 ± 5.44^{a}	89.97 ± 19.93^{b}	64.28 ± 7.96^{ab}	82.56± 10.65^{b}	67.27 ± 5.48^{ab}
TC (mg/dL)	96.75 ± 4.44	112.03 ± 6.29	98.48 ± 1.91	118.60 ± 7.03	107.62 ± 5.76
HDL-C	39.32 ± 4.45	20.22 ± 2.09	33.92 ± 5.77	33.21 ± 5.47	36.72 ± 5.03
Non-HDL-C	58.11 ± 10.72	83.62 ± 9.50	56.36 ± 7.10	93.22 ± 13.10	57.88 ± 5.24
HDL-C / non-HDL-C	0.85 ± 0.31	0.26 ± 0.05	0.80 ± 0.24	0.42 ± 0.17	0.67 ± 0.12
TC / HDL-C	2.85 ± 0.38	5.30 ± 0.65	2.72 ± 0.60	4.73 ± 0.78	2.69 ± 0.34

The values are the means±SEM (n=10). Statistical analyses were performed using one-way ANOVA with Scheffe's or Dunnett's T3 post hoc test when necessary. Different letters indicate the significant differences between groups of at least $p<0.05$.
Abbreviations: BW: Body weight; GLU: glucose; HOMA-IR: homeostasis model assessment-estimated insulin resistance, QUICKI: quantitative insulin sensitivity check index; TG: triglycerides; TC: total cholesterol; HDL-c: High-density lipoprotein cholesterol and non-HDL-c: All cholesterol not including the high-density lipoprotein cholesterol.

3.2. Daily administration of GSPE with a CAF diet prevents from the failure of the rWAT to expand

Histological images of the rWAT in the CAF diet-fed rats showed an increase in the size of adipocytes compared to those in the STD diet-fed rats. Nevertheless, the histological images of rWAT in the CAF diet-fed rats supplemented with GSPE were more similar those in the STD diet-fed rats (1A shows representative histological images from the 4 groups of animals).

A more detailed analysis of these images showed that the adipocyte area and volume of the rWAT were significantly higher when the animals were fed a CAF diet than the STD diet, even though the adipocyte number was not affected by the diet. This was further supported by the observation of the increase in the frequency of adipocytes with areas greater than 2800 μm^2 in the CAF diet-fed animals (Figure 1B-E). Additionally, the daily administration of GSPE together with the CAF diet prevented the increase in the area and volume of the rWAT adipocytes in a dose-dependent manner, resulting in a lower frequency of adipocytes with areas greater than 2800 μm^2 also in a dose-dependent manner, eventually reaching similar levels as those in the STD diet-fed rats at 200 mg GSPE /kg BW. Interestingly, the adipocyte number was increased when the rats were fed a CAF diet with the daily administration of GSPE for 12 weeks compared to the CAF diet-fed rats without GSPE. Particularly, at the dose of 200 mg GSPE/kg BW, the number of adipocytes showed an 82% increase compared with that observed with the CAF diet-fed rats without GSPE. Thus, when the rats were fed a CAF diet and daily supplemented with GSPE, the rWAT expansion was modified to increase hyperplasia and reduce hypertrophy compared to the rats without GSPE where only the adipocyte size increased but not the number.

Finally, we performed a correlation analysis of the adipocyte volume and number in rWAT with the BW, WATs weights and plasma parameters to test if the

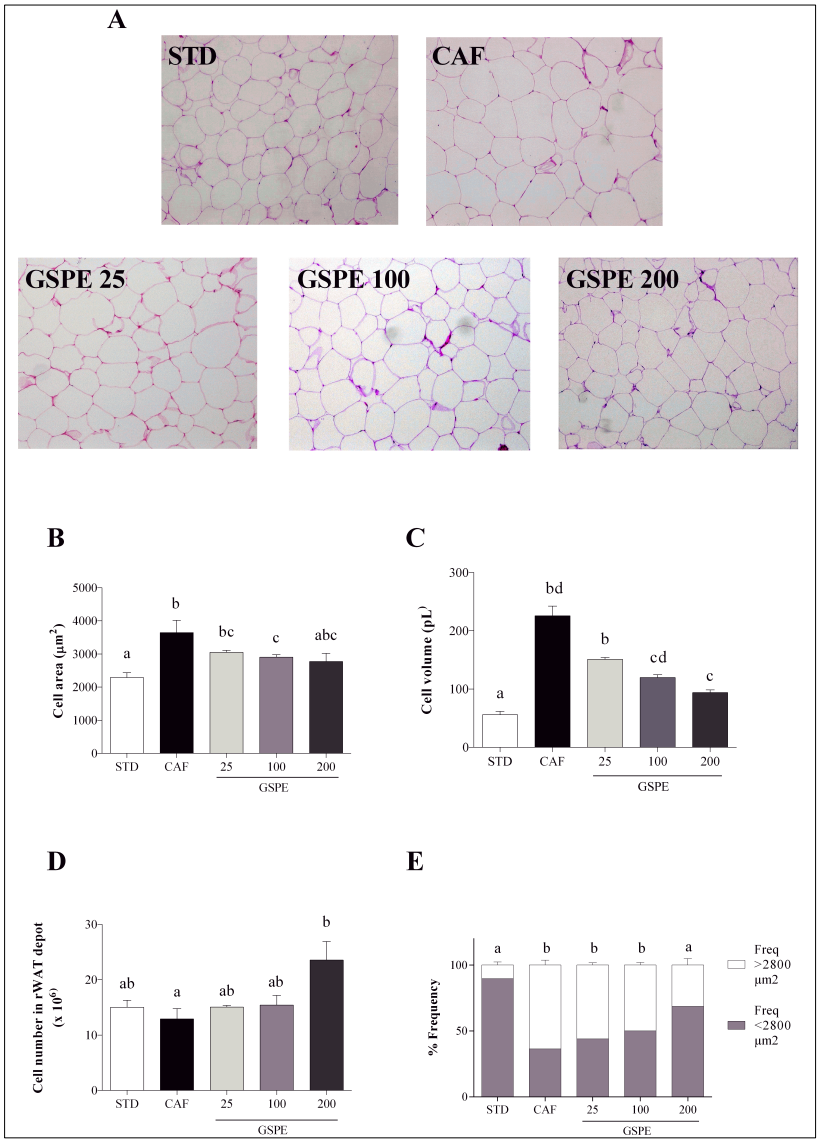

Figure 1. Effect of the long-term administration of a grape seed proanthocyanidin extract (GSPE) with an obesogenic diet on the size and number of visceral adipocytes. Rats were fed either a standard chow diet (STD group, n=10), a cafeteria diet (CAF group, n=10) or a cafeteria diet with daily administration of 25, 100 or 200 mg GSPE /kg BW (GSPE 25, GSPE 100 and GSPE 200, n=10 per group) for 12 weeks. (A) Representative light microscopy images of retroperitoneal white adipose tissue (rWAT) stained with hematoxylin and eosin from each group; (B) Adipocyte area measured form the representative light microscopy images from each group; (C) Adipocyte volume calculated from the adipocyte area; (D) Total adipocyte number extrapolated from the size of adipocytes and the weight of rWAT; (E) Frequency of adipocyte size calculated from the adipocyte area. The values are the means ± SEM of 5 fields per animal from 3 animals of each group. Different letters indicate significant differences between groups using one-way ANOVA. Significance was considered when $p \leq 0.05$.

morphological changes in the rWAT correlated with the improvement of dyslipidemia by GSPE. The results showed that the adipocyte volume was positively correlated with BW and eWAT weight and the adipocyte number was negatively correlated with the TC/HDL-c ratio (Supplementary Table 3).

3.3. Daily administration of GSPE with a CAF diet transcriptionally increases the differentiation of rWAT adipocytes

We also evaluated, at the gene expression level, whether the GSPE administration with the CAF diet can regulate adipocyte differentiation and energy homeostasis. For this, we quantified the mRNA levels of Pparγ, C/ebpβ and Sirt1 at the dose of 200 mg GSPE /kg BW since that was the dose that mostly increased hyperplasia and decreased hypertrophy. The nuclear receptor Pparγ, which is the master regulator of adipogenesis, was upregulated in the CAF diet-fed rats that were daily supplemented with 200 mg GSPE /kg BW compared with the CAF diet-fed rats without GSPE (Figure 2A). In contrast, C/ebpβ, which transactivates Pparγ during adipocyte differentiation[15], was not affected by GSPE (Figure 2B). Similarly, the mRNA level of Pgc1α, which coactivates Pparγ[16], was not altered by the administration of GSPE (Figure 2C). In concordance with the Pparγ upregulation by GSPE, Sirt1, which has been reported to repress Pparγ[17], was repressed by the administration of GSPE (Figure 2D).

As the administration of the highest dose of GSPE in the CAF diet-fed rats attenuated the increase in BW, we also evaluated the browning process of rWAT as a mechanism of the maintenance of the BW during the regulation of the energy balance by proanthocyanidins. The results showed that the daily administration of 200 mg GSPE /kg BW induced a substantial decrease on the level of Ucp1 mRNA compared to the CAF diet-fed rats without the proanthocyanidin administration. This decrease could also be appreciated at the protein level (Figure 2E), even though the differences were not statistically significant likely due to the high degree of variability among the animals. Moreover, the administration of GSPE decreased the expression of Prdm16 and increased the level of miR-133a, although the differences were not statistically significant (Figure 2F and 2G, respectively).

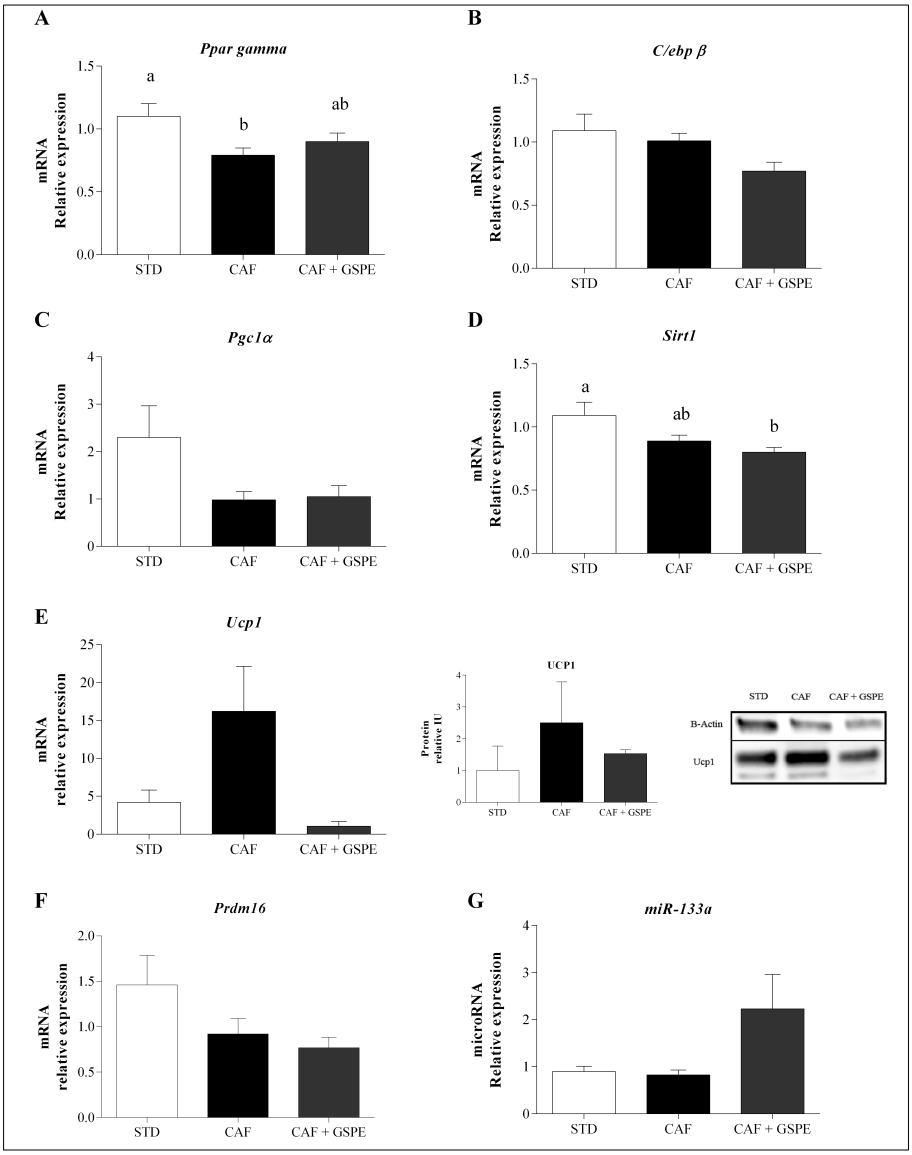

Figure 2. Effect of the long-term administration of a grape seed proanthocyanidin extract (GSPE) with an obesogenic diet on the expression of genes related to adipogenesis and browning in retroperitoneal white adipose tissue. Rats were fed either a standard chow diet (STD group, n=10), a cafeteria diet (CAF group n=10) or a cafeteria diet with daily administration of 200 mg GSPE /kg BW (CAF+GSPE, n=10) for 12 weeks. (A) Level of peroxisome proliferator activated receptor gamma (Pparγ) mRNA; (B) Level of CCAAT/enhancer binding protein (C/EBP) beta (C/ebpβ) mRNA; (C) Level of Pparγ coactivator 1 alpha (Pgc1α) mRNA; (D) Level of Sirtuin 1 (Sirt1) mRNA; (E) mRNA and protein levels of Uncoupling protein 1 (UCP1); (F) Level of PR domain containing 16 (Prdm16) mRNA; (G) microRNA-133a levels. The values are the means ± SEM. Statistical analyses were performed using the t-test. Different letters indicate significant differences between groups when p≤0.05.

3.4. Daily administration of GSPE with a CAF diet affect the rWAT functionality at the molecular level

Aside from the lipogenic modulation of rWAT by GSPE by the regulation of Pparγ, we further evaluated the functional capacity of the rWAT after 12 weeks administration of 200 mg GSPE /kg BW with the CAF diet. Since dysfunctional WAT is characterized by leptin resistance and immune cells infiltration among others, we analyzed the expression of leptin in the rWAT and found that was slightly increased in the CAF diet-fed animals supplemented with GSPE compared with the animals without GSPE (Figure 3A). Moreover, we demonstrated that the mRNA level of Adgre1, a marker of macrophage infiltration that is indicative of dysfunctional rWAT, was not altered by the administration of GSPE (Figure 3B).

We also studied some of the most important genes involved in lipid metabolism in WAT. Regarding the lipogenic markers, we studied the expression of Fasn, which is the principal enzyme involved the de novo synthesis of lipids, and Gpdh. Fasn was slightly downregulated in the CAF diet-fed animals supplemented with GSPE, although the reduction of the mRNA level of Fasn was not statistically significant (Figure 3C). However, the level of Gpdh mRNA was not modified by GSPE (Figure 3D). Similarly, other genes related to lipid metabolism in the rWAT such as Cpt1, which is the rate-limiting enzyme for fatty acid β-oxidation, or Lpl, which is the principal enzyme that hydrolyzes circulating TGs into free fatty acids and facilities their entry into adipocytes, were not altered with the administration of GSPE (Figure 3E and 3F, respectively).

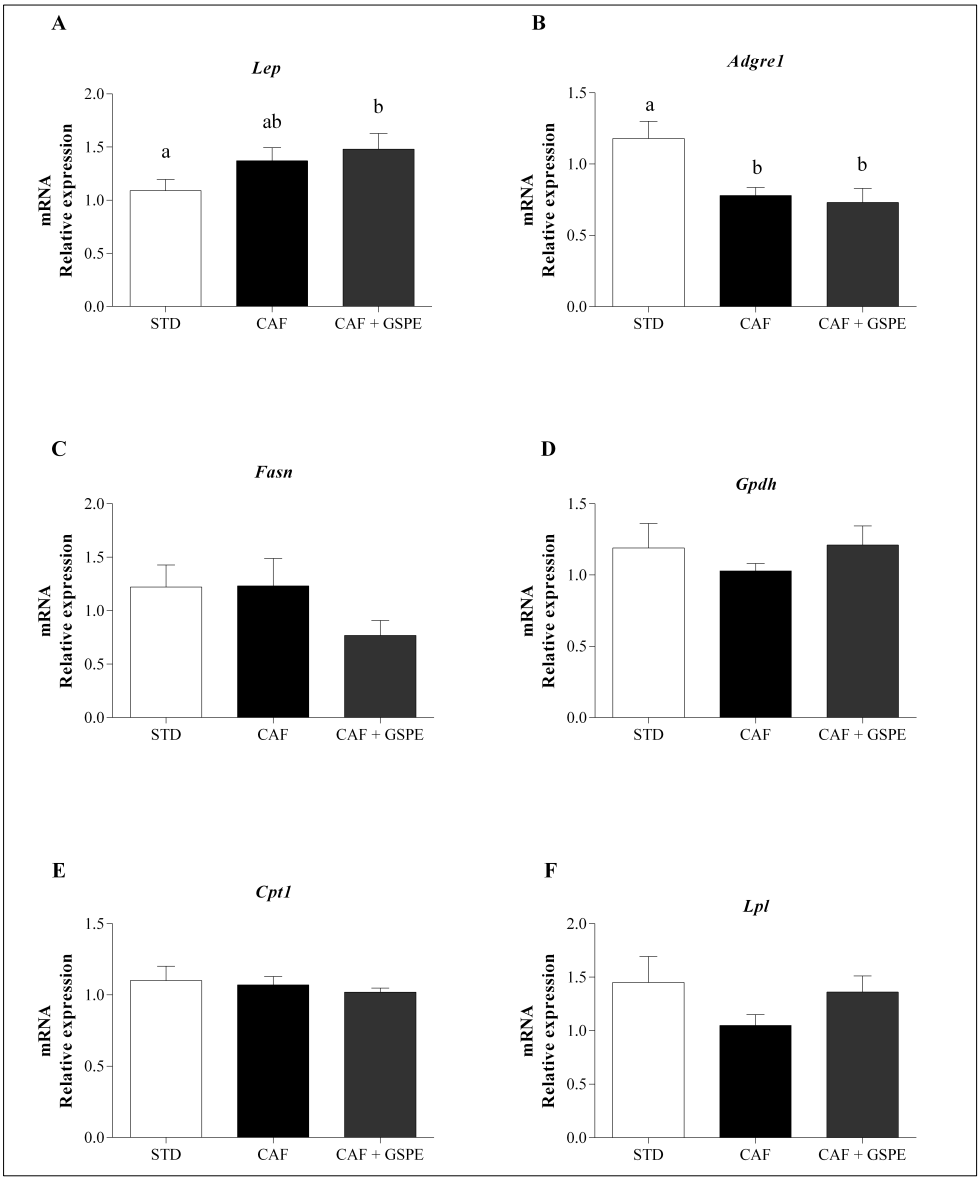

Figure 3. Effect of the long-term administration of a grape seed proanthocyanidin extract (GSPE) with an obesogenic diet on the expression of genes related to adipocyte function in retroperitoneal white adipose. Rats were fed either a standard chow diet (STD group, n=10), a cafeteria diet (CAF group, n=10) or CAF diet with daily administration of 200 mg GSPE /kg BW (CAF+GSPE group, n=10) for 12 weeks. (A) Level of Leptin (Lep) mRNA; (B) Level of the macrophage surface marker gene Adhesion G protein-coupled receptor E1 (Adgre1) mRNA; (C) Level of fatty acid synthase (Fasn) mRNA; (D) Level of glycerol-3-phosphate dehydrogenase (Gpdh) mRNA; (E) Level of carnitine palmitoyltransferase 1A (Cpt1) mRNA; (F) Level of Lipoprotein lipase (Lpl) mRNA. The values are the means ± SEM. Statistical analyses were performed using the t-test. Different letters indicate significant differences between groups when p≤0.05.

4. Discussion

Our research group had previously evaluated the progression of blood pressure, BW, waist parameter and plasma lipid levels in rats during 12 weeks of CAF diet together with the daily administration of different doses of GSPE (25, 100 and 200 mg/kg BW)[13]. The results demonstrated that the administration of GSPE with the CAF diet mitigated the obesogenic diet-induced hypertension and dyslipidemia in rats. However, only with the dose of 200 mg GSPE /kg BW, a slight protection against the CAF diet-induced BW gain was observed[13]. In this study, we used the same cohort of animals to study the effect of the CAF diet with or without GSPE at the end of the feeding period (after 12 weeks of dietary treatment). Our aim was to study whether the attenuation in the development of obesity-associated cardiometabolic risk factors by the long-term administration of GSPE together with a CAF diet is due to a healthier expansion of WAT that prevents the dysfunction of WAT and to evaluate the regulation of energy balance focusing on the browning process of this fat depot.

To this end, we first evaluated the metabolic damages induced in the rats by the 12 weeks of CAF diet and the preventive effect of this damage by the administration of GSPE. The prolonged feeding of CAF diet to the rats was seen to increase the GLU, leptin and TG levels, although the daily administration of GSPE only prevented the CAF diet-induced increase in TGs. Indeed, this metabolic damage induced by the obesogenic diet can be a consequence of WAT dysfunction leading to the inability to store the extra lipids from the diet[3]. Interestingly, the proanthocyanidin hypotriglyceridemic protective effect is in concordance with the preventive decrease in adipocyte hypertrophy in rWAT, since visceral adipocyte hypertrophy has been associated with dyslipidemia[18]. This is also in agreement with the well-defined hypolipidemic and cardioprotective effect of proanthocyanidins[9,11]. This study examined rWAT as a representative of visceral WAT because an excess of visceral fat appears to contribute more to the development of cardiovascular diseases than subcutaneous fat[19], and moreover, this tissue mass was slightly reduced after 12 weeks of CAF-diet with daily administration of 200 mg GSPE /kg BW.

The prevention of the increase in visceral adipocyte size and therefore the effect on adipocyte hypertrophy in visceral fat depots was also reported for other

polyphenols or polyphenol-rich extracts, such as isorhamnetin glycosides when they were administered with high-fat diet in mice[20]; resveratrol when it was administered with a high-fat and high-sugar diet in rhesus monkeys[21] and a blackcurrant anthocyanin-rich extract with high-fat and high-cholesterol diet in mice (in epididymal fat pad)[22]. However, to the best of our knowledge, the capacity of polyphenols or their extracts to modulate hyperplasia has not been previously reported.

Taken this together, this study demonstrated that the administration of GSPE with an obesogenic diet affected the rWAT expansion by increasing adipocyte hyperplasia and reducing hypertrophy, which might prevent the increase in plasma TG levels for the healthier expansion of rWAT, as hyperplasia is associated with the prevention of obesity-associated metabolic disorders. The increase in the adipocyte number in rWAT by preadipocyte differentiation with the supplementation of GSPE at the highest dose (200 mg/kg BW per day) is in concordance with the prevention of CAF diet-induced Pparγ downregulation. Pparγ is considered the master regulator of adipogenesis. However, GSPE has been reported to inhibit adipogenesis by the reduction of Pparγ mRNA levels[23]. This inhibition of adipogenesis was also observed with grape seed procyanidin B2[24]. Nevertheless, these studies were carried out in 3T3-L1 cells and not in animals. The differences between the *in vitro* and *in vivo* studies regarding adipogenesis may be because of differences in the compounds targeting the adipocytes. In fact, after the administration of GSPE in rats, flavanols are actively conjugated in the small intestine and liver and metabolized in the colon. Furthermore, after 2 h of administration of 250 mg GSPE /kg BW to the rats, flavanols and their metabolites were found to target mWAT[25]. Thus, the differences in the version of the molecules targeting the fat depots between the *in vivo* and *in vitro* studies could, at least in part, explain the differences in the results.

Sirt1, which is an NAD^+-dependent nuclear deacetylase, has been reported to repress Pparγ and thus adipocyte differentiation. Specifically, a downregulation of Sirt1 in 3T3-L3 cells resulted in an increase in the level of Pparγ without affecting the expression of C/ebp-β[17]. In concordance with the prevention of CAF diet-induced downregulation of Pparγ, GSPE also promoted the downregulation of

Sirt1 induced by the CAF diet. Moreover, the level of C/ebp-β was not affected by GSPE. To the best of our knowledge the effect of GSPE to Sirt1 mRNA levels has not been studied in adipocytes; however, it was reported to be upregulated in the livers of healthy rats that were supplemented with 5, 25 or 50 mg GSPE /kg BW for 21 days[26] and in the hypothalamus after 13 weeks of administration of 25 mg GSPE /kg BW with a CAF diet[27]. Therefore, the differential regulation of Sirt1 expression by GSPE in rWAT could have resulted from the different experimental conditions, such as the GSPE dose used, or from the different metabolites targeting these different tissues[25].

The CAF diet-induced increase in BW and rWAT was attenuated by the highest GSPE dose without an effect on food intake as previously reported by Pons et al.[13]. Moreover, Pparγ is also known to be related with the regulation of the browning process[28], which may prevent obesity-related disorders. Therefore, we further studied if the administration of GSPE with a CAF diet to the rats could regulate the browning of rWAT at the molecular level, thereby regulating the energy balance and BW retention. The remodeling of white-to-brown AT induces the appearance of brown-like adipocytes in the WAT depots, which express UCP1 and increase energy expenditure[29]. This in turn may prevent the occurrence of obesity and related disorders. Ucp1 mRNA and protein levels were not affected by the administration of GSPE, although the high interindividual variability may explain this lack of difference. In fact, other authors have previously shown that the expression of Ucp1 in rWAT is highly variable within groups that were evaluated after dietary treatment[30]. Aside from Ucp1, Prdm16 and Pgc1α are crucial transcriptional regulators of the browning process[31]. Furthermore, Pgc1α, which coactivates Pparγ, is involved in the induction of mitochondrial biogenesis and regulates thermogenesis and oxidative metabolism[32]. Additionally, the expression of Prdm16 is regulated by miR-133[33]. As proanthocyanidins are known to modulate the expression of miRNAs[9,34], we also studied whether GSPE can regulate miR-133. However, Prdm16, miR-133 and Pgc1α were not modulated by the administration of GSPE, pointing to the inability of this dietary treatment to induce rWAT browning or increase energy expenditure at the molecular level. Nevertheless, it is important to consider the high interindividual variability in the analysis of expression of the markers of the browning process. Thus, we cannot provide a conclusive statement regarding the effect of GSPE on rWAT browning.

Nonetheless, other dietary polyphenols have been reported to induce the browning of WAT. Along those lines, a combination of resveratrol with quercetin induced browning of rWAT in obesogenic diet-fed rats[35]. In that study, resveratrol alone and the combination of both polyphenols increased the protein expression of UCP1. Additionally, black tea, which contains catechins and theaflavins, has been shown to increase the levels of UCP1 mWAT[36].

It has been suggested that the failure of WAT to store extra energy affects some of the key factors involved in lipogenesis[4]. Pparγ is also a lipogenic regulator, and its activation in WAT is known to increase the storage of extra fatty acids[37]. Moreover, regarding WAT lipogenesis, although GSPE did not affect the expression of Gpdh, it may slightly mitigate the de novo synthesis of lipids as the CAF diet-induced increase in Fasn expression was slightly diminished, indicating a minor reduction in the lipogenic activity of rWAT pointing to the decrease in TG accumulation of the adipocytes. However, the expression of markers of lipid mobilization and fatty acid β-oxidation in the adipocytes were not affected by the administration of GSPE as seen by the lack of change in Lpl and Cpt1, respectively. Other polyphenols have been shown to downregulate FAS in adipocytes[38]. For instance, resveratrol has been reported to reverse the high-fat diet-induced upregulation in FASN in mouse eWAT[39].

These results suggest that the preventive effect of proanthocyanidins on cardiometabolic risk factors produced by an obesogenic diet could be consequence of the inhibition of the failure of visceral WATs, by increasing adipocyte hyperplasia and reducing adipocyte hypertrophy in a dose dependent manner. Although more studies in humans are necessary, the administration of proanthocyanidins in individuals consuming a high-fat and high-carbohydrate diet might prevent the development of obesity-related pathologies by improving WAT expansion.

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The authors declare no conflict of interest.

Author contributions

Conceived and designed the experiments: APS CB AAA. Performed the experiments: APS. Analyzed the data: APS CB MS AAA. Wrote the paper: APS CB AAA.

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ADIPOSE TISSUE REMODELLING BY POLYPHENOLS IN OBESE RATS
Aïda Pascual Serrano

SUPPLEMENTARY MATERIAL

Supplementary methods

Adipose tissue morphology

Small pieces of frozen rWAT (-80°C) were sent to ELDINE Patología (Tarragona, Spain) where they were thawed and fixed in 4% formaldehyde. After 24 hours of fixation, successive dehydration (alcohol/ethanol 70%, 96% and 100%; plus xylol/Dimethyl benzene) and paraffin infiltration and immersion at 52°C (Citadel 2000. Thermo Scientific) were performed. The paraffin blocks were subsequently cut into successive 2 µ-thick sections (Microm HM 355S, Thermo Scientific). The sections were deposited on slides (JP Selecta Paraffin Bath) and subjected to automated staining (Varistain Gemini, Shandon, Thermo) [1]. Images of the adipose tissue sections were acquired with AxioVision Zeiss Imaging software (Carl Zeiss Iberia, S.L, Madrid, Spain). Finally, the images taken at 20x were stored and analyzed with Adiposoft software (CIMA, University of Navarra, Spain) to quantify the adipocyte number and area. Five fields per sample and three samples from each group of animals (STD, CAF, GSPE 25, GSPE 100 and GSPE 200) were measured. The area was calculated from the average cell area in all the measured fields. Total number of cells in the rWAT fat depots was calculated from the values of fat cell volumes.

Fat cell volume was obtained for each captured field by using the formula $\frac{\pi}{6}x\left[3\sigma^2\times\bar{d}+\bar{d}^5\right]^2$ (where d is the mean diameter of 100 measured cells in the field and σ is the standard deviation of the diameter). Then, the fat cell density was applied (0.92 g/mL) to determine the fat cell weight[3]. Finally, the total number of fat cells in the whole rWAT depot of each animal was determined by dividing the total weight of the fat depot by the mean cell weight from all the captured fields.

Protein extraction and Western blotting

Briefly, 200 mg of rWAT were homogenized in 200 µl of RIPA lysis buffer (15 mM Tris-HCl, 165 mM NaCl, 0.5% Na-deoxycholate, 1% Triton X-100 and 0.1% SDS) containing protease inhibitor cocktail (1:1000, Sigma-Aldrich, Madrid, Spain)

and 1 mM of phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich 93482, Madrid, Spain).

Total protein was determined with the BCA kit (Thermo Scientific, 23227, Barcelona, Spain). The samples were then prepared for Western blotting with the addition of sample buffer (0.5 M Tris-HCl (pH 6.8), 10% glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 0.05% bromophenol blue). The protein samples were boiled for 5 min and 40 µg of protein was loaded and separated on a 10% SDS-polyacrylamide gel made with the TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad Laboratories, Barcelona, Spain). The samples were then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, 162-017, Barcelona, Spain) using a Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, 1658029, Barcelona, Spain) and blocked with 5% (w/v) non-fat milk in TTBS (Tris-buffered saline plus 0.5% (v/v) Tween-20) for 1 h. The membranes were incubated at 4°C overnight with primary monoclonal antibodies directed against Ucp1 (ab23841, Abcam, Cambridge, UK) and β-actin (A2066, Sigma, Madrid, Spain) at 1:1000 dilution in the blocking solution. The membranes were then washed in TTBS (Tris-buffered saline including Tween-20) and incubated with a peroxidase-conjugated monoclonal anti-rabbit secondary antibody (Sigma-Aldrich, A1949, Madrid, Spain) at 1:10000 dilution for 1.5 hours at room temperature. The membranes were then washed in TTBS followed by a single wash in TBS (Tris-buffered saline). Chemiluminescence reaction was carried out using the ECL plus kit (Amersham Biosciences, GE Healthcare, RPN2132, Barcelona, Spain) for protein detection. Images were obtained with the GBOX Chemi XL 1.4 image system (Syngene, UK). Bands were quantified with ImageJ software (NIH, USA). The results were expressed as the relative intensity UCP1/β-actin, where the signal for β-actin was used as the loading control.

Supplementary Tables

Supplementary Table 1. Main polyphenols of the grape seed proanthocyanidin extract (GSPE).

Compound	Concentration (mg/g)
Gallic acid	31.07 ± 0.08
Protocatechuic acid	1.34 ± 0.02
Vanillic acid	0.77 ± 0.04
Proanthocyanidin dimer B2	33.24 ± 1.39
Proanthocyanidin dimer B1	88.80 ± 3.46
Proanthocyanidin dimer B3	46.09 ± 2.07
Catechin	121.32 ± 3.41
Epicatechin	93.44 ± 4.27
Dimer gallate	8.86 ± 0.14
Epicatechin gallate	21.24 ± 1.08
Epigallocatechin gallate	0.03 ± 0.00
Epigallocatechin	0.27 ± 0.03
Proanthocyanidin trimer	4.90 ± 0.47
Proanthocyanidin tetramer	0.05 ± 0.01

Adapted from Margalef *et al* [4]. The results are expressed as mg of phenolic compound/g of GSPE on a wet basis as the mean ± SD (n=3).

Supplementary Table 2. Primer sequences used for real time qPCR analysis.

Gene	Primer sequences (5' - 3')	Product size (bp)	Gen Bank accession no/reference
Adgre1	CTTTGGCTATGGGCTCCCAGTC GCAAGGAGGGCAGAGTTGATCGTG	165	NM_001007557.1
Cpt1	TAT CGT CGC ACA TTA GAC C CAT CTA TGA CCT CCT GGC A	751	NM_031559.2
C/EBPβ	CCA CGA CTT CCT TTC CGA CC CGT AGT CGG ACG GCT TCT TG	71	NM_024125.4
Fasn	TCC CAG GTC TTG CCG TGC GCG GAT GCC TAG GAT GTG TGC	260	Sawano T. *et al.* [5]
Gpdh	CCC TTC CTC CAG GCT ACT CT GAG CTC GGA AAG GTC ACA CA	138	NM_022215.2
Lep	CAT TTC ACA CAC GCA GTC GG GCA AGC TGG TGA GGA TCT GT	137	NM_013076.3
Lpl	ACT GGT GGG ACA GGA TGT GG CCG TTC TGC ATA CTC AAA GTT AGG	196	Kroupa O. *et al.* [6]
Pgc1α	TGC CCC TGC CAG TCA CAG GA GCT CAG CCG AGG ACA CGA GG	177	Distel E. *et al.* [7]
Pparγ	AGG ATT CAT GAC CAG GGA GTT AGC AAA CTC AAA CTT AGC CTC CAT	79	Dovinová I. *et al.* [8]
Ppia	CTT CGA GCT GTT TGC AGA CAA AAG TCA CCA CCC TGG CAC ATG	138	NM_017101.1
Prdm16	GTT CTG CGT GGA TGC CAA TC TGG CGA GGT TTT GGT CAT CA	89	XM_008764418.1
Sirt1	TTG GCA CCC ATC CTC GAA ACA GAA ACC CCA GCT CCA	217	XM_006223877.1
Ucp1	CGA GCC AAG ATG GTG AGT TCG ACA GTG ATG GTC CCT AAG ACA CCT	200	NM_012682.2

Abbreviations: Adgre1: Adhesion G protein-coupled receptor E1; Cpt1: carnitine palmitoyl transferase I; C/EBPβ: CCAAT/enhancer binding protein beta; Fasn: fatty acid synthase; Gpdh: glycerol-3-phosphate dehydrogenase; Lep: leptin; Lpl: lipoprotein lipase; Pgc1α: PPARG coactivator 1 alpha; Pparγ: peroxisome proliferator-activated receptor gamma; Ppia: peptidylpropyl isomerase A (Cyclophilin A); Prdm16: PR domain containing 16; Sirt1: Sirtuin 1; Ucp1: uncoupling protein 1.

Supplementary Table 3. Correlation analysis of the different parameters analyzed in the study with the rWAT adipocyte volume and number of rats fed during 12 weeks with a standard (STD), a cafeteria (CAF) diet or a cafeteria diet with the daily administration of 25, 100 or 200 mg GSPE /Kg body weight.

	Adipocyte volume		**Adipocyte number (x10^6)**	
Variables	*Correlation coefficient*	*p value*	*Correlation coefficient*	*p value*
Adipocyte volume			-0.431	0.108
Adipocyte number (x10^6)	-0.431	0.108		
Body weight	**0.554**	**0.040**	-0.030	0.918
rWAT weight	0.330	0.270	0.126	0.681
eWAT weight	**0.558**	**0.038**	-0.171	0.559
mWAT weight	0.259	0.392	0.407	0.167
sWAT weight	0.312	0.277	0.402	0.154
Adiposity index	0.473	0.102	0.136	0.657
GLU (mg/dL)	0.312	0.299	-0.258	0.394
Insulin (ng/mL)	0.306	0.287	0.018	0.951
Leptin (ng/mL)	-0.147	0.665	0.270	0.421
HOMA-IR	0.211	0.489	-0.177	0.563
QUICKI	-0.125	0.684	0.047	0.878
TG	0.235	0.461	-0.125	0.700
TC	0.222	0.511	-0.080	0.815
HDL-c	-0.292	0.445	0.349	0.357
Non-HDL-c	0.149	0.751	-0.676	0.095
HDL/non-HDL-c	-0.541	0.210	0.696	0.082
TC / HDL-c	0.463	0.296	**-0.758**	**0.048**

Correlations were calculated using all values obtained for all animals for each variable. The table shows the correlation coefficient which illustrate the level of correlation between variables between 0 (no correlation) and 1 (maximum correlation) level. Bold numbers indicate significant correlations ($p \leq 0.05$).

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2. Capacity of dietary polyphenols to counteract adipose tissue dysfunction by remodelling adipocytes in obesity

2.1. Grape seed proanthocyanidin supplementation reduces adipocyte size and increases adipocyte number in obese rats (Manuscript 2)

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Grape seed proanthocyanidin supplementation reduces adipocyte size and increases adipocyte number in obese rats

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Running title: GSPE remodels adipose tissue

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ABSTRACT

Objectives: White adipose tissue (WAT) expands through hypertrophy (increased adipocyte size) and/or hyperplasia (increased adipocyte number). Hypertrophy has been associated with insulin resistance and dyslipidemia independently of body composition and fat distribution. In contrast, hyperplasia protects against metabolic alterations. Proanthocyanidins, which are the most abundant flavonoids in the human diet, improve metabolic disturbances associated with diet-induced obesity without reducing body weight or adiposity. The aim of this study was to determine whether grape seed proanthocyanidin extract (GSPE) can modulate WAT expandability. Because GSPE also contains gallic acid (GA), we also studied the capacity of GA to remodel WAT.

Design: Male Wistar rats were fed a standard chow diet (n=6) or a cafeteria diet (CAF) for 13 weeks. After 8 weeks, the CAF-fed animals were supplemented with 25 mg GSPE/kg body weight (n=6), 7 mg GA/kg body weight (n=6) or the vehicle (n=6) for 3 weeks. Histological analyses were performed in the retroperitoneal (rWAT) and inguinal (iWAT) WAT to determine adipocyte size and number. Specific markers for adipogenesis and WAT functionality were analyzed in rWAT using quantitative RT-PCR.

Results: GSPE or GA supplementation did not reduce weight gain or reverse and adiposity. However, GSPE reduced adipocyte size significantly in rWAT and moderately in iWAT and tripled the adipocyte number in rWAT. GA slightly reduced adipocyte size in rWAT and iWAT and doubled the adipocyte number in both WATs. In accordance with this adipogenic activity, Pref-1 and PPARγ tended to be overexpressed in rWAT of rats supplemented with GSPE. Moreover, GSPE supplementation increased Plin1 and Fabp4 expression and restored adiponectin expression completely, indicating a better functionality of visceral WAT.

Conclusions: GSPE supplementation has anti-hypertrophic and hyperplasic activities in rats with established obesity, mainly in visceral WAT inducing a healthier expansion of WAT to match the surplus energy provided by the cafeteria diet.

INTRODUCTION

Obesity is a risk factor for many metabolic diseases, such as dyslipidemia, hypertension, type 2 diabetes and cardiovascular disease[1]. It has been assumed that the prevalence of metabolic diseases increases with white adipose tissue (WAT) accretion. However, some obese individuals do not display metabolic alterations and are referred to as metabolically healthy obese[2]. Therefore, new theories linking obesity and metabolic diseases have been proposed. One theory suggests that metabolic disorders appear when the storage capacity of WAT is exceeded, thereby generating dysfunctional adipose tissue[3]. This phenomenon is even called WAT failure by some authors[4]. Thus, the expansion of WAT to match the surplus energy may confer metabolic benefits despite making individuals more obese.

WAT expansion occurs by hypertrophy (increase in adipocyte size by lipid accumulation in adipocytes) and/or hyperplasia (increase in adipocyte number by preadipocyte differentiation, i.e., adipogenesis). Several studies have shown that adipocyte size is an independent predictor of metabolic diseases, and adipocyte hypertrophy has been associated with insulin resistance[5] and dyslipidemia[6] independently of body composition and fat distribution. In fact, hypertrophic adipocytes have greater capacity to attract inflammatory cells[7] and are more lipolytic and resistant to insulin action than are small adipocytes[8]. Moreover, the capacity of adipocytes to store and mobilize lipids is disturbed in dysfunctional WAT. In this regard, a reduced expression of peroxisome proliferator-activated receptor γ (PPARγ) and fatty acid synthase (FASN) has been observed in obese humans with insulin resistance[4]. Thus, it is assumed that hyperplasia protects against metabolic alterations, whereas adipocyte hypertrophy results in metabolic dysfunctions. Consequently, adipogenesis is a key factor in preserving suitable WAT expandability, and several studies have shown that obese subjects exhibit lower adipogenic potential[4].

Hypoxia is also associated with impaired WAT functionality; thus, WAT expansion requires angiogenesis. Vascular endothelial growth factors (VEGFs) promote angiogenesis, and the expression of VEGF-A in WAT is higher in obese subjects with low insulin resistance than in those with insulin resistance[4]. In addition, mice overexpressing VEGF-A in WAT have an increased number and size

of blood vessels and are consequently protected against hypoxia and insulin resistance induced by a high-fat diet[9].

Proanthocyanidins, which are flavonoids that are very abundant in the human diet, have been defined as healthy bioactive compounds[10]. Several studies have reported many beneficial effects of grape-seed proanthocyanidin extract (GSPE) on various obesity-associated diseases, such as insulin resistance[11], dyslipidaemia[12], hypertension[13], inflammation[14] and leptin resistance[15]. Interestingly, these beneficial effects of GSPE have been observed without significant reductions in body weight and adiposity. Thus, the objective of this study was to determine whether GSPE can improve expandability in retroperitoneal (rWAT) and inguinal (iWAT) adipose tissues in the case of obesity and thereby avoid metabolic diseases associated with high-fat diet-induced obesity. In addition to proanthocyanidins, GSPE also contains gallic acid (GA). Thus, the objective of this work was further extended to investigate the contribution of GA to the capacity of GSPE to remodel WAT.

MATERIALS AND METHODS

Grape seed proanthocyanidin extract and gallic acid

GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The composition of the GSPE was previously characterized by Margalef et al.[16] and is described in Supplementary Table 1. Gallic acid (98.5% purity) was purchased from Sigma-Aldrich (G7384, Madrid, Spain).

Animal experimental procedure

The investigation was carried out in accordance with the ethical standards and the Declaration of Helsinki and was approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 7959 by Generalitat de
Catalunya).

Five-week-old male Wistar rats were purchased from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22°C with a

light/dark period of 12 h (light from 8:00 am to 20:00 pm). Animals were housed for one week and fed chow diet for adaptation. The animals were then divided into four groups randomly: the standard group (STD, n=6) was fed a standard chow diet (STD Panlab A04, Panlab, Barcelona, Spain) and tap water *ad libitum*, and all other groups were fed a cafeteria diet (CAF) consisting of sausage, bacon, biscuits with paté, cheese, ensaïmada (sweetened pastry), carrots and sweetened milk (20% sucrose w/v) in addition to the standard chow diet. The composition of the cafeteria diet was 14% protein, 35% fat, and 51% carbohydrates. This highly palatable diet is able to induce voluntary hyperphagia. CAF was freshly provided to the animals daily, and animals could choose and eat *ad libitum*. After 8 weeks, CAF-fed animals were divided into three new groups: GSPE group (GSPE, n=6), which received a supplementation of CAF diet with an oral dose of 25 mg GSPE/kg body weight in sweetened milk; GA group (GA, n=6), which received an oral dose of 7 mg GA/kg body weight in sweetened milk; and CAF group, which received sweetened milk alone. All groups received the compounds or the vehicle throughout the remainder of the experiment. After three weeks, the rats were fasted for 3 hours after the oral dose and then sacrificed by live decapitation. Total blood was collected using heparin (DeltaLab, Barcelona, Spain) as an anticoagulant. Plasma was obtained by centrifugation (1500 *g*, 15 minutes, 4°C), and all adipose tissue depots were excised and immediately frozen in liquid nitrogen. Both plasma and tissues were stored at -80°C until further use.

The adiposity index was computed for each animal as the sum of the mesenteric, retroperitoneal and epididymal white adipose tissue depot weights expressed as a percentage of the total body weight.

Quantification of plasma parameters

The plasma levels of insulin, adiponectin and leptin were measured using an immunometric sandwich enzyme-linked immunosorbent assay (ELISA) using a rat/mouse insulin ELISA kit (EZRMI-13K), rat leptin ELISA kit (EZRL-83K) and rat adiponectin ELISA kit (EZRADP-62K) purchased from Millipore Ibérica (Madrid, Spain). Plasma samples were diluted, and immunoassays were performed in duplicate according to the manufacturer's protocols. Plasma triglycerides (TGs), total cholesterol (TC), HDL-cholesterol (HDL-C) and glucose were measured with enzymatic colorimetric kits following the manufacturer's protocols (QCA,

Barcelona, Spain). Plasma NEFAs were analyzed with the enzymatic colorimetric HR NEFA series kit (Wako, CA, USA).

Adipose tissue morphology

Small pieces of frozen rWAT and iWAT (-80°C) were sent to ELDINE Patología (Tarragona, Spain), where they were thawed and fixed in 4% diluted formaldehyde. After 24 hours of fixation, tissues underwent successive dehydration (Alcohol / Ethanol 70%, 96% and 100%; plus xylol / Dimethyl benzene) and paraffin infiltration and immersion at 52°C (Citadel 2000. Thermo Scientific, HistoStar). The paraffin blocks were subsequently cut into successive 2 µ-thick sections (Microm HM 355S. Thermo Scientific). The sections were deposited on slides (JP Selecta Paraffin Bath) and subjected to automated hematoxylin-eosin staining (Varistain Gemini. Shandom. Thermo)[17]. Images of the adipose sections were acquired using AxioVision Zeiss Imaging software (Carl Zeiss Iberia, S.L, Madrid, Spain). Finally, the images captured at 20x magnification were stored and analyzed with the Adiposoft software (CIMA, University of Navarra, Spain) to quantify adipocyte number and areas. Five fields per sample and three samples from each group (STD, CAF, GSPE and GA) were measured. The area was calculated from the average value of the cell area in all measured fields. The total cell number in the rWAT and iWAT fat depots was calculated from the fat cell volume.

Fat cell volume was obtained for each captured field using the formula $\frac{\pi}{6} x\left[3\sigma^2 \times \bar{d} + \bar{d}^5\right]$[18] (where d is the mean diameter of 100 measured cells in the field, and σ is the standard deviation of the diameter). Then, fat cell density was applied (0.92g/mL) to determinate fat cell weight. Finally, the total fat cell number in the whole rWAT and iWAT depot of each animal was determined by dividing the total fat depot weight by the mean cell weight of all captured fields.

RNA extraction and mRNA quantification by real-time qRT-PCR

Total RNA was isolated from the frozen rWAT and iWAT using trizol reagent (Ambion, MA, USA) according to the manufacturer's protocol. The quality of total RNA was checked using a NanoDrop 1000 Spectrophotometer (Thermo Scientific Wilmington, DE, USA).

Relative mRNA levels of Perilipin 1 (Plin1), Fatty acid binding protein 4 (Fabp4), Peroxisome proliferator-activated receptor gamma (Pparγ), Fatty acid synthase (Fasn), Wnt family member 10 Beta (Wnt10β), Collagen type VI alpha 2 chain (Col6A2), Vascular endothelial growth factor A (Vegfa), Leptin, Uncoupling protein 1 (Ucp1), Interleukin 6 (IL-6), Tumor necrosis factor alpha (TNFα), Adiponectin (Adipoq), Preadipocyte factor-1 (Pref-1) and Adhesion G protein-coupled receptor E1 (Adgre1) were analyzed by real-time PCR in rWAT using cyclophilin (Ppia) as the endogenous control.

Total RNA was reverse transcribed using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, MA, USA) according to the manufacturer's protocol. Gene expression was evaluated with the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Barcelona, Spain) using the SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Barcelona, Spain) and gene-specific SYBR primers designed for each gene using the FastPCR software (Supplementary Table 2). The results were normalized to PPIA. Amplification was performed following the temperature steps of 95°C for 30 seconds followed by 40 cycles at 95°C for 5 seconds and 60°C for 5 seconds. The fold-change in the mRNA level was calculated in the log 2 scale using the equation $2^{-\Delta\Delta Ct}$ (where ΔCt = Ct mRNA − Ct Ppia and ΔΔCt = ΔCt treated samples − ΔCt untreated controls).

Statistical analysis

The results are reported as the mean ± SEM of 6 animals per group for mRNA levels and as the mean ± SEM of 3 animals per group for histology Adiposoft analysis. Group means were compared using one-way analysis of variance (ANOVA) with IBM SPSS statistics 20.0 software (SPSS, Inc, Chicago, IL, USA). The comparisons were considered significant at $P \leq 0.05$.

RESULTS

GSPE or GA supplementation did not reduce body weight and adiposity but improved hyperglycemia and dyslipidemia in cafeteria diet-fed rats

GSPE or GA supplementation for 3 weeks did not reduce body weight or reverse the subcutaneous and visceral fat-pad accretion induced by the cafeteria diet (Table 1). However, GSPE and GA supplementations were effective in modulating the plasma level of hormones and metabolites related to glucose and lipid homeostasis (Table 2). Specifically, GSPE and GA reduced plasma TGs, TC and non-HDL-C levels by more than 20%. This resulted in a more than 20% reduction of the atherogenic index TC/HDL-C in the animals supplemented with GSPE. Moreover, GSPE and GA reduced the plasma glucose levels by 11% and 21% and insulin levels by 27% and 23%, respectively. Thus, GSPE or GA supplementation improved the insulin resistance indexes HOMA-IR, QUICKI and R-QUICKI. However, the adiponectin and leptin levels were not affected by GSPE or GA administration.

Table 1. Effects of grape seed proanthocyanidins (GSPE) or gallic acid (GA) supplementation on body weight and fat accretion in obese rats.

	STD	*CAF*	*CAF +GSPE*	*CAF + GA*
Body Weight (g)	438.5 ± 17.02^{a}	518.4 ± 14.66^{b}	538.7 ± 45.48^{b}	521.6 ± 19.21^{b}
iWAT Weight (g)	1.00 ± 0.12^{a}	5.49 ± 0.71^{b}	6.63 ± 2.18^{b}	5.13 ± 0.71^{b}
rWAT Weight (g)	3.54 ± 0.08^{a}	9.69 ± 1.51^{b}	9.22 ± 1.69^{b}	12,53 ± 2.10^{b}
eWAT Weight (g)	8.99 ± 0.95^{a}	18.64 ± 1.42^{b}	18.60 ± 3.27^{b}	24.11 ± 2.43^{b}
Adiposity index	2.95 ± 0.10^{a}	6.35 ± 0.61^{b}	6.20 ± 0.76^{b}	7.72 ± 0.68^{b}

Rats were fed a cafeteria diet for 11 weeks and supplemented with GSPE (25 mg GSPE/kg body weight) or GA (7 mg GA/kg body weight) for the last 3 weeks. Each value is the mean of 6 animals ± SEM. Different letters denote significant differences between groups by one-way ANOVA followed by Tukey or Dunnett's T3 post hoc analysis according to Levene's test.

Abbreviations: iWAT: inguinal fat depot; rWAT: retroperitoneal fat depot; eWAT: epididymal fat depot.

Table 2. Effects of grape seed proanthocyanidins (GSPE) or gallic acid (GA) supplementation on plasma metabolites, hormones, and atherogenic and insulin resistance indexes in obese rats.

	STD	*CAF*	*CAF + GSPE*	*CAF + GA*
Glucose (mg/dL)	126.12 ± 4.05^{a}	186.52 ± 14.81^{b}	165.51 ± 12.83^{ab}	147.12 ± 5.85^{ab}
Insulin (ng/mL)	2.68 ± 0.22^{a}	7.48 ± 1.14^{b}	5.43 ± 1.00^{ab}	5.74 ± 0.69^{b}
HOMA-IR	21.35 ± 2.73^{a}	66.93 ± 5.79^{b}	58.14 ± 14.16^{ab}	42.18 ± 5.65^{ab}
QUICKI	0.255 ± 0.004^{a}	0.222 ± 0.005^{b}	0.234 ± 0.007^{ab}	0.237 ± 0.004^{ab}
R-QUICKI	0.274 ± 0.008^{a}	0.229 ± 0.009^{b}	0.242 ± 0.009^{ab}	0.239 ± 0.004^{ab}
Adiponectin (µg/mL)	28.15 ± 2.17^{a}	57.73 ± 5.46^{b}	53.40 ± 4.28^{b}	47.71 ± 4.25^{b}
Leptin (ng/mL)	10.88 ± 0.40^{a}	40.84 ± 4.40^{b}	34.90 ± 3.76^{b}	50.10 ± 5.18^{b}
Triglyceride (mg/dL)	69.27 ± 12.70^{a}	151.99 ± 7.23^{b}	128.95 ± 18.09^{ab}	98.97 ± 9.04^{a}
NEFAs (mmol/L)	0.55 ± 0.05	0.78 ± 0.13	0.96 ± 0.1	0.81 ± 0.05
Total cholesterol (TC) (mg/dL)	79.63 ± 4.52^{ab}	99.74 ±7.17^{a}	76.57 ± 5.60^{b}	79.25 ± 2.19^{ab}
HDL-C	40.96 ± 4.06^{a}	24.81 ± 1.42^{b}	26.47 ± 2.39^{b}	18.97 ± 2.89^{b}
Non-HDL-C	34.91 ± 1.73^{a}	70.97 ± 8.82^{b}	51.69 ± 4.38^{ab}	56.92 ± 7.17^{ab}
HDL-C / non-HDL-C	1.24 ± 0.11^{a}	0.42 ± 0.01^{b}	0.49 ± 0.04^{b}	0.35 ± 0.07^{b}
TC / HDL-C	1.20 ± 0.18^{a}	4.08 ± 0.70^{b}	3.11 ± 0.20^{ab}	3.67 ± 0.44^{b}

Rats were fed a cafeteria diet for 11 weeks and supplemented with GSPE (25 mg GSPE/kg body weight) or GA (7 mg GA/kg body weight) for the last 3 weeks. Each value is the mean of 6 animals ± SEM. Different letters denote significant differences between groups by one-way ANOVA followed by Tukey or Dunnett's T3 post hoc analysis according to Levene's test.

Altogether, these results indicate that GSPE and GA supplementation improved hyperglycemia and dyslipidemia induced by the cafeteria diet without affecting body weight and fat mass accretion. Because dysfunctional adipose tissues can be determinants of metabolic impairments associated with obesity, we next explored whether GSPE or GA supplementation could modulate adipocyte morphology and WAT expansion in obesity.

GSPE or GA supplementation reduced adipocyte size and increased adipocyte number.

There are metabolic and functional differences between WAT depots. Thus, adipocyte morphology was studied in rWAT and iWAT fat depots representing visceral and subcutaneous WAT, respectively (Figures 1 and 2). We evaluated hyperplasia in visceral and subcutaneous WAT by extrapolating the total number of adipocytes from the size of adipocytes (via histology) and the weight of the fat pad in rWAT and iWAT. Figures 1A and 2A show a representative histological image of rWAT and iWAT for each group of rats.

Feeding rats, the cafeteria diet for 11 weeks significantly increased adipocyte area and volume in both rWAT (Figures 1B and 1C) and iWAT (Figures 2B and 2C) relative to the control lean animals, resulting in an elevated frequency of adipocytes of higher than 3800 μm^2, mainly in rWAT (Figures 1D and 2D). In contrast, the cafeteria diet almost doubled adipocyte number in iWAT alone, although this increase was not statistically significant (Figures 1E and 2E). These values indicate that the cafeteria diet induced visceral and subcutaneous fat accretion mainly by hypertrophy.

GSPE supplementation for the last 3 weeks, when obesity was already established, totally reversed the adipocyte hypertrophy of rWAT induced by the cafeteria diet alone (Figures 1B and 1C). Thus, the adipocyte area was normalized due to an increased frequency of adipocytes to lower than 3800 μm^2 (Figure 1D). Moreover, GSPE supplementation tripled the adipocyte numbers in the rWAT depot (Figure 1E). In contrast, GSPE supplementation tended to reduce adipocyte area and volume in iWAT without any significant effect on adipocyte number (Figures 2B, 2C, 2D and 2E).

Effects of GA supplementation on adipocyte morphology were not as evident as those of GSPE supplementation. Rats fed cafeteria diet supplemented

with GA for the last 3 weeks displayed a slight reduction in adipocyte area and volume (approximately 15-20%) compared to those of rats fed with cafeteria diet alone in both rWAT (Figures 1B, 1C and 1D) and iWAT (Figures 2B, 2C and 2D). Moreover, GA supplementation doubled the adipocyte number in both rWAT and iWAT. However, this increase was only significant in iWAT when compared to lean rats (Figures 1E and 2E).

Altogether, these results indicate that the supplementation of GSPE or GA was able to modulate visceral and subcutaneous WAT, reducing hypertrophy and increasing hyperplasia. However, rWAT was more sensitive than iWAT to GSPE, whereas iWAT was more sensitive than rWAT to GA.

To determine whether these morphological changes in WAT expansion induced by GSPE or GA supplementation could be related to the improvement of dyslipidemia and hyperglycemia, we calculated the correlation between the volume and number of adipocytes with plasma metabolic parameters (Supplementary Table 3). Interestingly, the iWAT adipocyte volume correlated negatively with the QUICKI index and positively with TG and the TC/HDL-C ratio. In addition, the adipocyte number in iWAT correlated negatively with HDL-C and the HDL-C/non-HDL-C ratio. In contrast, no significant correlation was found between adipocyte number and size in rWAT.

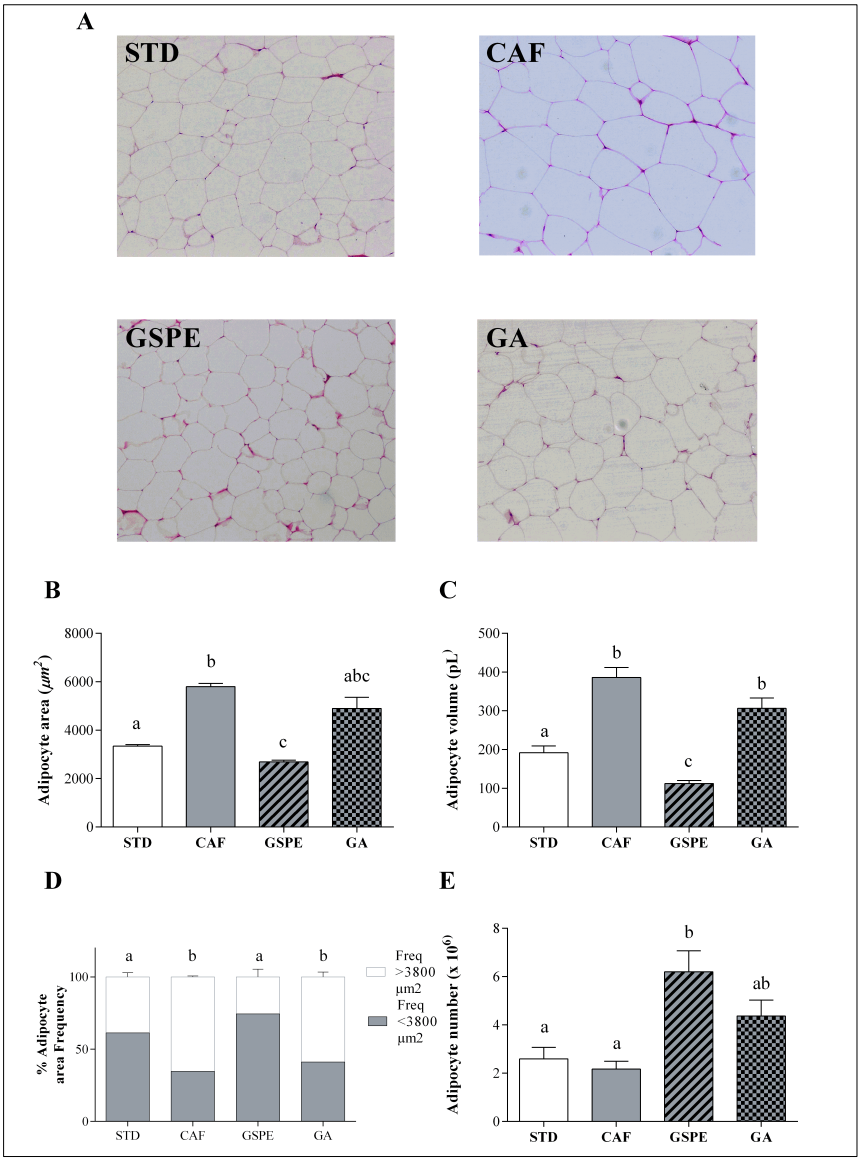

Figure 1: Effect of grape seed proanthocyanidin extract (GSPE) or gallic acid (GA) supplementation on adipocyte size and number in retroperitoneal white adipose tissue. Rats were fed a standard chow diet (STD group) or cafeteria diet (CAF) for 11 weeks. After 8 weeks, CAF-fed animals were supplemented with 25 mg GSPE/kg body weight (GSPE group), 7 mg GA/kg body weight (GA group) or the vehicle (CAF group) for 3 weeks. Samples of retroperitoneal white adipose tissue (rWAT) were stained with hematoxylin and eosin. Representative light microscopy images (A) from each group were used to measure adipocyte area (B). Adipocyte volume (C) and the frequency of adipocyte size (D) were calculated from adipocyte area. Total adipocyte number (E) was extrapolated from the size of adipocytes and the weight of rWAT. The values are the mean ± SEM of five fields per animal from three animals of each group. Different letters indicate significant differences between groups at $P \leq 0.05$ using one-way ANOVA.

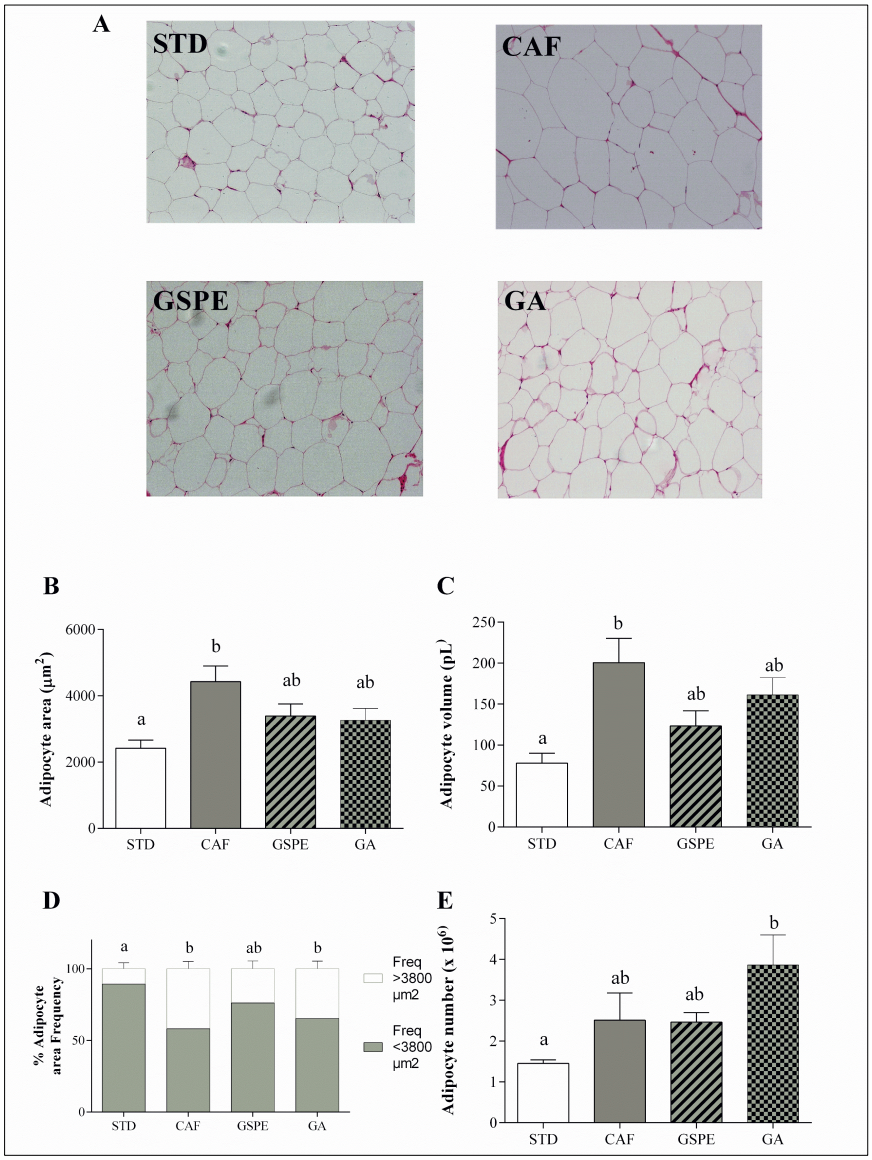

Figure 2: Effect of grape seed proanthocyanidin extract (GSPE) or gallic acid (GA) supplementation on adipocyte size and number in inguinal white adipose tissue. Rats were fed a standard chow diet (STD group) or cafeteria diet (CAF) for 11 weeks. After 8 weeks, CAF-fed animals were supplemented with 25 mg GSPE/kg body weight (GSPE group), 7 mg GA/kg v (GA group) or the vehicle (CAF group) for 3 weeks. Samples of inguinal white adipose tissue (iWAT) were stained with hematoxylin and eosin. Representative light microscopy images (A) from each group were used to measure adipocyte area (B). Adipocyte volume (C) and the frequency of adipocyte size (D) were calculated from adipocyte area. Total adipocyte number (E) was extrapolated from the size of adipocytes and the weight of iWAT. The values are the mean ± SEM of five fields per animal from three animals of each group. Different letters indicate significant differences between groups at $P \leq 0.05$ using one-way ANOVA.

Mechanisms by which GSPE or GA supplementation could remodel visceral adipose tissue

The large modifications of adipocyte size and number in rWAT induced by GSPE supplementation, and to a lesser extent by GA supplementation, prompted us to investigate some of the processes involved in adipose remodeling by these compounds. These processes include adipogenesis (pre-adipocyte differentiation), extracellular matrix and vascularization. Moreover, visceral WAT, in contrast to subcutaneous WAT, is more closely associated with obesity-related metabolic disorders[19].

The expression of preadipocyte factor-1 (Pref-1), a preadipocyte marker, was increased in the rWAT of rats supplemented with GSPE or GA, although the differences were not significant because of the high variability in expression between rats of the same group (Figure 3A). Moreover, the expression of PPARγ, which is considered the master regulator of adipogenesis, tended to be upregulated by GSPE supplementation but not by that of GA (Figure 3B). In contrast, the Wnt signaling pathway, which inhibits adipogenesis, was not affected by GSPE or GA supplementation (Figure 3C). These observations indicate that GSPE increased the number of preadipocytes and favored their differentiation into mature adipocytes.

GSPE or GA supplementation did not affect the extracellular matrix, as measured by the expression of Collagen 6A2 gene (Figure 3D). In contrast, GSPE, but not GA, increased the expression of the vascular endothelial growth factor A (VEGF-A) (Figure 3E), suggesting increased angiogenesis in the rWAT of rats supplemented with GSPE.

Furthermore, we also investigated whether GSPE or GA supplementation could modulate the fate of preadipocytes to the beige phenotype by analyzing UCP1 expression (Figure 3F). However, because of the high variability in UCP1-expression between rats of the same group, no significant differences were observed.

Although the study was focused in rWAT, the PPARγ and UCP1 expression was also analysed in iWAT (Supplementary Figure 1). According to the less sensitivity of iWAT to these compounds, no statistical differences were observed in these gene expression in iWAT.

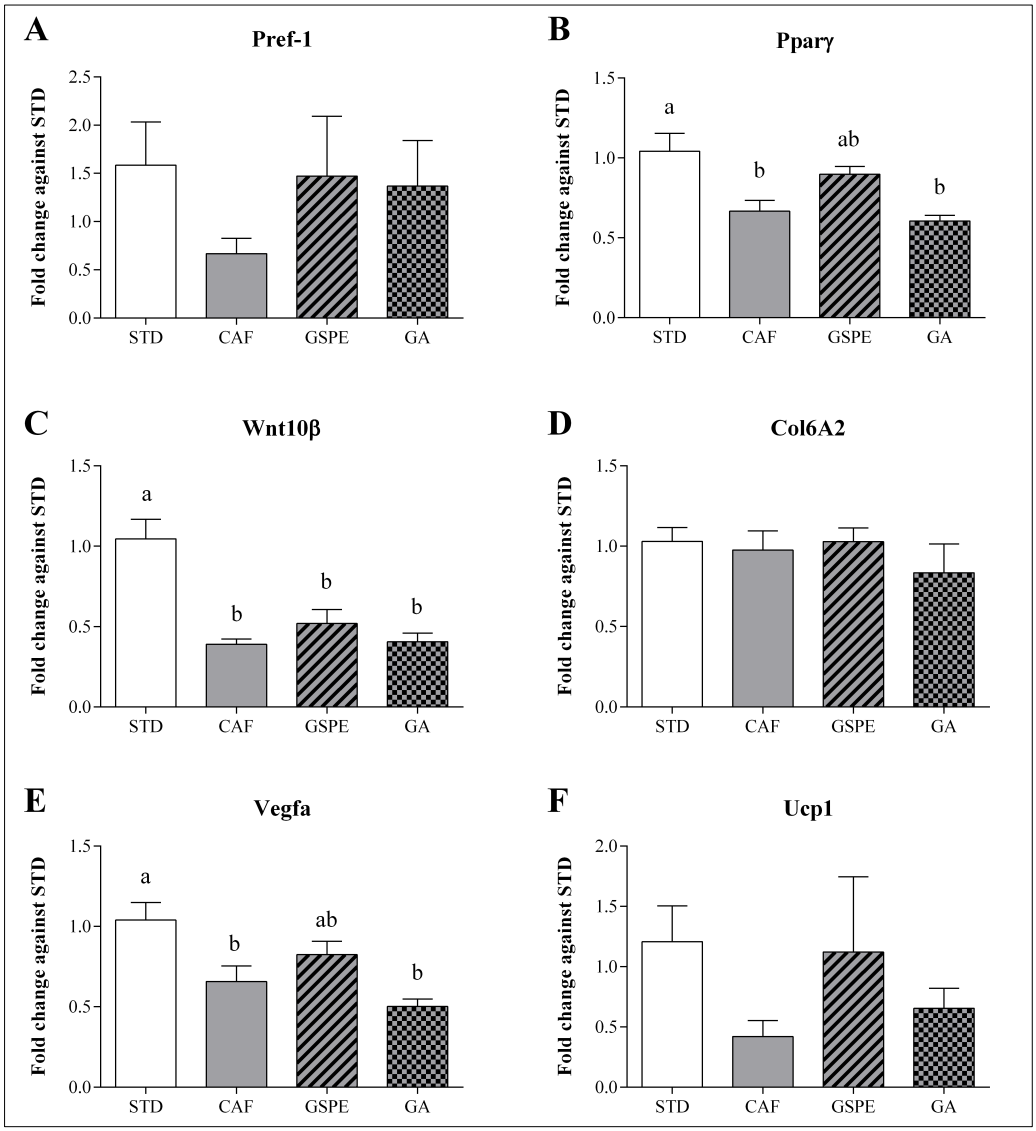

Figure 3. Effect of grape seed proanthocyanidin extract (GSPE) or gallic acid (GA) supplementation on the expression of genes related to adipose remodeling in retroperitoneal white adipose tissue.

Rats were fed a standard chow diet (STD group) or cafeteria diet (CAF) for 11 weeks. After 8 weeks, CAF-fed animals were supplemented with 25 mg GSPE/kg body weight (GSPE group), 7 mg GA/kg body weight (GA group) or the vehicle (CAF group) for 3 weeks. (A) Preadipocyte factor-1 (Pref-1) mRNA levels; (B) Peroxisome proliferator-activated receptor gamma (Pparγ) mRNA levels; (C) Wnt family member 10 Beta (Wnt10β) mRNA levels; (D) Collagen type VI alpha 2 chain (Col6A2) mRNA levels; (E) Vascular endothelial growth factor A (Vegfa) mRNA levels; (F) Uncoupling protein 1 (Ucp1) mRNA levels. The values are the mean ± SEM of 6 animals per group. Statistical analyses were performed using one-way ANOVA. Different letters (a, b, c) indicate significant differences between groups considering $P \leq 0.05$.

GSPE supplementation improved visceral adipose functionality

Adipose hypertrophy is associated with adipose tissue dysfunction. Thus, we investigated the capacity of GSPE or GA supplementation to improve several markers of adipose tissue functionality, focusing on rWAT.

A reduced capacity to store and mobilize lipids is associated with WAT dysfunction. Thus, we analyzed the expression of Fasn, Plin1 and Fabp4 in rWAT (Figures 4A, 4B, and 4C). Interestingly, GSPE supplementation significantly increased the expression of Plin1 and Fabp4, thereby indicating an increased capacity of visceral fat to store and mobilize triglycerides. In contrast, GA supplementation did not modify these processes.

Moreover, GSPE supplementation also completely restored adiponectin expression and partially restored leptin expression (Figures 4D and 4E). Thus, GSPE normalized visceral functionality relative to adipokine secretion. However, GA supplementation only partially restored both leptin and adiponectin expressions.

Inflammation and macrophage infiltration are characteristics of dysfunctional adipose tissue. However, feeding rats a cafeteria diet did not increase IL-6 or TNFα expression in these animals (Figures 4F and 4G), and GSPE supplementation did not alter the expression of these markers any further. Nevertheless, it is important to note that GA worsened inflammation, as its supplementation significantly increased IL-6 expression in rWAT. The expression of Adgre1 (Figure 4H), a macrophage marker, indicated that dietary treatments did not induce immune cell infiltration.

Taken together, these results clearly indicate that GSPE supplementation improved visceral adipocyte functionality. In contrast, GA supplementation was not as effective as GSPE and even worsened inflammation markers in rWAT.

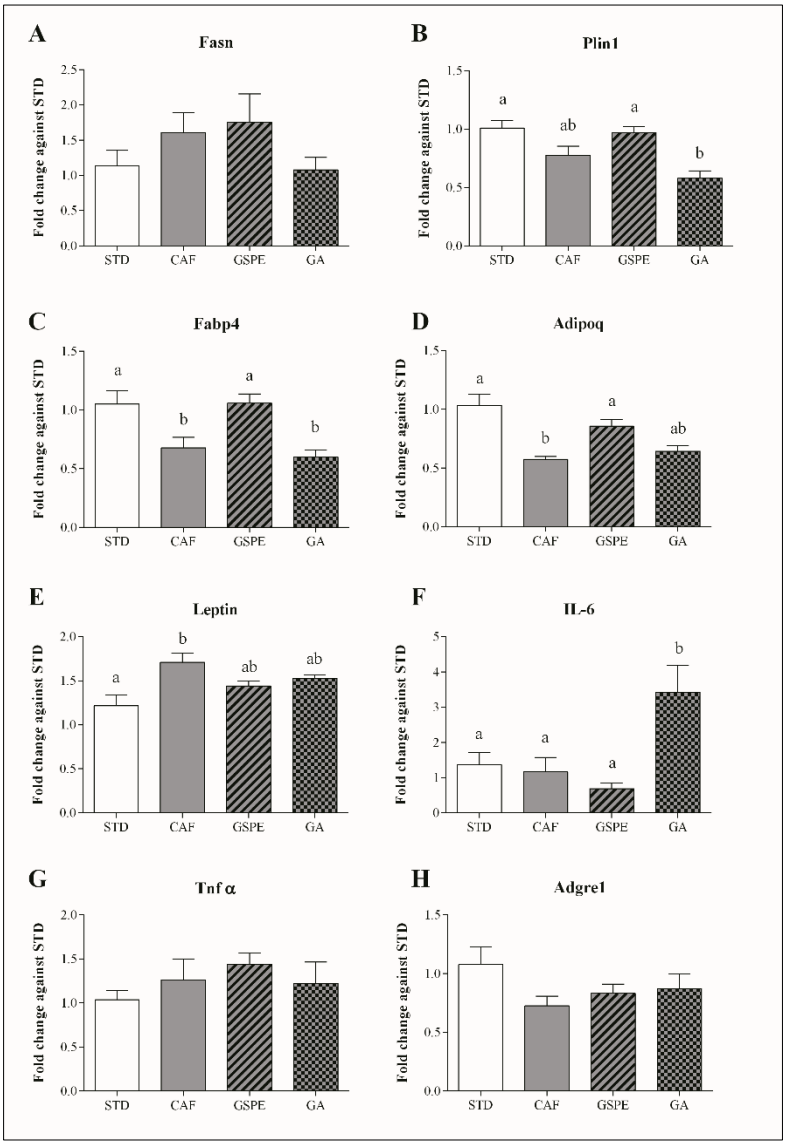

Figure 4. Effect of grape seed proanthocyanidin extract (GSPE) or gallic acid (GA) supplementation on the expression of genes related to white adipose tissue functionality in the retroperitoneal fat depot. Rats were fed a standard chow diet (STD group) or cafeteria diet (CAF) for 11 weeks. After 8 weeks, CAF-fed animals were supplemented with 25 mg GSPE/kg body weight (GSPE group), 7 mg GA/kg body weight (GA group) or the vehicle (CAF group) for 3 weeks. (A) Fatty acid synthase (Fasn) mRNA levels; (B) Perilipin 1 (Plin1) mRNA levels; (C) Fatty acid binding protein 4 (Fabp4) mRNA levels; (D) Adiponectin (Adipoq) mRNA levels; (E) Leptin mRNA levels; (F) Interleukin 6 (IL-6) mRNA levels; (G) Tumor necrosis factor alpha (TNF-α) mRNA levels; and (H) the macrophage surface marker gene Adhesion G protein-coupled receptor E1 (Adgre1) mRNA levels. The values are the mean ± SEM of 6 animals per group. Statistical analyses were performed using one-way ANOVA. Different letters indicate significant differences between groups considering $P \leq 0.05$.

DISCUSSION

Previous studies have demonstrated that GSPE improves hyperglycemia, dyslipidemia and even the disruption of central leptin signaling induced by a cafeteria diet without affecting body weight or adipose index[11,12,15]. Impaired WAT expandability is considered causative of metabolic disorders associated with obesity. Thus, in this study, we investigated whether the beneficial effects of GSPE could be associated with improved WAT expandability.

Hyperplasia and hypertrophy can contribute to WAT expansion in obesity. In our model of obesity using cafeteria diet-fed rats, rWAT expanded by hypertrophy, whereas iWAT expanded by a combination of hypertrophy and hyperplasia. Studies in rodents fed a high-fat diet indicate that hyperplasia is relevant mainly in subcutaneous WAT expansion[20], whereas others demonstrate that hyperplasia is significant only in visceral WAT expansion[21]. This discrepancy has been ascribed to the methodology used to measure WAT hyperplasia[22].

Importantly, the supplementation of cafeteria diet with GSPE for 3 weeks, when obesity was noticeable, deeply altered the pattern of the CAF-induced rWAT expansion by normalizing adipocyte size and increasing the number of adipocytes. Thus, to match surplus energy, GSPE induced a healthier expansion of rWAT than the cafeteria diet alone. This could be concluded because GSPE supplementation clearly increased the capacity to store and mobilize TGs and restored adiponectin and leptin expression in rWAT. In contrast, in iWAT, GSPE supplementation modulated adipocyte number and size to a much lower extent than in rWAT. These results highlight that visceral fat was more sensitive than subcutaneous fat to GSPE.

GA, a non-proanthocyanidin component of GSPE, is known to have beneficial effects on metabolic syndrome[23] and to modulate 3T3-L1 adipocyte differentiation[24]. Thus, we reasoned that GA could also participate in the modulation of WAT morphology induced by GSPE. To test this, a group of rats was supplemented with 10 times the quantity of GA present in 25 mg of GSPE. GA supplementation affected the adipocyte number more than it affected the adipocyte size, suggesting that the anti-hypertrophic activity of GSPE could be ascribed mainly to proanthocyanidins, whereas GA and proanthocyanidins could contribute to the hyperplasic activity of GSPE.

Other authors have focused on the capacity of pure polyphenols and polyphenol-rich extracts to modulate adipocyte hypertrophy, but not hyperplasia, in rodent models of diet-induced obesity. For example, polyphenol-rich grape pomace[25], resveratrol[26] or piceatannol[27] supplementation reduces epididymal adipocyte size. Moreover, consistent with our results, long-term resveratrol supplementation reduces adipocyte size in visceral, but not subcutaneous, WAT in rhesus monkeys fed a high-fat, high-sugar diet[28]. The capacity of resveratrol to reduce adipocyte size has also been confirmed in abdominal subcutaneous WAT of healthy humans[29]. However, in all these animal studies, polyphenols were administered from the beginning of the diet, indicating that these polyphenols prevented the development of hypertrophy associated with obesity. In contrast, in our study, GSPE was only administered when obesity was noticeable, demonstrating that GSPE had clear anti-hypertrophic and hyperplasic activities.

Visceral WAT is more closely related than subcutaneous WAT to metabolic disorders associated with obesity[19]. Thus, by mainly targeting rWAT expandability, GSPE could have a high potential to avoid metabolic disturbances. The anti-hypertrophic action of GSPE on visceral fat could restore glucose and lipid homeostasis because hypertrophic adipocytes are associated with insulin resistance[30] and with an increased risk of having metabolic syndrome[31]. Moreover, the hyperplasic action of GSPE could also increase insulin sensitivity because thiazolidinedione drugs lead to insulin sensitivity by increasing adipogeneis[32]. This suggests that having more adipocytes can be beneficial. Notably, this modification of adipocyte number and size induced by GSPE in rWAT was concomitant with the overexpression of VEGF, a factor that induces angiogenesis and increases WAT adipose vasculature[33], indicating that GSPE supplementation increased the oxygen supply to adipocytes and thus improved rWAT functionality. In accordance with these results, measurements of plasma parameters also indicate an improvement of dyslipidemia and hyperglycemia by GSPE, even though the correlation found between adipocyte number and size in rWAT and these plasma parameters was not significant. In contrast, iWAT adipocyte volume correlated positively with TG and the TC/HDL-C ratio, and iWAT adipocyte number correlated negatively with HDL-C and the HDL-C/non-HDL-C ratio. Therefore, moderate changes in iWAT expandability induced by GSPE and GA could be relevant to the improved lipid and glucose homeostasis by supplementation with these dietary compounds.

Interestingly, the effect of GSPE on increasing the adipocyte number and reducing adipocyte size was very fast, i.e., within 3 weeks of GSPE supplementation. Although the life span of adipocytes in rodents is not well defined, this speed of action of GSPE is in agreement with the results of some studies investigating epididymal fat that indicate a very rapid adipogenesis after cold exposure[21].

Until now, the mechanisms that control adipogenesis have been studied mainly *in vitro,* and little information is known under *in vivo* conditions. Some studies have identified adipocyte precursors in the WAT stromal-vascular fraction that are able to differentiate into mature adipocytes[22]. In the present study, the rWAT of rats supplemented with GSPE showed a tendency to increase the expression of Pref-1, which is a preadipocyte marker[34], suggesting that GSPE supplementation increased the number of adipocyte precursors in visceral WAT. Although Pref-1 has been considered an adipogenic inhibitor[35], the concomitant overexpression of PPARγ, the master regulator of adipogenesis, indicated that adipogenesis was active in the rWAT of rats supplemented with GSPE.

The adipogenic activity of GSPE observed in this study contradicts previous *in vitro* studies using 3T3-L1 cells, which postulated proanthocyanidins and GSPE as anti-adipogenic agents[36]. Although *in vitro* and *in vivo* conditions are not equivalent, several factors can account for these contradictory results. One important factor could be the specific molecules that reach adipocytes under each condition, i.e., parental proanthocyanidins or their metabolites. Under *in vivo* conditions, proanthocyanidins are actively metabolized by the intestine, the liver and the microbiota, generating a large set of metabolites, such as phenyl-valerolactones[37,38,39]. Thus, the adipocytes *in vivo* are in contact with proanthocyanidin metabolites and not with parental proanthocyanidins. In contrast, in the *in vitro* studies, the preadipocytes were cultured directly with GSPE, i.e., with parental proanthocyanidin. An additional factor that could explain this disparity is the presence of mature adipocytes under *in vivo* conditions. These mature adipocytes secrete adipogenic signals that can affect adipocyte formation either positively or negatively[40–42] and even control adipocyte size[43]. Thus, GSPE could increase adipogenesis *in vivo* by modulating some of these factors in mature adipocytes without directly affecting the differentiation of preadipocytes, which is the process observed under *in vitro* conditions.

In this experiment, GSPE was administered at 25 mg of GSPE/kg of body weight. This dose, using a translation of animal to human doses[44] and estimating the daily intake for a 70 kg human, correspond to an intake of 284 mg of GSPE/day. This GSPE intake can be achieved in humans with a polyphenol-rich diet. For example, in Spanish adults, the mean dietary flavonoid intake was 313.26 mg/day, with proanthocyanidins comprising 60.1%[45]. Although experimental data obtained in rats cannot be directly translatable to humans, the fact that proanthocyanidins reverse adipocyte hypertrophy suggests that the inclusion of proanthocyanidin-rich foods in the diets of obese humans could be a good strategy for improving their metabolic alterations.

To summarize, GSPE supplementation has anti-hypertrophic and adipogenic activities in rats with established obesity, mainly in visceral WAT. Because hypertrophy is associated with insulin resistance and metabolic syndrome, GSPE supplementation induced a healthier expansion of WAT to match the surplus energy provided by the cafeteria diet. Moreover, GSPE supplementation improved visceral WAT functionality, increasing the capacity of visceral WAT to store and to mobilize TGs and restoring the expression of adiponectin.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Grape seed proanthocyanidin composition

Compound	Concentration (mg/g)
Gallic acid	31.07 ± 0.08
Protocatechuic acid	1.34 ± 0.02
Vanillic acid	0.77 ± 0.04
Proanthocyanidin dimer B2	33.24 ± 1.39
Proanthocyanidin dimer B1	88.80 ± 3.46
Proanthocyanidin dimer B3	46.09 ± 2.07
Catechin	121.32 ± 3.41
Epicatechin	93.44 ± 4.27
Dimer gallate	8.86 ± 0.14
Epicatechin gallate	21.24 ± 1.08
Epigallocatechin gallate	0.03 ± 0.00
Epigallocatechin	0.27 ± 0.03
Proanthocyanidin trimer	4.90 ± 0.47
Proanthocyanidin tetramer	0.05 ± 0.01

Adapted from Margalef *et al*, 2016 [1]. The results are expressed as mg of phenolic compound/g of GSPE on a wet basis as the mean ± SD (n=3).

Supplementary Table 2. Primer sequences used for real time qPCR analysis.

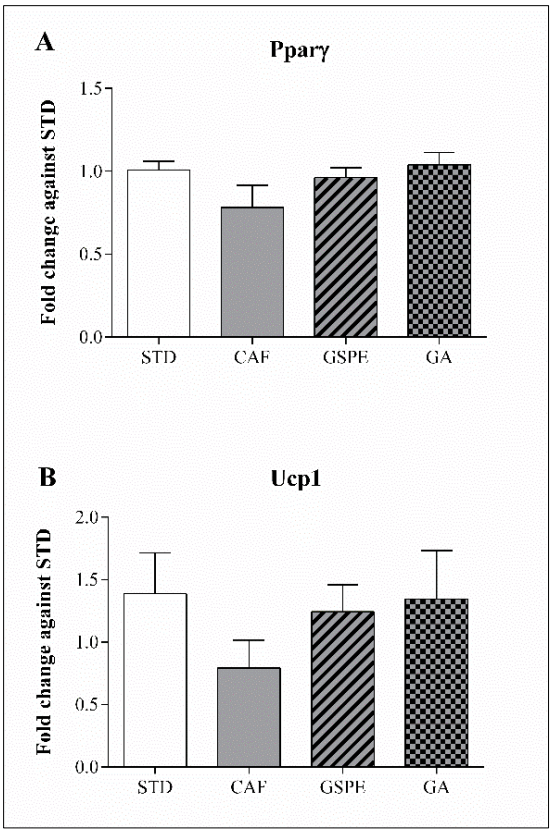
Gene	*Primer sequences (5' - 3')*	*Product size (bp)*	*Gen Bank accession no/reference*
Adipoq	GTTCCAGGACTCAGGATGCT CGTCTCCCTTCTCTCCCTTC	226	NM_144744.3
Adgre1	CTTTGGCTATGGGCTCCCAGTC GCAAGGAGGGCAGAGTTGATCGTG	165	NM_001007557.1
Axin2	AGTCAGCAGAGGGACAGGAA CTTGGAGTGCGTGGACACTA	127	NM_024355.1
Col6A2	TGACGCTGTTCTCTGACCTG TGTAGAAGTTCTGCTCGCCC	195	NM_001100741.1
Fabp4	AGAAGTGGGAGTTGGCTTCG ACTCTCTGACCGGATGACGA	103	NM_053365.1
Fasn	TCC CAG GTC TTG CCG TGC GCG GAT GCC TAG GAT GTG TGC	260	Sawano T. *et al.* [2]
Il6	CTCTCCGCAAGAGACTTCCA GGTCTGTTGTGGGTGGTATCC	122	NM_012589.2
Lep	CAT TTC ACA CAC GCA GTC GG GCA AGC TGG TGA GGA TCT GT	137	NM_013076.3
Plin1	GTGGCTCTCAGCTGCATGT CTGGAAGCACTCACAGGTCC	144	NM_001308145.1
Pparγ	AGG ATT CAT GAC CAG GGA GTT AGC AAA CTC AAA CTT AGC CTC CAT	79	Dovinová I. *et al.*[3]
Ppia	CTT CGA GCT GTT TGC AGA CAA AAG TCA CCA CCC TGG CAC ATG	138	NM_017101.1
Tnfα	TGCCTCAGCCTCTTCTCATT GCTTGGTGGTTTGCTACGAC	209	NM_012675.3
Ucp1	CGA GCC AAG ATG GTG AGT TCG ACA GTG GTG ATG GTC CCT AAG ACA CCT	200	NM_012682.2
Vegfa	CTTGAGTTGGGAGGAGGATG TGGCAGGCAAACAGACTTC	116	NM_001287114.1

Abbreviations: Adgre1: adhesion G protein-coupled receptor E1; Adipoq: adiponectin, C1Q and collagen domain containing; Axin2: Axin 2; Col6A2: collagen type VI alpha 2 chain; Fasn: fatty acid synthase; Il6: interleukin 6; Lep: leptin; Plin1: Perilipin 1; Pparγ: peroxisome proliferator-activated receptor gamma; Ppia: peptidylpropyl isomerase A (Cyclophilin A); Tnfα: tumor necrosis factor; Ucp1: uncoupling protein 1, Vegfa: vascular endothelial growth factor A

Supplementary Table 3. Correlations

Variables	***rWAT***				***iWAT***			
	Adipocyte volume		***Adipocyte number (x10^6)***		***Adipocyte volume***		***Adipocyte number (x10^6)***	
	Correlation coefficient	*P value*	*Correlation coefficient*	*P value*	*Correlation coefficient*	*P value*	*Correlation coefficient*	*P value*
Adipocyte volume rWAT			**-0,668**	**0,017**	*0,592*	*0,071*	0,352	0,262
Adipocyte number rWAT (x10^6)	**-0,668**	**0,017**			0,133	0,715	0,166	0,606
Adipoc. volume iWAT	0,592	0,071	0,133	0,715			0,332	0,291
Adipocyte number iWAT (x10^6)	0,352	0,262	0,166	0,606	0,332	0,291		
GLU (mg/dL)	0,490	0,150	-0,029	0,937	*0,630*	*0,051*	0,099	0,716
Insulin (ng/mL)	0,396	0,291	0,070	0,857	*0,636*	*0,065*	0,272	0,307
HOMA-IR	0,467	0,205	-0,025	0,950	0,646	0,083	0,138	0,638
QUICKI	-0,505	0,166	-0,072	0,854	**-0,741**	**0,022**	-0,208	0,457
QUICKI-R	-0,371	0,326	-0,255	0,508	**-0,750**	**0,020**	-0,242	0,385
Adiponectin	0,374	0,232	0,086	0,790	**0,615**	**0,033**	0,252	0,299
TG	0,309	0,329	0,108	0,738	**0,644**	**0,032**	0,298	0,245
NEFAs	0,112	0,759	0,385	0,272	0,424	0,222	0,226	0,400
TC	0,364	0,270	-0,182	0,593	0,413	0,207	-0,098	0,708
HDL-c	-0,213	0,529	-0,338	0,310	-0,365	0,244	**-0,617**	**0,006**
non-HDL-c	0,447	0,145	0,020	0,952	0,528	0,078	0,266	0,302
HDL/non-HDL	-0,287	0,392	-0,365	0,269	*-0,540*	*0,070*	**-0,636**	**0,008**
TC / HDL-c	0,244	0,497	0,460	0,181	**0,674**	**0,023**	*0,484*	*0,058*
Body weight	0,564	0,056	0,003	0,992	0,517	0,104	0,312	0,207
rWAT weight	0,166	0,626	0,364	0,271	0,355	0,284	**0,562**	**0,015**
iWAT weight	0,395	0,204	0,222	0,488	**0,647**	**0,023**	**0,720**	**0,001**
Liver weight	0,235	0,487	0,323	0,332	*0,569*	*0,067*	0,362	0,153
eWAT weight	0,369	0,238	0,263	0,409	0,460	0,154	**0,504**	**0,033**
Adiposity index	0,184	0,588	0,373	0,258	0,400	0,252	**0,642**	**0,006**

Supplementary Figure 1. Effect of grape seed proanthocyanidin extract (GSPE) or gallic acid (GA) supplementation on the expression of genes in the inguinal fat depot.



Rats were fed a standard chow diet (STD group) or cafeteria diet (CAF) for 11 weeks. After 8 weeks, CAF-fed animals were supplemented with 25 mg GSPE/kg body weight (GSPE group), 7 mg GA/kg body weight (GA group) or the vehicle (CAF group) for 3 weeks. (A) Peroxisome proliferator-activated receptor gamma (Pparɣ) mRNA levels; (B) Uncoupling protein 1 (Ucp1) mRNA levels. The values are the mean ± SEM of 6 animals per group. Statistical analyses were performed using one-way ANOVA. Different letters indicate significant differences between groups considering $P \leq 0.05$.

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UNIVERSITAT ROVIRA I VIRGILI
ADIPOSE TISSUE REMODELLING BY POLYPHENOLS IN OBESE RATS
Aïda Pascual Serrano

2. Capacity of dietary polyphenols to counteract adipose tissue dysfunction by remodelling adipocytes in obesity

2.2. Resveratrol remodels visceral adipose tissue and down-regulates hepatic miR-33 in obese rats. (Manuscript 3)

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Resveratrol remodels visceral adipose tissue and down-regulates hepatic miR-33 in obese rats.

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Keywords: *resveratrol; adipocyte; adipogenesis; non-flavonoids; hyperplasia; hypertrophy polyphenol.*

Manuscript in preparation

Abstract

White adipose tissue (WAT) expansion occurs via adipocyte size increase (hypertrophy) and/or adipocyte number increase (hyperplasia). Hypertrophied adipose tissue is associated with the development of metabolic obesity-related complications. Conversely, hyperplasia has been regarded as protective against metabolic disturbances by improving WAT expandability. Resveratrol (RSV) is one of the most studied non-flavonoid. Many studies have demonstrated that RSV has several health benefits, such as reducing body fat accumulation and preventing obesity comorbidities through modulating lipid metabolism. Previous studies have shown that RSV protects against adipocyte hypertrophy induced by obesogenic diets. However, the capacity of RSV to remodel the anomalous WAT expansion typical of obesity has never been tested. Thus, the aim of this study was to evaluate whether RSV can correct dysfunctional WAT in obesity through improving its expandability. Because RSV also regulates metabolism through miRNA modulation, we also studied the effect of RSV on miRNAs, and their target genes, involved in lipid metabolism in visceral WAT and liver. For this, male wistar rats were fed a standard chow diet or a cafeteria diet (CAF) for 8 weeks. Next, CAF-fed animals were administered oral doses of 50, 100 or 200 mg RSV/kg BW or the vehicle carrying on with their obesogenic diets for 3 more weeks. Adipocyte size and number in visceral retroperitoneal WAT (rWAT) were determined by histological analyses. The expression levels of markers of adipogenesis, lipogenesis and browning were quantified by qPCR in rWAT as well as lipid regulators miR-33a and miR-122 levels in rWAT and/or liver together with their target genes. Results showed that pharmacological doses of RSV reduced hypertrophy and increased hyperplasia in visceral WAT, remodelling it in a dose-dependent manner. Consistent with improved rWAT expandability, RSV restored glucose and insulin homeostasis as well as leptin expression levels. RSV increased adipogenesis and reduced lipolysis in rWAT. Furthermore, RSV modified lipid metabolism through miR33a modulation in the liver whereas this miRNA was not affected in rWAT. In conclusion, RSV administration induces a healthier WAT expandability in rats with stablished obesity through anti-hypertrophic and hyperplasic activities. This could explain the attributed effects to RSV in improving obesity-related disorders.

1. Introduction

Obesity is a widely spread health problem around the world and is characterised by an imbalance in energy intake and expenditure leading to the increase in fat accumulation in the body [1]. Once obesity is stablished, adipose tissue may be pathogenic through the unfavourable consequences of the excessive fat mass. This increase in size of fat depots has been associated with the development of obesity-related diseases such as dyslipidaemia, cardiovascular diseases, systemic inflammation, insulin resistance and type 2 diabetes among others[2,3]. However, some obese individuals do not suffer from metabolic complications and are described as metabolically healthy obese[4,5].

White adipose tissue (WAT) expansion is one of the mechanisms by which the body manages the energy surplus during obesity development. This expansion is carried on by an increase in adipocyte size (hypertrophy) to store more triglycerides (TAG) and/or by an increase in adipocyte number (hyperplasia). Adipocytes in WAT store energy but, when they reach a threshold in terms of storage capacity, adipocyte hypertrophy is stablished and this can lead to alterations in adipocyte proper functionality. A different pattern of adipokines and inflammation markers are secreted from this hypertrophic adipose tissue and there is an impairment in the whole adipose tissue depot function[6,7]. Concretely, it has been described that visceral hypertrophic adipocytes are the most important factor influencing systemic deregulations such as insulin resistance and other obesity-related disorders mentioned above[8,9]. Furthermore, an increased hyperplasia has been defined as a protection against metabolic complications of adipose tissue in obesity due to the increased storage capacity of new adipocytes and their smaller size[10,11].

Given the relevance of WAT expansion in obesity and all the problems associated to it, scientific and medical communities are involved in the research of natural bioactive compounds present in foods, edible plants and species which could ameliorate obesity state through body weight (BW) reduction or improvement of other physiological and metabolic parameters. Regarding obesity, polyphenols, which are a wide family of natural compounds present in plants, species and natural foods, are one of the most important and studied compounds[12–14]. They have been attributed different beneficial effects such as counteracting dyslipidaemia[15], acting as anti-oxidant[16,17], anti-inflammatory[18] or

anti-hypertensive agents and exerting cardioprotective[19,20] and cardiopreventive effects.

Among all phenolic compounds, resveratrol (RSV) is one of the most studied molecules inside the non-flavonoid class of polyphenols. RSV is produced in plants in response to determined stress stimuli such as injury or biological attack. RSV can be found in different isoforms but the active agent is known to be trans-resveratrol and its glucoside forms. Notably, RSV has been described for being the effector of the beneficial health effects attributed to red wine[21]. Moreover, the effect of RSV reducing body fat accumulation has been described in several rodent models[22,23]and a huge number of studies evidence the preventive effect of RSV on obesity and obesity-related disorders in *in vitro* and *in vivo* models [24–28]. RSV has been also described as preventive anti-hypertrophic modulator in some animal studies [29]. However, to our knowledge, there are no evidences regarding RSV anti-obesity effects once the obesity is already stablished. Other polyphenols, such as proanthocyanidins from grape seed, have been described to have anti-hypertrophic and hyperplasic activities in obese rats[30]. Thus, the objective of this study was to test whether RSV was able to counteract the anomalous WAT expansion characteristic of obesity by reducing adipocyte size and increasing adipocyte number in the visceral fat, thereby conferring to adipose tissue a better management of energy surplus once obesity has been stablished. Additionally, several miRNAs have been described to be involved in obesity [31]and to regulate associated processes such as glucose [32] and lipid metabolism [33,34]. Two of the best-studied miRNAs in lipid metabolism regulation are miR-33 and miR-122[33] and RSV modulates these miRNAs by directly binding them [35]. Therefore, we also aimed to determine the role of miR-33a (rWAT and liver) and miR-122 (liver) on the modulation of lipid metabolism exerted by RSV.

2. Materials and methods

2.1. Resveratrol

Resveratrol (trans-resveratrol) with a minimum of 98% purity was purchased from Fagron (Fagron Ibérica, S.A.U, Barcelona, Spain).

2.2. Animal experimental procedure

The research was conducted in accordance with the ethical standards and the Declaration of Helsinki and was approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 7959 by Generalitat de
Catalunya).

Five-week-old male Wistar rats were purchased from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22°C with a light/dark period of 12 h (light from 8:00 am to 20:00 pm). Animals stayed for one week and were fed chow diet in order to adapt. Rats were then divided into five groups: the standard group (STD, n=6) was fed a standard chow diet (STD Panlab A04, Panlab, Barcelona, Spain) and tap water *ad libitum*, and all other groups were fed a cafeteria diet (CAF) with a composition of 14% protein, 35% fat, and 51% carbohydrates. CAF diet was consisting of sausage, bacon, biscuits with paté, cheese, ensaïmada (sweetened pastry), carrots and sweetened milk (20% sucrose w/v) and it was given in addition to the standard chow diet. This highly palatable diet is able to induce voluntary hyperphagia. CAF was freshly provided to the animals daily and animals had free access to the CAF diet which they could eat *ad libitum*. After 8 weeks, CAF-fed animals were divided into four groups: RSV 50 group (RSV 50, n=6), which received a daily oral administration together with CAF diet of a dose of 50 mg RSV/kg BW; RSV 100 group (RSV 100, n=6), which received an oral dose of 100 mg RSV/kg BW; RSV 200 group (RSV 200, n=6), which received an oral dose of 200 mg RSV/kg BW and CAF group, which received sweetened milk alone. RSV was diluted in sweetened milk as the vehicle. All groups received the specific dose of RSV or the vehicle throughout the remainder of the experiment. After three weeks, rats were fasted for 3 hours after the oral dose and then sacrificed by live decapitation. Total blood was collected using heparin (DeltaLab, Barcelona, Spain) as an anticoagulant. Plasma was obtained by centrifugation (1500 *g*, 15 minutes, 4°C), and retroperitoneal adipose tissue depot and liver were excised and immediately frozen in liquid nitrogen. Both plasma and tissues were stored at -80°C until further use.

2.3. Adiposity

Adiposity index was determined by the sum of the iWAT, retroperitoneal WAT (rWAT) and epididymal WAT (eWAT) depot weights. Results were expressed as percentage of total BW. In addition, the body composition was measured one day before sacrifice using an choMRI-700 (Echo Medical Systems, LLC., TX, USA). Direct measurements of fat mass were obtained in triplicate for each animal of each group (n=6) using the analyser, and expressed as a percentage of total BW.

2.4. Quantification of plasma parameters

The plasma levels of insulin, adiponectin and leptin were measured using an immunometric sandwich enzyme-linked immunosorbent assay (ELISA) using a rat/mouse insulin ELISA kit (EZRMI-13K), rat leptin ELISA kit (EZRL-83K) and rat adiponectin ELISA kit (EZRADP-62K, Millipore Ibérica, Madrid, Spain). Appropriate range of dilutions to be used was previously tested and then immunoassays were performed in duplicate according to the manufacturer's protocols. Plasma NEFAs were analysed with the enzymatic colorimetric HR NEFA series kit (Wako, CA, USA). Plasma TAG, total cholesterol (TC), HDL-cholesterol (HDL-C) and glucose were measured with enzymatic colorimetric kits following the manufacturer's protocols (QCA, Barcelona, Spain).

2.5. Adipose tissue morphology analysis

Pieces of frozen rWAT (-80°C) were sent to ELDINE Patología (Tarragona, Spain), where they were thawed and fixed in 4% diluted formaldehyde. After 24 hours of fixation, tissues underwent successive dehydration (Alcohol/Ethanol 70%, 96% and 100%; plus Xylol/Dimethyl benzene) and paraffin infiltration and immersion at 52°C (Citadel 2000. HistoStar, Thermo Scientific. Madrid, Spain). The paraffin blocks were subsequently cut into successive 2 µ-thick sections (Microm HM 355S. Thermo Scientific. Madrid, Spain). The sections were deposited on slides (JP Selecta Paraffin Bath) and subjected to automated hematoxylin-eosin staining (Varistain Gemini. Shandom. Thermo)[36]. Images of the adipose sections were acquired using AxioVision Zeiss Imaging software (Carl Zeiss Iberia, S.L.,Madrid, Spain). Finally, the images captured at 20x magnification were analysed with the

Adiposoft software (CIMA, University of Navarra, Spain) to quantify adipocyte number and areas. Five fields per sample and three samples from each group (STD, CAF, RSV50, RSV100 and RSV200) were measured. The area was calculated from the average value of the cell area in all measured fields. The total cell number in the rWAT fat depots was calculated from the fat cell volume values. Fat cell volume was obtained for each analysed field using the formula $\frac{\pi}{6} x\left[3\sigma^2 \times \bar{d} + \bar{d}^5\right]$[37] (where d is the mean diameter of 100 measured cells in the field, and σ is the standard deviation of all diameters). Subsequently, fat cell density was applied to determinate fat cell weight. Finally, the total fat cell number in the whole rWAT depot of each animal was determined by applying the quotient between total fat depot weight and mean cell weight of all captured fields.

2.6. RNA extraction

Total RNA was isolated from the frozen rWAT and liver using trizol reagent (Ambion, MA, USA) according to the manufacturer's protocol including a step of incubation with isopropanol at -20ºC overnight instead of the 10 minutes at room temperature in order to obtain in the RNA pellet also small RNAs for a subsequent miRNA quantification. The quantity, quality and integrity was checked with Agilent 2100 Bioanalyzer system (Agilent Technologies, Madrid, Spain).

2.7. mRNA quantification by real-time qRT-PCR

Relative mRNA levels of ATP binding cassette subfamily A member 1 (Abca1), carnitine palmitoyltransferase 1B (Cpt1b), Fatty acid synthase (Fasn), Fatty acid binding protein 4 (Fabp4), Insulin receptor substrate 2 (Irs2), Leptin (Lep), Lipoprotein Lipase (Lpl), Peroxisome proliferator-activated receptor gamma (Pparɣ) and Uncoupling protein 1 (Ucp1) were analysed by real-time PCR in rWAT using cyclophilin (Ppia) as the endogenous control.

Relative mRNA levels of ATP binding cassette subfamily A member 1 (Abca1), carnitine palmitoyltransferase 1A (Cpt1a), Fatty acid synthase (Fasn) and sterol regulatory element binding transcription factor 2 (Srebp2) were analyzed by real-time PCR in liver samples using Ppia as an endogenous control.

Total RNA was reverse transcribed using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, MA, USA) according to the manufacturer's protocol. Gene expression was evaluated with the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Barcelona, Spain) using the SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Barcelona, Spain) and gene-specific SYBR primers designed for each gene using the FastPCR software (Supplementary Table 1). The results were normalized to Ppia. Amplification was performed following the temperature steps of 95°C for 30 seconds followed by 40 cycles at 95°C for 5 seconds and 60°C for 5 seconds. The fold-change in the mRNA level was calculated by the $2^{-\Delta\Delta Ct}$ method, where ΔCt = Ct mRNA − Ct Ppia and ΔΔCt = ΔCt treated samples − ΔCt untreated controls.

2.8. miRNA quantification by real-time qRT-PCR

Single-stranded cDNAs were synthesized by using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) and the miRNA specific reverse transcription primers (Applied Biosystems. Madrid, Spain). The reaction was performed in a final volume of 7.5µl at the following temperature cycles: 16ºC for 30 minutes, 42ºC for 30 minutes and 85ºC for 5 minutes. The cDNA products of the reaction were mixed with the appropriate volume of TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain) following manufacturer's recommendations and with the associated specific PCR primers provided in the Taqman MicroRNA kit (Applied Biosystems, Madrid, Spain). Then the mixture was used for a quantitative polymerase chain reaction (qPCR) amplification. A specific Taqman probe was used for the microRNA analysis: 5'-UGGAGUGUGACAAUGGUGUUUG -3' for microRNA-122 (002245, Applied Biosystems, Madrid, Spain), 5'-GUGCAUUGUAGUUGCAUUGCA-3' for microRNA-33a (002135, Applied Biosystems, Madrid, Spain) and U87 small nuclear RNA (001712, Applied Biosystems, Madrid, Spain) was used as an endogenous control and the results were normalized to its expression. The amplification reaction was run on an ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems, Madrid, Spain) at 95ºC for 10 minutes followed by 40 cycles at 95ºC for 15 seconds and 60ºC for 1 minute. Fold change in the miRNA level was calculated according to the equation: $2^{-\Delta\Delta Ct}$; where ΔCt = Ct miRNA − Ct U87 and ΔΔCt = ΔCt treated samples − ΔCt untreated controls.

2.9. Statistical analyses

Results are reported as the mean ± SEM of 6 animals per group for mRNA, miRNA and metabolites. Regarding Adiposoft histology analysis, results are reported as the mean ± SEM of 3 animals per group. Group means were compared using one-way analysis of variance (ANOVA) with IBM SPSS statistics 20.0 software (SPSS, Inc, Chicago, IL, USA). In all cases the comparisons were judged to be significant at $P \leq 0.05$.

3. Results

3.1. Resveratrol administration improved body fat percentage, hypertriglyceridaemia and glucose and insulin metabolisms without counteracting the body weight increase induced by cafeteria diet.

RSV administration during 3 weeks after obesity induction by CAF diet did not significantly reduce body weight (BW) that had been previously increased by CAF diet (Table 1). However, the higher dose of RSV (200mg/kg BW) was able to reverse in a significant manner the fat accumulation in subcutaneous inguinal WAT (iWAT) and the body fat percentage induced by CAF. It is of note that depending on the RSV dose, it affects differently to the fat depots. RSV at a dose of 50mg/Kg reduced subcutaneous iWAT depot accumulation produced by CAF diet, while visceral rWAT and eWAT fat mass was increased and overall, body fat percentage was increased with RSV50. RSV100 group showed less differences when compared to CAF group although it showed a slightly increased rWAT weight. Regarding RSV200 group, it tended to reduce both subcutaneous and visceral depots compared to CAF obese group.

RSV was also effective in normalising TAG levels in plasma at all doses, acting only in significant way in the 100 and 200mg/kg oral doses (Table 2). Glucose and Insulin levels were reduced compared to CAF group in more than 20% for glucose with both doses and in a 20% and 40% in insulin with RSV 100 and RSV 200 respectively and thus, these doses improved HOMA-IR and R-QUICKI indexes, which are strongly related to insulin resistance. Adiponectin levels, which were found to be increased with CAF diet were also normalised to the standard group levels by the highest dose of RSV. Leptin and cholesterol levels were not

Table 1. Effects of resveratrol (RSV) administration on body weight and fat deposition after diet-induced obesity.

	STD	*CAF*	*CAF + RSV 50 mg/kg*	*CAF + RSV 100 mg/kg*	*CAF + RSV 200 mg/kg*
Body weight (g)	438.5 ± 17.02^{a}	518.4 ± 14.66^{bc}	635.5 ± 30.77^{c}	550.7 ± 24.18^{bc}	500.3 ± 16.32^{ab}
Liver Weight (g)	12.85 ± 0.90^{a}	14.87 ± 0.29^{a}	19.38 ± 0.84^{b}	17.14 ± 1.15^{ab}	15.79 ± 0.59^{ab}
iWAT weight (g)	1.00 ± 0.12^{a}	5.49 ± 0.71^{b}	2.82 ± 0.44^{ab}	4.75 ± 0.93^{ab}	1.85 ± 0.24^{a}
rWAT weight(g)	3.54 ± 0.08^{a}	8.40 ± 0.95^{b}	9.10 ± 1.87^{b}	9.82 ± 1.50^{b}	7.06 ± 0.70^{b}
eWAT weight(g)	8.99 ± 0.95^{a}	18.64 ± 1.42^{b}	26.01 ± 2.87^{c}	19.22 ± 1.31^{abc}	14.62 ± 1.43^{ab}
Adiposity index (%)	2.95 ± 0.10^{a}	6.35 ± 0.61^{b}	5.61 ± 0.28^{b}	5.82 ± 0.46^{b}	4.48 ± 0.29^{b}
Body fat (%)	12.60 ± 1.24^{a}	26.06 ± 1.44^{b}	29.48 ± 3.36^{bc}	26.71 ± 2.82^{bc}	18.93 ± 0.77^{c}

Rats were fed a standard chow diet (STD group) or cafeteria diet (CAF) for 11 weeks. After 8 weeks, CAF-fed animals were supplemented with 50 mg RSV/kg body weight (RSV 50 group), 100 mg RSV/kg body weight (RSV 100 group), 200 mg RSV/kg body weight (RSV 200 group) or the vehicle (CAF group) for 3 weeks. Each value is the mean of 6 animals ± SEM. Different letters denote significant differences between groups by one-way ANOVA followed by Tukey or Dunnett's T3 post hoc analysis according to Levene's test.

Abbreviations: iWAT: inguinal fat depot; rWAT: retroperitoneal fat depot; eWAT: epididymal fat depot.

Table 2. Effects of different doses of resveratrol (RSV) administration on plasma metabolites, hormones and insulin resistance and atherogenic indexes in diet-induced obese rats.

Plasma parameters	*STD*	*CAF*	*CAF + RSV 50 mg/kg*	*CAF + RSV 100 mg/kg*	*CAF + RSV 200 mg/kg*
GLU (mg/dl)	126.12 ± 4.05^{a}	186.52 ± 14.81^{b}	152.96 ± 10.19^{ab}	152.19 ± 10.55^{ab}	139.52 ± 3.99^{ab}
Insulin (ng/mL)	2.68 ± 0.22^{a}	7.48 ± 1.14^{ab}	8.76 ± 1.45^{b}	5.36 ± 0.88^{ab}	4.38 ± 0.45^{ab}
HOMA-IR	21.35 ± 2.73^{a}	66.93 ± 5.79^{b}	83.06 ± 15.60^{ab}	48.51 ± 9.56^{ab}	36.80 ± 4.84^{a}
QUICKI	0.26 ± 0.00^{a}	0.22 ± 0.00^{b}	0.22 ± 0.01^{b}	0.23 ± 0.01^{b}	0.24 ± 0.00^{ab}
R-QUICKI	0.27 ± 0.01^{a}	0.23 ± 0.01^{b}	0.23 ± 0.01^{b}	0.25 ± 0.01^{ab}	0.25 ± 0.01^{ab}
Adiponectin (µg/mL)	28.15 ± 2.17^{a}	57.73 ± 5.46^{b}	49.08 ± 4.02^{bc}	41.34 ± 2.99^{bc}	34.74 ± 3.36^{ac}
Leptin (ng/mL)	10.88 ± 0.19^{a}	40.84 ± 0.79^{b}	58.23 ± 0.80^{b}	47.62 ± 0.66^{b}	25.29 ± 0.39^{b}
TAG (mg/dl)	69.27 ± 12.70^{a}	151.99 ± 7.23^{b}	109.15 ± 17.2^{ab}	58.56 ± 6.24^{a}	67.56 ± 6.83^{a}
NEFAs (mmol/L)	0.55 ± 0.05	0.78 ± 0.13	0.73 ± 0.09	0.56 ± 0.05	0.66 ± 0.05
TC (mg/dl)	79.63 ± 4.52	99.74 ±7.17	100.98 ± 2.75	81.14 ± 6.25	81.14 ± 0.56
*HDL-**C***	40.96 ± 4.06^{a}	24.81 ± 1.42^{b}	23.32 ± 3.24^{b}	22.95 ± 2.08^{b}	22.53 ± 2.46^{b}
Non-HDL-c	34.91 ± 1.73^{a}	70.97 ± 8.82^{b}	67.21 ± 4.76^{b}	53.94 ± 3.67^{ab}	59.03 ± 2.54^{b}
HDL-c / non-HDL-c	1.24 ± 0.11^{a}	0.42 ± 0.01^{b}	0.35 ± 0.05^{b}	0.42 ± 0.03^{b}	0.39 ± 0.05^{b}
TC / HDL-c	2.01 ± 0.18^{a}	4.08 ± 0.70^{b}	4.21 ± 0.62^{b}	3.58 ± 0.22^{ab}	4.37 ± 0.66^{b}

Rats were fed a standard chow diet (STD group) or cafeteria diet (CAF) for 11 weeks. After 8 weeks, CAF-fed animals were supplemented with 50 mg RSV/kg body weight (RSV 50 group), 100 mg RSV/kg body weight (RSV 100 group), 200 mg RSV/kg body weight (RSV 200 group) or the vehicle (CAF group) for 3 weeks. Each value is the mean of 6 animals ± SEM. Different letters denote significant differences between groups by one-way ANOVA followed by Tukey or Dunnett's T3 post hoc analysis according to Levene's test.

Abbreviations: GLU: glucose; HOMA-IR: homeostasis model assessment-estimated insulin resistance; QUICKI: quantitative insulin sensitivity check index; R-QUICKI: revised quantitative insulin sensitivity check index; TAG: triglycerides; NEFAs: non-esterified fatty acids; TC: total cholesterol; HDL-c: high density lipoprotein cholesterol; non-HDL-c: non-high-density lipoprotein cholesterol; HDL-c/non-HDL-c: ratio between HDL-c and non-HDL-c levels; TC/HDL-c: ratio between TC and HDL-c levels.

significantly affected by any dose and thus, all the different cholesterol measured forms were not modified either.

These results indicate that RSV administration was able to ameliorate TAG and glycaemic profile altered by CAF diet when administered in the 100 and 200mg/kg doses. As visceral adipose tissue accumulation is more related to metabolic disorders and RSV reduced the total fat percentage, we focused on visceral rWAT depot to further investigate the mechanisms of RSV in adipose tissue.

3.2. Resveratrol administration reverses hypertrophy originated by CAF diet and increases hyperplasia in visceral rWAT

We tested how visceral rWAT depot was modified by CAF diet and how the different RSV doses affect its morphology. The study was performed by analysing adipocyte area, volume and total number of cells in the fat depot from histologic adipose tissue cuts (Fig 1). Representative histological images from all animal groups are shown in figure 1A.

Animals fed with the CAF diet along 11 weeks showed a significant increase in both adipocyte area and volume (Figures 1B and 1C). All the doses of RSV used in this study were able to reduce adipocyte size and volume after 3 weeks of RSV treatment together with a CAF diet, showing a reduced hypertrophy that was completely counteracted with the highest dose. Frequency of adipocyte area to be under or over a threshold of 3800µm^2 was also calculated (Figure 1D) and results show an increase in the number of adipocytes overpassing the size threshold in the rats fed with CAF diet; in this case only the dose of 200mg/kg of RSV was able to return to a pattern of adipocyte size comparable to lean rats (STD) while 50 and 100mg/kg doses did not show any significant changes.

As pronounced hypertrophy has been associated with dysfunctional adipose tissue while hyperplasia has been related to a well-functioning fat pad, we also quantified adipocyte number in the whole visceral rWAT fat pad (Figure 1E). Adipocyte number remained unchanged when CAF diet was given to rats but after three weeks of RSV administration at all doses a tendency to increase adipocyte number was observed, being again more pronounced with the dose of 200mg/kg of RSV, which increased adipocyte number significantly in a 150%.

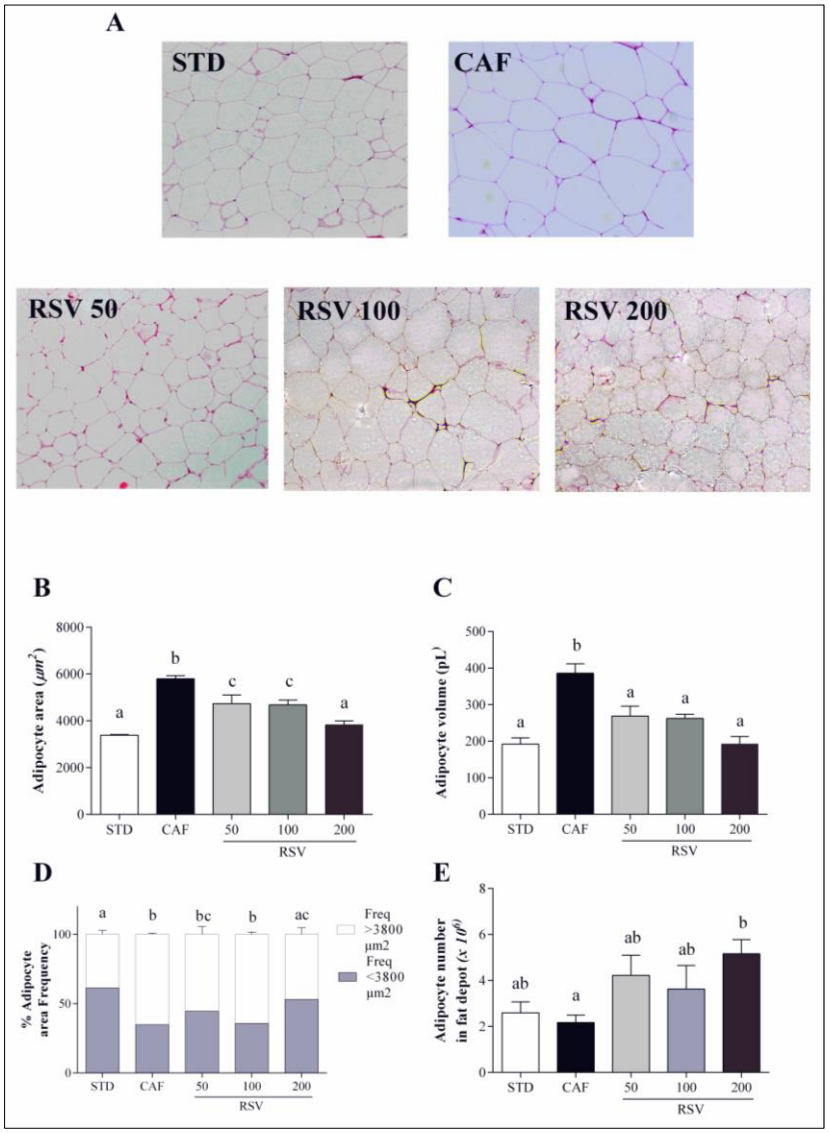

Figure 1: Effect of three different doses of resveratrol administration on adipocyte size and number in retroperitoneal white adipose tissue of obese rats.

Rats were fed a standard chow diet (STD group) or cafeteria diet (CAF) for 11 weeks. After 8 weeks, CAF-fed animals were administered with 50 mg RSV/kg body weight (BW) (RSV 50 group), 100 mg RSV/kg BW (RSV 100 group), 200 mg RSV/kg BW (RSV 200 group) or the vehicle (CAF group) for 3 weeks. Samples of retroperitoneal white adipose tissue (rWAT) were stained with hematoxylin and eosin. Representative light microscopy images from each group (A) were used to measure adipocyte area (B), adipocyte volume (C) and the frequency of adipocyte size (D). Total adipocyte number (E) was extrapolated from the size of adipocytes and the weight of rWAT. The values are the mean ± SEM of five fields per animal from three animals per group; different letters indicate significant differences calculated using one-way ANOVA statistical analysis. Differences were judged to be significant when $p \leq 0.05$.

Given these modifications of adipocyte size and number in rWAT induced by RSV administration that suggest an improvement in adipose tissue functionality, some of the processes involved in adipose remodelling and lipid metabolism at gene expression level were evaluated to study the mechanisms by which adipose tissue remodelling could be improving whole body metabolism.

3.3. Resveratrol administration is able to modify mRNA expression of genes involved in adipogenesis and lipolysis in rWAT

Firstly, we studied PPARγ gene expression, which is really involved in adipogenesis regulation. A 30% reduction in expression was shown when CAF diet was administered (Figure 2D) and these levels were normalized again with the oral dose of 50mg/kg of RSV even though none of these changes were statistically significant. In lipogenesis gene expression, a dose-response pattern seems to be acting Firstly, we studied mRNA expression of some genes involved in lipogenesis adipogenesis and browning of rWAT in order to determine rWAT functionality. Then we evaluated the state of lipogenesis involved genes Fabp4, Lpl and then Leptin mRNA expression which is expressed according to adipose tissue quantity. In CAF diet fed rats, no significant changes were observed on Fabp4 expression (Figure 2A), which is involved in fatty acid uptake, and RSV doses did not show any significant effect either. CAF diet did not affect Lpl (Figure 2B) gene expression but RSV doses tended to reduce its expression at 100mg/kg and reduction was significant after 3 weeks of 200mg/kg RSV administration compared with the CAF diet group. Leptin mRNA levels in this adipose depot were not changed by the CAF diet in a significant manner (Figure 2C), although the higher doses of RSV showed a tendency to normalise its levels being only statistically relevant with the 200mg/kg RSV dose.

Regarding browning markers, the capacity of RSV administration to modulate changes of preadipocytes towards a beige/brown phenotype through Ucp1 mRNA expression analysis was evaluated. Nevertheless, no changes were seen in the analysis of Ucp1 mRNA levels due to the big inter-individual variations (Figure 2E).

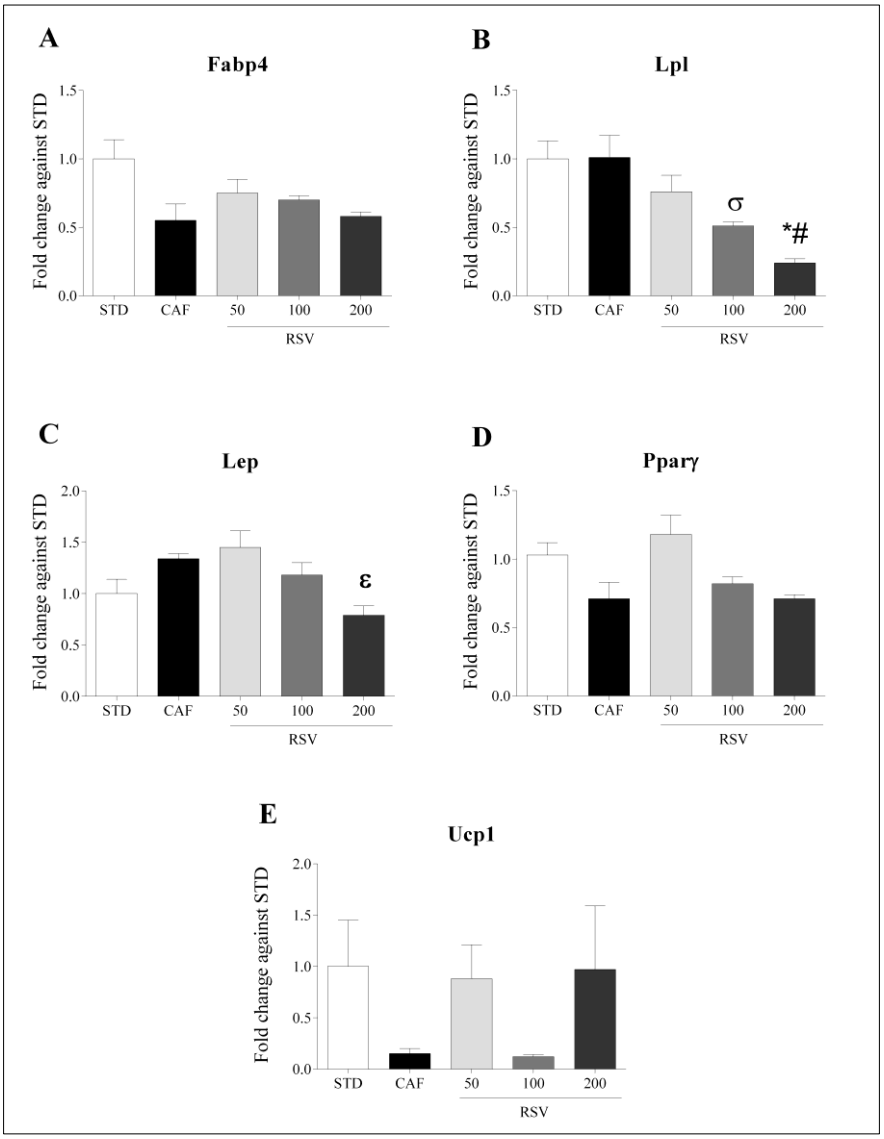

Figure 2: Effect of resveratrol administration on mRNA levels of genes related to adipogenesis and browning in retroperitoneal white adipose tissue of obese rats.

Rats were fed a standard chow diet (STD group) or cafeteria diet (CAF) for 11 weeks. After 8 weeks, CAF-fed animals were administered with 50 mg RSV/kg body weight (BW) (RSV 50 group), 100 mg RSV/kg BW (RSV 100 group), 200 mg RSV/kg BW (RSV 200 group) or the vehicle (CAF group) for 3 weeks. (A) Fatty acid binding protein 4 (Fabp4) mRNA levels; (B) Lipoprotein Lipase mRNA levels; (C) Leptin (Lep) mRNA levels; (D) Peroxisome proliferator-activated receptor gamma (PPARγ) mRNA levels and Uncoupling protein1 (Ucp1) mRNA levels. The values are the mean ± SEM of 6 animals per group. Statistical analyses were performed using one-way ANOVA. Symbols indicate significant differences against standard group (*) when p≤0.05 and (σ) when there was a tendency p≤0.1, significant differences against CAF group (#) when p≤0.05 and tendency against CAF group (ε) when p≤0.1.

3.4. Resveratrol administration modifies mRNA levels of lipid metabolism genes differently in rWAT and liver.

Gene expression was also analysed in both liver and rWAT samples to study the effect of CAF diet and RSV doses on lipid metabolism, this study was carried by analysing mRNA changes in Fasn, implied in fatty acid synthesis; Cpt1 (Cpt1b as the rWAT isoform and Cpt1a as the liver isoform) which is essential in metabolising fat in order to obtain energy; Abca1, relevant in reverse cholesterol transport.

In rWAT, CAF diet feeding for eight weeks slightly enhanced the expression of Fasn (Fig 3A) in rWAT and liver. Moreover, all doses of RSV normalised this induction by CAF diet in rWAT. No changes were observed in Cpt1b (Figure 3B) in rWAT neither by the diet nor by the RSV administration. CAF diet repressed Abca1 mRNA levels in rWAT (Figure 3C) and only a 50mg/kg of RSV administration was able to counteract this effect. Moreover, we wanted to study the effect of diet and/or RSV administration on the expression of Irs2 in rWAT (Figure 3D), which mediates insulin effects in adipose tissue. Irs2 was reduced in a 30% with CAF diet while RSV administration together with CAF diet of 100 and 200 mg/kg doses was able to normalize its levels back.

Regarding liver, Fasn mRNA levels were significantly enhanced by CAF diet (Figure 3E) and administration of 100mg/kg and 200mg/kg of RSV normalised this increase in mRNA levels. Furthermore, Cpt1a expression was reduced in around 40% in CAF fed animals compared with the standard diet group (Figure 3F) and when RSV was administered for three weeks it increased its levels in a dose-response manner, reaching the higher and significant increase with the 200mg/kg oral dose of RSV. Finally, Abca1 mRNA in hepatic tissue showed no changes with diet and/or RSV administration (Figure 3G).

Thus, these results indicate that RSV administration altered, at molecular level, mediator genes of lipid metabolism differently in liver and rWAT.

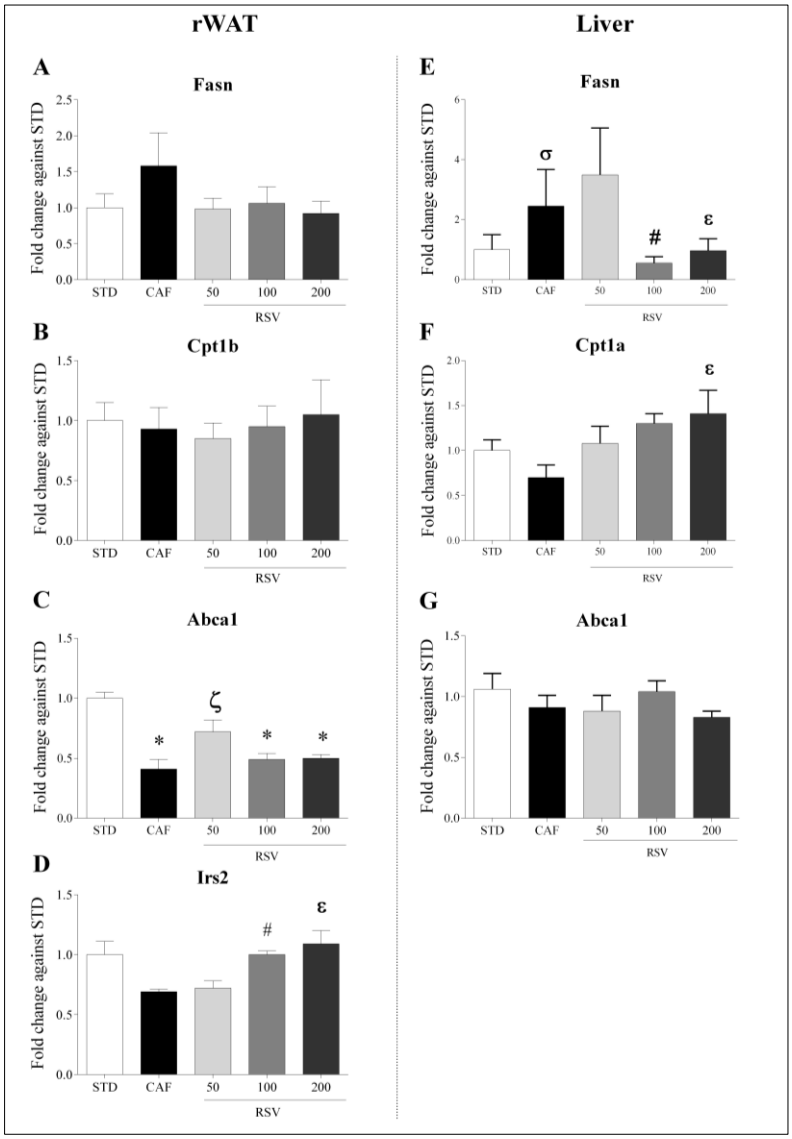

Figure 3: Effect of resveratrol administered on mRNA levels of genes related to lipid metabolism in retroperitoneal white adipose tissue and in liver of diet-induced obese rats. Rats were fed a standard chow diet (STD group) or cafeteria diet (CAF) for 11 weeks. After 8 weeks, CAF-fed animals were administered with 50 mg RSV/kg body weight (BW) (RSV 50 group), 100 mg RSV/kg BW (RSV 100 group), 200 mg RSV/kg BW (RSV 200 group) or the vehicle (CAF group) for 3 weeks. (A) Fatty acid synthase (Fasn) mRNA levels in rWAT; (B) carnitine palmitoyltransferase 1B (Cpt1b) mRNA levels; (C) ATP binding cassette subfamily A member 1 (Abca1) mRNA levels; (D) Insulin receptor substrate 2 (Irs2) mRNA levels; (E) Fasn mRNA levels in liver; (F) Carnitine palmitoyltransferase 1A (Cpt1a) mRNA levels and (G) Abca1 mRNA levels in liver. The values are the mean ± SEM of 6 animals per group. Statistical analyses were performed using one-way ANOVA. Symbols indicate significate differences against standard group (*) when p≤0.05, significant differences against CAF group (#) when p≤0.05 and tendency against CAF group (ε) when p≤0.1.

3.5. Resveratrol regulated lipid metabolism by miRNA modulation in liver but not in rWAT

The expression of Fasn, Cpt1 and Abca1 are modulated, among other mechanisms, by miR-33a. Thus, we further investigated whether RSV could modulate lipid metabolism through the modulation of this miRNA in both rWAT and liver.

In rWAT no significant changes were observed with CAF diet over miR-33a although its levels were increased in a 30%. RSV administration of 50 and 200 mg/kg reduced the CAF increase in miRNA levels but only the higher dose was able to reduce its levels significantly compared to CAF diet (Figure 4A). Srebp2, which has been described to be the host gene of miR-33a, having it embedded within its introns, was not strongly modulated by CAF diet and only dose of 50mg/kg of RSV showed a tendency to increase its expression when compared to STD group (Figure 4B).

In liver, miR-33a levels were increased by the CAF diet and the doses of 100 and 200 mg/kg of RSV were significantly decreased (Figure 4C). In concordance, Srebp2 showed a slightly increase in expression with CAF diet and the three oral doses of RSV were able to reduce Srebp2 mRNA expression compared to CAF diet in a significant manner (Figure 4D) which suggests that the modulation of miR-33a levels by RSV is by means of the modulation of its host gene expression

The liver levels miR-122 were also determined as an important specific hepatic lipid regulator. CAF diet did not induce any significant increase in liver miR-122 levels and only 100mg/kg of RSV administration showed a tendency to decrease its levels compared to CAF group (Figure 4E).

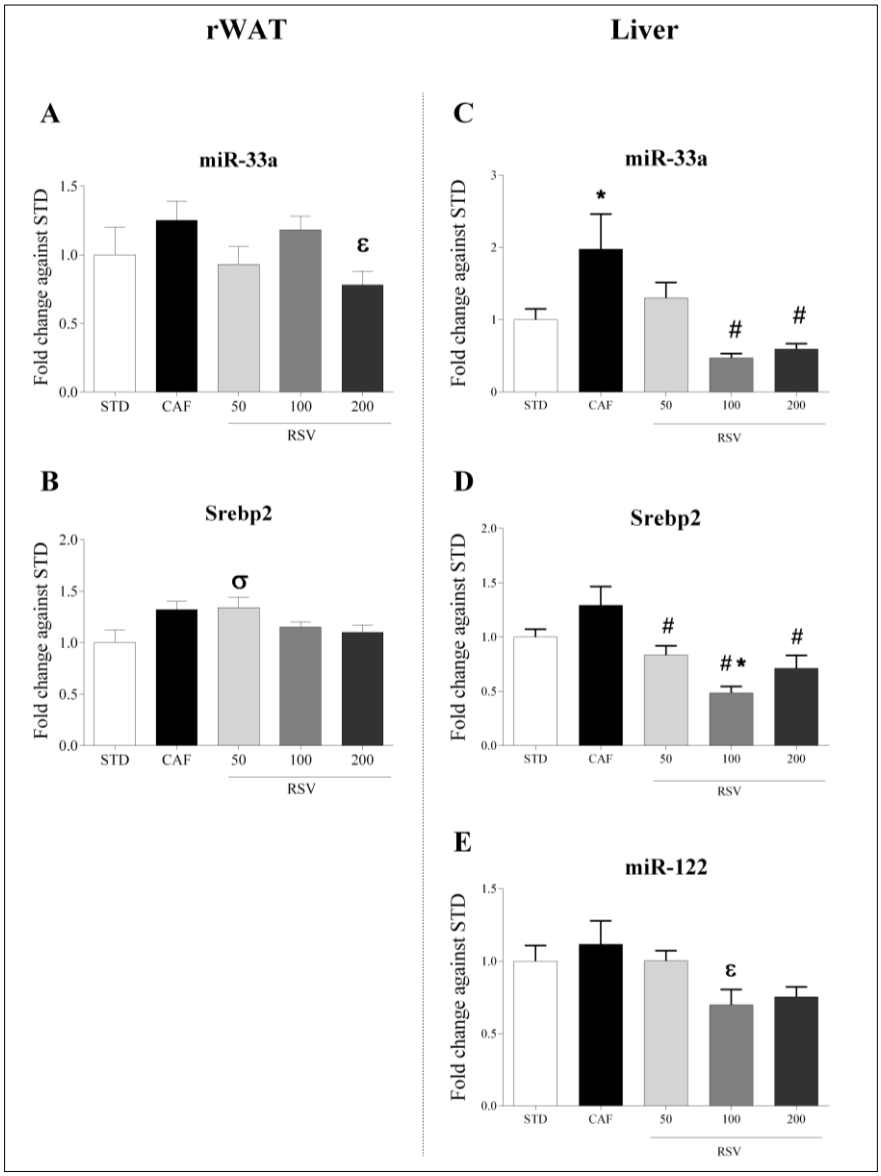

Figure 4: Effect of resveratrol administration on levels of microRNAs miR-33a and its host gene SREBP2 and miR-122 in retroperitoneal white adipose tissue and in liver of obese rats.
Rats were fed a standard chow diet (STD group) or cafeteria diet (CAF) for 11 weeks. After 8 weeks, CAF-fed animals were administered with 50 mg RSV/kg body weight (BW) (RSV 50 group), 100 mg RSV/kg BW (RSV 100 group), 200 mg RSV/kg BW (RSV 200 group) or the vehicle (CAF group) for 3 weeks. A) miR-33a levels in rWAT; (B) Sterol regulatory element binding protein 2 (Srebp2) mRNA levels; (C) miR-33a mRNA levels in liver; (D) Srebp2 mRNA levels in liver and (E) miR-122 levels in liver. The values are the mean ± SEM of 6 animals per group. Statistical analyses were performed using one-way ANOVA. Symbols indicate significate differences against standard group (*) when p≤0.05 and (σ) when there was a tendency p≤0.1, significant differences against CAF group (#) when p≤0.05 and tendency against CAF group (ε) when p≤0.1.

4. Discussion

In situations with an excessive energy surplus, like in obesity, the additional lipid accumulation can lead to dysfunctional hypertrophic adipocytes and to an impaired expandability of the adipose tissue. This process inside visceral fat depots has been correlated to a high risk of developing obesity-related diseases [38]. RSV has been demonstrated to ameliorate health state in obesity without affecting BW [23,39–42] and thus, we considered as seen with other polyphenols, that RSV could exert its beneficial effects on obesity through adipose tissue remodelling by counteracting hypertrophy and increasing adipocyte number once obesity is stablished. We tested rWAT as representative of visceral fat because it was shown to be more sensitive to modulation of adipocyte size and number by phenolic compounds than subcutaneous fat[30]. Furthermore, we wanted to see if miR-33a and miR-122 and their target genes related to lipid metabolism were involved in the mentioned positive effects of RSV.

The administration of RSV for 3 weeks together with CAF diet, once obesity was established, ameliorated the aberrant expansion of visceral rWAT, normalising adipocyte size and volume by reducing CAF-induced hypertrophy. Although BW was not modified, the expansion of adipose tissue when RSV was administered was produced by an increase in hyperplasia.

These results are in concordance with the effect of a grape seed proanthocyanidin extract on reduction of the hypertrophy induced by CAF diet and increased adipocyte hyperplasia [30]. Some other studies support these findings regarding phenolic compounds and the reduced visceral WAT hypertrophy in different models and also in human [43–46] but nothing is reported about increased hyperplasia. Furthermore, these works with RSV administered it since the beginning of experiments together with high-fat diet feeding, suggesting hypertrophic-preventive effects [47]. Otherwise, in the present work, RSV was administered during 21 days after obesity had been developed, showing marked RSV anti-hypertrophic and hyperplasic properties.

In order to analyse at the molecular level these results, PPARγ mRNA levels, which is known for being one of the central adipogenesis regulators was determined. PPARγ downregulation by CAF diet was reverted by 50mg/kg RSV dose although it was not significant some dose-response behaviour could be seen and this could be an explanation for the increased adipocyte number in RSV

groups. Many studies reported the contrary effects in PPARγ, as many of them reported reduced adipogenesis, although all of them were or *in vitro* or preventive *in vivo* studies with RSV. However, one of them reported adipogenesis upregulation in humans and they related it with increased an ameliorated adipogenic potential resulting in an increased adipose tissue expandability and insulin sensitivity [46].

Ucp1 is highly expressed in brown adipocytes but it has also been found UCP1-expressing adipocytes, called brite or beige, within WAT depots in response to determinate stimuli, and this increases energy expenditure inside WAT [48]. Some works have reported RSV could exert its anti-obesity effects through modulating energy expenditure in WAT by increasing Ucp1 expression [49]. Ucp1 mRNA levels were not affected by the RSV administration, however the interindividual variability was considerable and this could be masking some of the RSV effects. Regarding this fact, other groups have reported the same variability between individuals in Ucp1 mRNA levels in rWAT [50].

As Visceral WAT has been strongly related obesity-related complications and to metabolic syndrome, RSV remodelling of rWAT could have implications in ameliorating metabolic complications. In our results, rWAT healthier expandability could be supported by the results in TAG serum levels normalisation and improved glucose and insulin metabolisms. Furthermore, the higher RSV dose also normalised adiponectin levels suggesting improved adipose tissue state.

In view of the WAT improved expandability and the general metabolic state of animals after RSV administration, we studied RSV mechanisms at gene expression level. Gene expression analysis showed a tendency of RSV to reduce in a dose dependent manner the increased levels of, Lpl and Leptin by the CAF diet. These results are in agreement with the improved insulin sensitivity and restored TAG and glucose levels after the 3 weeks of RSV administration. Furthermore, leptin down-regulation by RSV suggests an improved adipose tissue state, given the fact that other works have related Leptin increased levels to adipose tissue inflammation [51]. In consistence with our results Lpl expression was also shown to be reduced by RSV administration exerting anti-obesogenic effects in animal models although RSV administration was given since the start of the studies [22,25].

Many phenolic compounds have been described for modulating the expression of miRNAs [52–54]. Concretely, an *in vitro* model in HepG2 showed RSV could modulate miR-33 and miR-122 by directly binding them [35]. Thus, we studied whether the improvement of lipid metabolism by RSV could by modulated by the regulation of miR-33 in both liver and rWAT as well as its host gene Srebp2 and miR-122 in liver. Consequently, some of their target genes involved in lipid and glucose metabolism could be modulated through these mechanisms. Cpt1, Abca1, Irs2 and Fasn have been shown to be targets of miR-33 or miR-122 [55–58]. In the present study, mRNA levels of Abca1, which is known to regulate reverse cholesterol transport [59], were down-regulated by CAF diet and were only restored when a dose of 50mg/kg of RSV was administered , however, miR-33a levels were not in concordance with these results . Fasn and Cpt1b (WAT isoform) [60] mRNA expression in rWAT showed a tendency to be normalised by RSV administration after CAF diet-induced dysregulations, suggesting a reduction in lipogenesis and an increase in fat oxidation, which could be the reason why fat depots showed a reduction in weight even though they were not statistically significant. The lack of changes in rWAT Fasn levels was supported by the work of Lopes K. *et al.* which could not find significant changes with HFD or RSV administration [61]. However, miRNA modulation of these three genes by RSV was not evident in rWAT suggesting that RSV effects were not driven via miRNA modulation in rWAT. For this reason, we wanted to test miR-33a and miR-122 levels in liver because they are well-described as hepatic lipid regulators and liver is the main organ involved in lipid metabolism. Regarding liver, Abca1 has been previously described by our group to be modulated by GSPE in diet-induced obese rats playing important roles in cholesterol homeostasis [59]. Fasn and Cpt1a hepatic levels were dysregulated by CAF diet and RSV restored them being more effective again in higher doses supporting the findings in rWAT with respect to lipogenesis and lipid oxidation status suggesting the effects are not only happening in rWAT but also in liver. Abca1, Cpt1a and Fasn are miR-33 target genes. Considering this, miR-33 showed a concordance with Cpt1a levels, down-regulated miR-33 was associated with increased levels of Cpt1a and vice-versa. In addition, Fasn has also been described to be an indirect miR-122 target gene in liver. Fasn and miR-122 showed a similar expression pattern although it was not significant at all RSV doses. Furthermore, once seen improvements by pharmacological doses of RSV in lipid metabolism and

given the improvement regarding glucose and insulin metabolites in serum, we evaluated Irs2 gene expression in rWAT, a miR-33a target which participates in insulin signalling. Irs2 was overexpressed with RSV higher doses compared to CAF diet group demonstrating beneficial effect in insulin sensitivity and endorsing the recovery of glucose and insulin metabolites in serum with 100 and 200 mg/kg/BW RSV doses. Matching these results, other polyphenols have also been described to improve insulin sensitivity through Irs2 modulation in enterocytes and aorta of rats [62,63] . Irs2 levels showed some kind consistency with miR-33a levels concerning CAF effect and RSV administration but only at the higher dose of RSV (200mg/kg).

All in all, it seems that liver is still more sensible to pharmacological doses of RSV in gene expression-induced changes regarding lipid metabolism when compared to rWAT response. Furthermore, miRNA modulation by RSV appears to be more evident in liver although only high doses are effective. Moreover, Srebp2 is described to be the miR-33a host gene and to be implied on its regulation [64] however, in rWAT changes in miR-33a levels were not according to those in Srebp2 mRNA expression while in liver, miR-33a levels demonstrated the same expression pattern as Srebp2. However, previous studies showed no relationship between hepatic Srebp2 and miR-33 levels[35]. The differences could be explained because the mentioned study was performed *in vitro* with supplementation of the HEPG2 cell culture media with the pure RSV compound. It has been demonstrated that polyphenols can reach the tissues in some hours after ingestion but they arrive with different metabolised structures[65], which could exert different effects although there is not much research surrounding RSV effects on miR-33 levels and its repercussions on lipid metabolism.

This outcome illustrates again the higher sensitivity of liver gene expression modulation by CAF diet and RSV compared to rWAT.

In conclusion, RSV administration after a diet-induced obesity exerts improvements on the pathological state induced by obesity through reducing adipocyte hypertrophy and increasing adipocyte hyperplasia in visceral rWAT fat depot acting in a dose-dependent manner. The fat depot remodelling influences in a healthier rWAT expandability and this can be observable by increasing TAG storage capacity and glucose and insulin metabolism improvements, although it is not yet visible in body weight.

RSV does not modify lipid metabolism genes through modulating miRNA lipid regulators miR-33a and miR-122 in rWAT. Target genes of these miRNAs were modulated by RSV but showed unclear relationship with miRNA levels. This suggests that these genes in rWAT are modulated by other mechanisms or via other pathways in which RSV could be acting. However, liver showed to be closer related to miRNAs regarding lipid metabolism regulation.

We could also elucidate that liver is more prone to changes under RSV effects when talking about Srebp2 gene expression. This situation is also reflected in the levels of the encoded miRNA, miR-33a. This brings to mind a possible indirect relationship between RSV administration and miR-33a in liver through Srebp2 modulation.

It is relevant to keep in mind that other studies use RSV administration without significant anti-obesity results in similar dose administration and length experiments [40,66]. In the present study, an oral dose of RSV during 21 days was enough to exert clear improvements at physiological and gene expression levels, even with oral dose of 50mg/kg some benefits were already tangible.

Further research concerning oral dosage of RSV should be conducted in *in vivo* models of diet-induced obesity before recommending its consumption to humans. Even though beneficial effects are observed, different doses of RSV can exert diverse responses at gene expression level. As it is reviewed by Fernández-Quintela *et al.* [67] , several works have shown that RSV does not always follow a dose-response pattern when exerting anti-obesity effects.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Primer sequences used for real time qPCR analysis.

Gene	*Primer sequences (5' - 3')*	*Product size (bp)*	*Gen Bank accession no/reference*
Abca1	TTGGATGGATTATATTGGACTGC TGGTCTCATTGAAAGCTTCTCTC	118	NM_178095.2
Cpt1a	TATCGTCGCACATTAGACC CATCTATGACCTCCTGGCA	751	NM_031559.2
Cpt1b	GCAAACTGGACCGAGAAGAG CCTTGAAGAAGCGACCTTTG	180	NM_013200.1
Fasn	TCCCAGGTCTTGCCGTGC GCGGATGCCTAGGATGTGTGC	260	Sawano T. *et al.* [1]
Fabp4	AGAAGTGGGAGTTGGCTTCG ACTCTCTGACCGGATGACGA	103	NM_053365.1
Irs2	CTCTTTGCCCCGCTCTTACA GGAAGGCACTGCTGAGTGAT	193	NM_001168633.1
Lep	CATTTCACACACGCA GTC GG GCA AGC TGG TGA GGA TCT GT	137	NM_013076.3
Lpl	ACTGGTGGGACAGGATGTGG CCGTTCTGCATACTCAAAGTTAGG	196	Kroupa O. *et al.* [2]
Pparγ	AGGATTCATGACCAGGGAGTT AGCAAACTCAAACTTAGCCTCCAT	79	Dovinová I. *et al.* [3]
Ppia	CTTCGAGCTGTTTGCAGACAA AAGTCACCACCCTGGCACATG	138	NM_017101.1
Ucp1	CGAGCCAAGATGGTGAGTTCGACA GTGGTGATGGTCCCTAAGACACCT	200	NM_012682.2
Srebp2	GTCCTCACCTTCCTGGGTCT CAGCAGTAGAGTCGGCATCA	168	NM_001033694.1

Abbreviations: Abca1: ATP binding cassette subfamily A member 1; Cpt1a: carnitine palmitoyltransferase 1 Alpha; Cpt1b: carnitine palmitoyltransferase 1 Beta; Fabp4: Fatty acid binding protein 4; Fasn: fatty acid synthase; Irs2: insulin receptor substrate 2, Lep: Leptin; Lpl: Lipoprotein Lipase; Pparγ: peroxisome proliferator-activated receptor gamma; Ppia: cyclophilin; Ucp1: Uncoupling protein 1 and Srebp2: sterol regulatory element binding transcription factor 2.

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V.GENERAL DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
ADIPOSE TISSUE REMODELLING BY POLYPHENOLS IN OBESE RATS
Aïda Pascual Serrano

Obesity is considered the epidemic of the 21st century[1] and additionally its prevalence is increasing worldwide. Obesity is a risk factor for many metabolic diseases, such as dyslipidemia, hypertension, type 2 diabetes and, eventually, cardiovascular disease (CVD) [2]. Remarkably, dysfunctional adipose tissue is strongly related to the development of these metabolic complications associated with obesity [3]. White adipose tissue (WAT) expands via hyperplasia and/or hypertrophy to store the energy surplus in the body. However, under conditions of a continuously excess of energy supply and/or decline of energy expenditure, excessive adipocyte hypertrophy occurs which triggers dysfunctional WAT. In this sense, several studies indicate that hypertrophy is linked to increased risk of developing obesity-related metabolic diseases [4,5]. Otherwise, hyperplasia protects against metabolic alterations [6,7]. Thus, hyperplasia is a healthier way to expand WAT than via hypertrophy.

Polyphenols are bioactive compounds that improve pathologies associated to obesity even without a significant body weight or fat mass reduction. Thus, in this Thesis it was hypothesised that the remodelling of WAT could be a mechanism by which polyphenols could improve metabolic diseases related to obesity. In this context, it has been studied the capacity of different types of polyphenols (i.e.; proanthocyanidins, gallic acid and resveratrol) to prevent and /or counteract WAT dysfunction associated to obesity.

Proanthocyanidins are one of the more abundant polyphenols in the human diet [8] and the Nutrigenomics group has an extensive experience in this class of polyphenols. Previous studies of our group have evidenced that a grape seed proanthocyanidin extract (GSPE), can improve several disorders associated

to obesity such as insulin resistance [9], hypertension and CVD risk [10,11], leptin resistance[12], inflammation[13] and dyslipidaemia[14,15] among others [16].
Nevertheless, GSPE did not reduce the body weight significantly. Hence, this Thesis firstly addressed whether adipose tissue remodelling could participate on the GSPE-mediated beneficial effects in obesity.

Chapter 1 studies the capacity of GSPE to prevent WAT dysfunctionality induced by an obesogenic diet. For that, rats were treated simultaneously with a cafeteria diet and GSPE for 12 weeks, a time long enough to develop obesity and adipocyte hypertrophy in control animals. In addition, we were also interested in determining whether proanthocyanidins could be effectivity preventing WAT dysfunctionality at pharmacological doses or at doses equivalents to those of human diets rich in proanthocyanidins. Thus, rats were administered with 25, 100 or 200mg of proanthocyanidins/kg of body weight. Because of the strong correlation between visceral WAT and metabolic complications associated to obesity [17], the study was centred in retroperitoneal WAT as representative for visceral WAT.

The daily administration of GSPE at any dose did not reduce the adiposity index. Nevertheless, GSPE administration prevented the increase in the area and volume of adipocytes in a dose-dependent manner. Remarkably, the adipocyte number was increased when the rats were fed a cafeteria diet with the GSPE, reaching an 82% increase in adipocyte number at 200 mg GSPE/kg body weight. Thus, GSPE administration together with the cafeteria diet improved retroperitoneal WAT expansion by preventing adipocyte hypertrophy and increasing adipocyte hyperplasia during obesity development in a dose-dependent manner. Therefore, the increase in the capacity of WAT to expand by GSPE may be a good strategy to prevent the failure of WAT and to avoid lipid

accumulation in other organs such as liver [18]. Many studies have linked hypertrophy with dyslipidaemia [5] and, accordingly, animals supplemented with GSPE improved the hypertriglyceridemia induced by the cafeteria diet.

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In order to find out the possible mechanism by which proanthocyanidins remodel WAT we used the highest dose of GSPE to study the expression of genes related to adipogenesis and browning. At molecular level, GSPE enhanced the expression of the adipogenesis master regulator PPARγ in agreement with the hyperplasia induced by these polyphenols. Remarkably, Sirt1, which is a repressor of PPARγ [19], was downregulated by GSPE. Indeed, other studied have also indicated that Sirt1 is a target of proanthocyanidins in the liver and hypothalamus [20,21]. Therefore, proanthocyanidins could modulate PPARγ expression via Sirt1 modulation. Although remodelling of white to brown adipose tissue could be one of the ways by which GSPE remodels WAT and increases energy expenditure, no changes were observed in mRNA expression of Ucp1, Pgc1a, or Prdm16, which are considered regulators of the browning process in WAT [22,23]. Additionally, Prdm16 has been reported to be regulated by miR-133 expression [24–26]. However, neither Prdm16 nor miR-133 were modulated by GSPE.

Proanthocyanidins are able to remodel WAT in conditions of an excess of energy preventing WAT hypertrophy despite maintaining adipose index. From human perspective, it is important to find out new strategies to combat obesity and its related pathologies. Thus, the aim of the next study was to determine if proanthocyanidins could reverse adipocyte dysfunctionality characteristic of obesity. To reach this objective, rats were supplemented with GSPE once obesity was already stablished. Rats were fed with a cafeteria diet and once obesity was

noticeable, a dietary dose of GSPE (25mg/kg/bw) was administered for three additional weeks together with the cafeteria diet. Besides proanthocyanidins, GSPE also contains gallic acid, a phenolic acid from the family of non-flavonoid polyphenols. Therefore, the contribution of gallic acid to the GSPE capability to remodel WAT was also investigated. Thus, another group of rats was supplemented with a proportional dose of the gallic acid present in GSPE (7mg/kg/bw). Visceral and subcutaneous fat depots have different metabolic and functional characteristics and can respond differently to proanthocyanidins. Hence, we studied retroperitoneal WAT and inguinal WAT as representative of visceral and subcutaneous fat depots, respectively.

GSPE or gallic acid supplementation for 3 weeks, when obesity was already established, did not reduce body weight or reverse the subcutaneous and visceral fat-pad accretion. However, GSPE reversed adipocyte hypertrophy and tripled the adipocyte number in retroperitoneal WAT. In contrast, GSPE supplementation only tended to reduce adipocyte area and volume in inguinal WAT without any significant effect on adipocyte number. These results highlight that visceral fat was more sensitive than subcutaneous fat to GSPE. This fact is relevant since visceral adipose tissue dysfunction has been associated with dyslipidaemia and the development of CVD [27,28]. Otherwise, gallic acid was not as effective as GSPE reducing hypertrophy, suggesting that the anti-hypertrophic effect of GSPE could be mainly attributed to proanthocyanidins while both proanthocyanidins and gallic acid could contribute to the hyperplasic GSPE activity. This anti-hypertrophic effect of GSPE was associated to an amelioration of glucose and lipid homeostasis. Therefore, GSPE is also effective at improve WAT expansion and recover WAT functionality when obesity is already stablished.

Further, we focus on the processes involved in adipose remodeling including adipogenesis, extracellular matrix and vascularization. Because visceral WAT

showed more sensitivity to GSPE, we studied the expression of key genes of these processes in retroperitoneal WAT.

None significant effects were observed on the expression of Pref-1 and PPARγ by GSPE because of the high variability in expression between rats of the same group. However, the results suggest that GSPE increased the number of preadipocytes and favored their differentiation into mature adipocytes. A pathological adipose tissue enlargement brings limited angiogenesis resulting in hypoxia [29]. Remarkably, GSPE induced VEGF expression, indicating enhanced angiogenesis and WAT vascularization thereby avoiding hypoxic conditions associated to WAT dysfunctionality.

Furthermore, GSPE supplementation overexpressed Plin1 and Fabp4, indicating an increased capacity of visceral fat to store and mobilize triglyceride, and restored adiponectin expression. Altogether, these results indicated that a three-week GSPE supplementation deeply improves visceral WAT expandability and functionality once obesity is completely stablished.

Proanthocyanidins and gallic acid are compounds from flavanol and phenolic acid groups. Thus, we further studied resveratrol as a representative polyphenol of the stilbene family. Even resveratrol is not as abundant in human diet, it has been widely studied and holds well-described beneficial effects on health and specifically on metabolic syndrome [30,31] .

Thus, we tested pharmacological doses of resveratrol in diet-induced obese animals. After rats developed obesity by cafeteria diet feeding, animals were administered with 50, 100 or 200 mg resveratrol/kg body weight together with the obesogenic diet for three weeks. This study was restricted to

retroperitoneal adipose tissue as it was the most sensitive fat pad to proanthocyanidins.

Similarly, as the other studied polyphenols, resveratrol reduced hypertrophy and increased hyperplasia in visceral WAT remodelling it in a dose-dependent manner. This remodelling seems to induce a healthier expandability of adipocytes because resveratrol also increased the triacylglycerol storage capacity and repressed leptin expression in visceral fat as well as improved serum glucose and insulin level. Regarding molecular mechanisms by which resveratrol induce hyperplasia, resveratrol also targets PPARγ like proanthocyanidins. However, the effect of resveratrol on PPARγ was not clear because only the dose of 50mg resveratrol/kg body weight was able to restore PPARγ levels in adipocytes.

As resveratrol modulates lipid metabolism in adipocytes and it has been described to modulate specific miRNAs *in vitro*[32] and *in vivo*[33], we further evaluated if these effects were mediated by the modulation of the lipid regulator miR-33 in visceral adipocytes. However, resveratrol did not significantly modulate miR-33 levels. The liver is a key organ for lipid homeostasis, thus the effects of resveratrol on hepatic lipid metabolism was evaluated by measuring the expression of miR-33 and miR-122 and their target genes in the liver. Results showed that resveratrol controlled some of these target genes improving lipid metabolism via miR-33 repression that was significant from 100 mg resveratrol/Kg body weight. Remarkably, Srebp2, which is the host gene of miR-33, showed the same profile expression patter than miR-33, indicating that resveratrol represses miR-33 via Srebp2 modulation.

These studies demonstrated that three different types of polyphenols, of both flavonoids and non-flavonoids types, have the capacity to remodel adipose

tissue in a healthier phenotype indicating a more functional adipose tissue that, in turn, can contribute to the beneficial effects ascribed to polyphenols in obesity-associated pathologies.

Diet and exercise are the conventional therapies for obesity treatment and its related pathologies. However, these interventions are not successful because of the difficulty of reaching a significant body weight loss and, specially, keeping this loss for long-time[34,35]. Therefore, the supplementation of regular diets with polyphenol-rich foods able to improve obese-related pathologies could be a good approach against obesity.

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VI. CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
ADIPOSE TISSUE REMODELLING BY POLYPHENOLS IN OBESE RATS
Aïda Pascual Serrano

1. Chronic consumption of proanthocyanidins prevents visceral aberrant adipocyte morphology induced by an obesogenic diet through increasing adipocyte number and reducing adipocyte size at both dietary and pharmacological doses, in a dose-dependent manner. According to these results, proanthocyanidins also prevented the hypertriglyceridemia, pointing out a healthier state after proanthocyanidins consumption.

2. A dietary dose of proanthocyanidins administered in obese animals counteracts the anomalous adipose tissue expansion characteristic of obesity through reducing hypertrophy in visceral and subcutaneous white adipose tissue and by increasing hyperplasia in visceral adipose tissue. Thus, proanthocyanidins are more effective remodelling visceral adipose tissue than subcutaneous fat.

3. A dietary dose of proanthocyanidins overexpress VEGF in visceral adipose tissue in obesity. Therefore, proanthocyanidins could also remodel adipose tissue by increasing angiogenesis and restoring the oxygen supply to adipocytes. Remarkably, according to the overall improvement of adipose tissue expansion, proanthocyanidins also normalise Plin1, Fabp4 and adiponectin expression in adipose tissue, increase insulin sensitivity and restore glucose and lipid homeostasis and in obese rats.

4. Proanthocyanidins induce PPARγ expression in visceral adipose tissue in both corrective and preventive animal models. Thus, PPARγ could be a target of proanthocyanidins that link with the hyperplasic effect of these polyphenols.

5. A dose of gallic acid, equivalent to the amount of gallic acid in the extract of proanthocyanidins, increases visceral adipose tissue hyperplasia without any significant effect on adipocyte size in obese animals. Thus, gallic acid seems to contribute to the increase in adipocyte number induced by proanthocyanidins contained in grape seed whereas it does not participate to the anti-hypertrophic effect.

6. Pharmacological doses of resveratrol counteracts hypertrophy and induce hyperplasia in visceral white adipose tissue in a dose-dependent manner correcting the anomalous adipose tissue expansion characteristic of obesity. According to these results, resveratrol also increases triacylglycerol storage capacity in adipose tissue, improves plasmatic glucose and insulin levels and repress leptin gene expression in adipocytes, pointing out a healthier state after resveratrol consumption.

7. Resveratrol modulates miR-33 expression in the liver but not in adipose tissue. The modulation of miR-33 by resveratrol is through a regulation of its host gene Srebp2. Moreover, resveratrol modulates miR-33 target genes in the liver, which indicate an improved lipid metabolism via Srebp2 and in turn miR-33 regulation.


VII. ANNEX

UNIVERSITAT ROVIRA I VIRGILI
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1. Resum

Els trastorns metabòlics vinculats a l'obesitat es correlaciona amb una disfunció en la capacitat d'expansió del teixit adipós blanc (TAB), la qual es produeix a través de la hipertròfia (augment de mida d'adipòcits) i/o la hiperplàsia (augment del nombre d'adipòcits). La hipertròfia s'associa a trastorns relacionats amb la obesitat mentre que la hiperplàsia protegeix envers alteracions metabòliques com la resistència a la insulina i la dislipèmia. Els polifenols presents en els aliments han mostrat millorar diferents components de la síndrome metabòlica (SM) tals com el metabolisme lipídic i l'acumulació de greix tot i no reduir el pes corporal. L'objectiu de la present tesi era avaluar l'efectivitat de diferents polifenols en prevenir o contrarestar la disfunció del TAB en la obesitat mitjançant la seva remodelació. Per assolir-lo es van emprar dosis dietètiques o farmacològiques d'un extracte de proantocianidines de pinyol de raïm (GSPE), d'Àcid Gàl·lic (GA) o de Resveratrol (RSV) en estudis *in vivo* en rates amb obesitat induïda per la dieta. Es van realitzar anàlisis histològics en TAB per avaluar canvis en mida i nombre d'adipòcits. A més, es va analitzar l'expressió gènica de marcadors d'adipogènesi, lipòlisi, "browning", oxidació lipídica i inflamació en els TABs així com els metabòlits circulants per observar l'impacte de la remodelació del TAB a nivell de l'organisme complet. Els resultats mostraren que diferents polifenols de diverses famílies prevenen i/o reverteixen la disfunció del TAB induïda per l'excés d'energia en la obesitat. Aquesta remodelació del TAB s'associa amb millores en l'homeòstasi dels metabolismes lipídic i glucídic. Per tant, la remodelació del TAB podria contribuir als efectes beneficiosos dels polifenols en les malalties associades a la obesitat i la SM. Per tant, la incorporació d' aliments rics en polifenols a la dieta o nutricèutics podria servir com estratègia en el tractament de trastorns metabòlics en l'obesitat.

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