A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME

Miguel Martin González

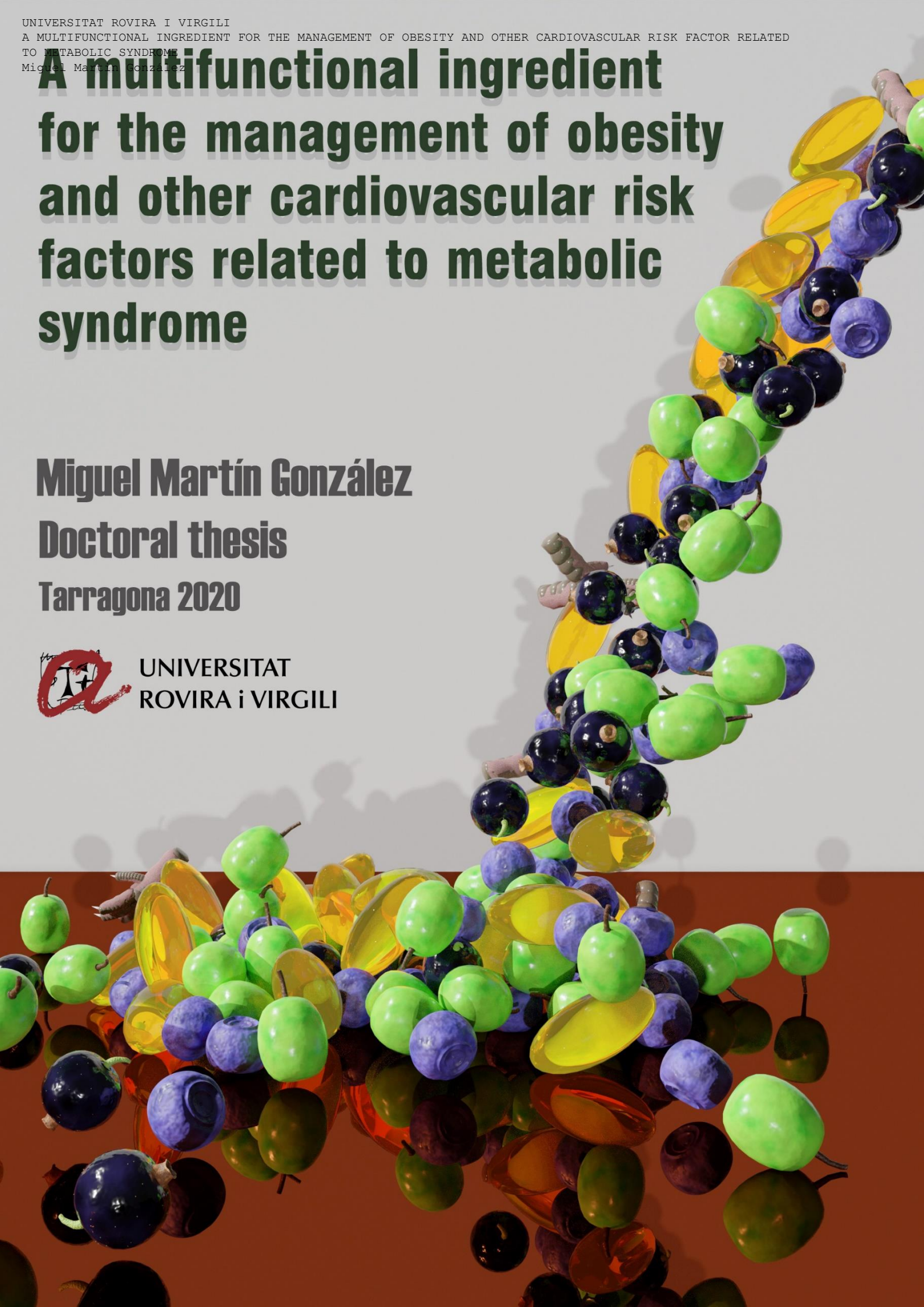
A multifunctional ingredient for the management of obesity and other cardiovascular risk factors related to metabolic syndrome

Miguel Martín González

Doctoral thesis

Tarragona 2020

UNIVERSITAT ROVIRA i VIRGILI



UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Miguel Martín González

A multifunctional ingredient for the management of obesity and other cardiovascular risk factors related to metabolic syndrome

Doctoral Thesis

Supervised by Dr. Begoña Muguerza Marquínez

And

Dr. Gerard Aragonès Bargalló

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY
NUTRIGENOMICS RESEARCH GROUP

UNIVERSITAT ROVIRA i VIRGILI

2020

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI

DEPARTAMENT DE BIOQUÍMICA I BIOTECNOLOGIA
Campus Sescelades
43007-Tarragona (SPAIN)
(+34) 977 559 521

FEM CONSTAR que aquest treball, titulat "**A multifunctional ingredient for the management of obesity and other cardiovascular risk factors related to metabolic syndrome**", que presenta Miguel Martín González per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica y Biotecnologia de la Universitat Rovira i Virgili i que compleix els requisits per a l'obtenció de la Menció Internacional de Doctorat.

HACEMOS CONSTAR que el presente trabajo, titulado "**A multifunctional ingredient for the management of obesity and other cardiovascular risk factors related to metabolic syndrome**", que presenta Miguel Martín González para la obtención del título de Doctor, ha sido realizado bajo nuestra dirección en el Departamento de Bioquímica y Biotecnología de la Universitat Rovira i Virgili i que cumple con los requisitos para la obtención de la Mención Internacional de Doctorado.

WE STATE that the present study, entitled "**A multifunctional ingredient for the management of obesity and other cardiovascular risk factors related to metabolic syndrome**", presented by Miguel Martín González for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology from the Universitat Rovira i Virgili and that is eligible to apply for the International Doctoral Mention.

Tarragona, 5 de febrer 2020

Els directors de la Tesi doctoral
Los directores de la Tesis doctoral
Doctoral Thesis supervisors

Dr. Gerard Aragonès Bargalló

Dr. Begoña Muguerza Marquínez

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

A mis padres, a Aurora y a Nessy
A mis amigos y compañeros
A Niurka y Rosa y a Rosa y Niurka

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Index

Index

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Summary

Obesity is defined as an excess of fat accumulation that represents a risk to health. It frequently occurs concurrently with other metabolic risk factors related to metabolic syndrome (MetS), such as dyslipidaemia, insulin resistance or non-alcoholic fatty liver disease (NAFLD). Many food bioactive compounds have been identified and further investigated for their ability to prevent obesity and its metabolic associated pathologies. Among them, conjugated linoleic acid (CLA) is one of the dietary bioactive compounds most investigated for weight management, although controversial metabolic results have been reported. However, the use of a single family of bioactive compounds could not be sufficient to correct multisystemic and highly regulated situations such as obesity and its associated metabolic pathologies. Thus, the aim of this Thesis was to evaluate whether the simultaneous co-administration of different bioactive compounds, as a multifunctional ingredient (MIX), including CLA, a mixture of grape-seed proanthocyanidins and berry anthocyanins, and the protein hydrolysate from chicken feet Hpp11, could reduce obesity and its associated cardiometabolic risk factors in a much more effective way than its individual administration. Our results demonstrated that the administration of an equal ratio of the CLA isomers *c9,t11* and *t10,c12* at low doses caused a decrease in the body weight gain induced by a cafeteria diet, and improved other cardiometabolic risk factors, without presenting any CLA-related adverse effects. In addition, the body weight lowering effect of CLA was higher when it was co-administered with the chicken feet hydrolysate Hpp11 and a mixture of proanthocyanidins and anthocyanidins. This anti-obesity effect, which could be mediated by an improvement of hypothalamic leptin sensitivity, was also not accompanied by any adverse effect of weight loss. On the contrary, MIX produced an improvement on glucose and lipid metabolism and exhibited antihypertensive properties. Thus, MIX could be a good candidate to be used as nutraceutical or to be included in functional foods for the management of metabolic syndrome.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Resumen

La obesidad se define como una acumulación anormal o excesiva de grasa que puede ser perjudicial para la salud. Con frecuencia aparece de forma conjunta con otros factores de riesgo metabólico relacionados con el síndrome metabólico, como son la dislipidemia, la resistencia a la insulina o el hígado graso no alcohólico. Se han identificado e investigado diferentes compuestos bioactivos debido a su capacidad para prevenir la obesidad y sus enfermedades relacionadas. Entre ellos, el ácido linoleico conjugado (CLA) es uno de los compuestos más investigados para el control del peso corporal, aunque se han notificado diferentes efectos adversos asociados a su consumo. Sin embargo, el uso de una sola familia de compuestos bioactivos podría no ser suficiente para corregir de forma eficaz enfermedades multisistémicas y altamente reguladas como la obesidad y el síndrome metabólico. Por ello, el objetivo de la presente Tesis ha sido evaluar si la co-administración simultánea (MIX) de diferentes compuestos bioactivos (incluido el CLA, una mezcla de proantocianidinas de pepita de uva y antocianinas de bayas, y el hidrolizado proteico de patas de pollo Hpp11) reduce la obesidad y/o sus factores de riesgo cardiometabólicos asociados de una manera mucho más eficaz que su administración individual. Nuestros resultados demostraron que la administración de los isómeros de CLA *c9,t11* y *t10,c12* en la misma proporción y en dosis bajas provoca una disminución de la ganancia de peso corporal inducido por una dieta de cafetería, y, a la vez, mejora otros factores de riesgo cardiometabólico sin presentar ningún efecto adverso relacionado con el propio consumo de CLA. Además, se observó que la reducción del peso corporal producida por el CLA fue mucho mayor cuando el CLA se administró conjuntamente con el hidrolizado de pata de pollo Hpp11 y la mezcla de proantocianidinas y antocianidinas. Este efecto sobre el peso corporal, que podría estar mediado por una mejora de la sensibilidad a la leptina a nivel hipotalámico, tampoco se acompañó de ningún efecto adverso asociado a la propia pérdida de peso. Al contrario, la co-administración de los diferentes compuestos produjo una mejora notable en el metabolismo de la glucosa y de los lípidos y mostró un efecto antihipertensivo muy marcado. Por lo tanto, nuestro ingrediente funcional podría ser un muy buen candidato para ser utilizado como nutracéutico o para ser incluido en alimentos funcionales para la prevención y control del síndrome metabólico.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Resum

L'obesitat es defineix com una acumulació anormal o excessiva de greix que pot ser perjudicial per a la salut. Sovint apareix de forma conjunta amb altres factors de risc metabòlic relacionats amb la síndrome metabòlica, com són la dislipidèmia, la resistència a la insulina o el fetge gras no alcohòlic. S'han identificat i investigat diferents compostos bioactius degut a la seva capacitat per a prevenir l'obesitat i les seves malalties relacionades. Entre ells, l'àcid linoleic conjugat (CLA) és un dels compostos més investigats per al control del pes corporal, tot i que s'han notificat diferents efectes adversos associats al seu consum. No obstant això, l'ús d'una sola família de compostos bioactius podria no ser suficient per a corregir de manera eficaç malalties multisistèmiques i altament regulades com l'obesitat i la síndrome metabòlica. Per això, l'objectiu de la present Tesi ha estat avaluar si la co-administració simultània (MIX) de diferents compostos bioactius (inclòs el CLA, una barreja de proantocianidines de llavor de raïm i antocianines de baies, i l'hidrolitzat proteic de pota de pollastre Hpp11) redueix l'obesitat i/o factors de risc cardiometabòlics associats a l'obesitat d'una manera molt més eficaç que la seva administració individual. Els nostres resultats van demostrar que l'administració dels isòmers de CLA *c9,t11* i *t10,c12* a la mateixa proporció i en dosis baixes provoca una disminució del guany de pes corporal induït per una dieta de cafeteria, i, alhora, millora altres factors de risc cardiometabòlics sense presentar cap efecte advers notori relacionat amb el propi consum de CLA. A més, es va observar que la reducció del pes corporal produïda pel CLA és molt més gran quan el CLA s'administra conjuntament amb l'hidrolitzat de pota de pollastre Hpp11 i la barreja de proantocianidines i antocianidines. Aquest efecte sobre el pes corporal, que podria estar mediat per una millora de la sensibilitat a la leptina a nivell hipotalàmic, tampoc es va acompanyar de cap efecte advers associat a la pròpia pèrdua de pes. Tot just al contrari, la co-administració dels diferents compostos va produir una millora notable en el metabolisme de la glucosa i dels lípids i va mostrar un efecte antihipertensiu molt marcat. Per tant, el nostre ingredient funcional podria ser un molt bon candidat per a ser utilitzat com a nutracèutic o per a ser inclòs en aliments funcionals per a la prevenció i control de la síndrome metabòlica.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

List of abbreviations

^{1}H-NMR: Proton nuclear magnetic resonance

ABCA1: ATP-binding cassette transporter 1

ABCG1: ATP-binding cassette sub-family G member 1

AC: Adenylyl cylclase

ACAT: Acyl-CoA:cholesterol acyltransferase

ACC: Acetyl-CoA carboxylase family

ACE: Angiotensin-converting enzyme

ACE 2: Angiotensin-converting enzyme 2

ACL: ATP-citrate lyase

AGrP: Agouti-Related Protein

ASBT: Apical sodium-dependent bile acid transporter

APOA: Apolipoprotein family

ARA: Arachidonic acid

ATGL: Adipose triglyceride lipase

ATP: Adenosine triphosphate

BAT: Brown adipose tissue

BCAA: Branched amino acid

BCAT: Branched-chain amino acid transferase

BCKA: Branched-chain α-keto acid

BCKD: Branched-chain α-keto acid dehydrogenase

BP: Blood pressure

CAF: Cafeteria diet

cAMP: Cyclic adenosine monophosphate

CD11C: Integrin, alpha X

CD163: Cluster of differentiation 163

CD36: Cluster of differentiation 36

ChREBP: Carbohydrate-responsive element-binding protein

CLA: Conjugated linoleic acid

CoA: Coenzyme A

CREB: cAMP response element-binding

CRP: C-reactive protein

CVD: Cardiovascular disease

CYP7A1: Cholesterol 7α-hydroxylase

DAPI: 4',6-diamidino-2-phenylindole

DHA: Docosahexaenoic acid

ELOV1: Elongation of very long chain fatty acids protein 1

eNOS: Endothelial nitric oxide synthase

EPA: Eicosapentaenoic acid

ET-1: Endothelin-1

eWAT: Epididymal white adipose tissue

F6P: Fructose-6-phosphate

FA: Fatty acids

FABPs: Fatty-acid-binding proteins

FAS: Fatty acid synthase

FATP: Fatty acid transport protein family

FGF21: Fibroblast growth factor 21

G6P: Glucose 6-phosphate

GCK: Glucokinase

GKRP: Glucokinase regulatory protein

GLUT2: Glucose transporter 2

GLUT4: Glucose transporter 4

GOT/AST: Glutamic oxaloacetic transaminase

GPT/ALT: Glutamic pyruvic transaminase

GSK-3: Glycogen synthase kinase 3

GSPE: Grape seed proanthocyanidin extract

HDL: High-density lipoprotein

HFD: High fat diet

HMGCR: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase

HOMA-β: Homeostasis model assessment of β-cell function

HOMA-IR: Homeostasis model assessment of insulin resistance

Hpp11: Chicken foot protein hydrolysate

HSD: High sucrose diet

HSL: Hormone-sensitive lipase

HTN: Hypertension

IL-1β: Interleukin 1-β

IL-6: Interleukin 6

IL-10: Interleukin 10

IRS-1: Insulin receptor substrate-1

JAK-2: Janus kinase 2

JNK: c-Jun N-terminal kinase

LC-PUFA: Long-chain polyunsaturated fatty acids

LCFAs: Long-chain fatty acids

LDL: Low-density lipoprotein

LPL: Lipoprotein lipase

MC4R: Melanocortin receptor 4

MCF: Mitochondrial carrier family

MCP1/CCL2: Monocyte chemoattractant protein 1

MetS: Metabolic syndrome

MGL: Monoglyceride lipase

MUFA: Monounsaturated fatty acids

mWAT: Mesenteric white adipose tissue

NAD+: Nicotinamide adenine dinucleotide

NAFL: Non-alcoholic fatty liver

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NAPDH: Nicotinamide adenine dinucleotide phosphate

NCEH: Neutral cholesteryl esterase

NEFA: Non-esterified fatty acids

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NO: Nitric oxide

NOX-4: NADPH oxidase

NPY: Neuropeptide Y

NMR: Nuclear magnetic resonance

OBRb: Obese gene receptor b

PA: Proanthocyanidins

PC: Pyruvate carboxylase

PDC: Pyruvate dehydrogenase complex

PDK4: Pyruvate Dehydrogenase Kinase 4

PEPCK-C: Cytoplasmic phosphoenolpyruvate carboxylase

PFK: 6-phosphofructo-1 kinase

PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K: Phosphatidylinositol 3-kinase

PK4: Protein kinase 4

PKA: Protein kinase A

PKCe: Protein kinase Ce

POMC: Pro-opiomelanocortin

PPAR: Peroxisome proliferator-activated receptors family

PPIA: Peptidylprolyl isomerase A

PTP1B: Protein tyrosine phosphatase 1B

PUFA: Polyunsaturated fatty acids

QUICKI: Quantitative insulin sensitivity check index

RAAS: Renin-angiotensin-aldosterone system

ROS: Reactive oxygen species

rWAT: Retroperitoneal white adipose tissue

SBP: Systolic blood pressure

SCDs: Stearoyl-CoA desaturases

SHR: Spontaneously hypertensive rat

SIRT-1: Sirtuin-1

SOC: Suppressors of cytokine signalling family

SREBP-1C: Sterol regulatory element-binding protein-1c

SREBP-2: Sterol regulatory element-binding protein-2

STAT3: Signal transducer and activator of transcription 3

STD: Standard diet

TCA cycle: Tricarboxylic acid cycle

TLR4: Toll-like receptor 4

TMAO: Trimethyl amino oxide

TNF-α: Tumor necrosis factor α

UCP1: Uncoupling protein 1

UCP3: Uncoupling protein 3

VCO_2: Volume carbon dioxide production

VEGF: Vascular Endothelial Growth Factor

VH: Vehicle

VLDL: Very low-density lipoprotein

VO_2: Volume oxygen consumption

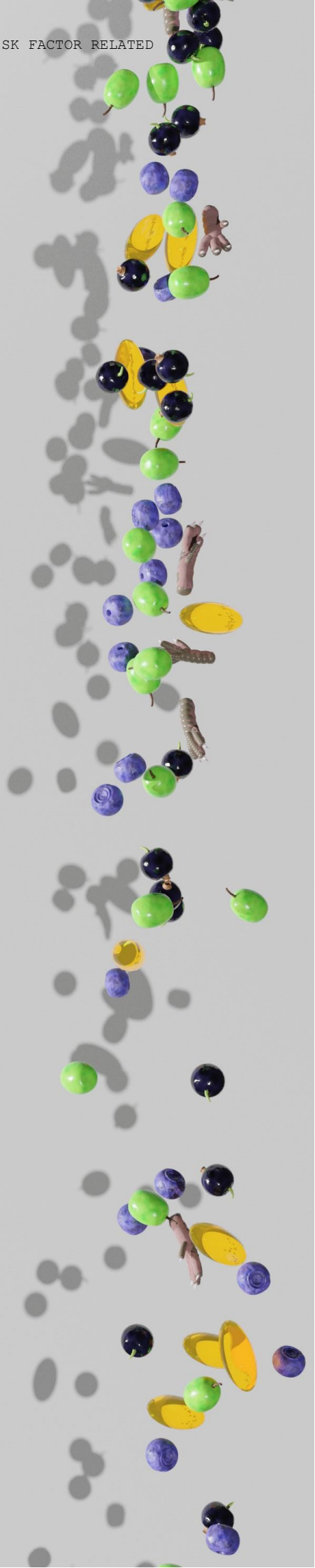
WAT: White adipose tissue

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

INTRODUCTION



UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

1. Definition, diagnosis and epidemiology of metabolic syndrome

Crucial changes have taken place in nutritional habits and food intake over the last few decades in western countries. The means of food production have increased, making it cheaper and more productive, implying an easier access to food and more abundance. Besides, markets have shifted towards more appetizing food, aiming to reach a larger market by including fats and sugars that improve tastes but increase calories as well [1].

A tendency has recently been found in western and developing countries, as more people are including processed, high-calorie content and sugar-rich foods that are changing the classic intake patterns and resulting in a higher consumption than actually needed for pure nourishment [2]. This excess food intake might not be bad by itself, but these changes in habits also include social aspects, as modern life is becoming more sedentary which reduces the need for energy consumption. These two mentioned issues have become increasingly correlated in recent years which has led towards a yearly increase in the percentage of obese people [3].

Obesity can be superficially understood as a positive imbalance in the individual's energy homeostasis, meaning that they are accumulating more energy than they are consuming. As metabolism stores energy in the form of fat, this results in an increase in fat mass. The positive imbalance, therefore, means an excess in food intake, or energy intake, that is not compensated by an equal increase in energy expenditure, being malnourishment the opposite side of the imbalance [4]. This simple equation explains the origin of obesity but does not take into account important facts that alters both energy intake and expenditure in the development of this illness as it is affected by genetics, other diseases and/or stress [5,6].

Obesity is, in fact, considered an illness by the World Health Organization and is being labelled as an epidemic due to the speed at which it is increasing in prevalence in western and developing countries [7]. An accumulation of energy reserves could not be health detrimental by itself, but it has been found that obesity is associated with several pathologies that greatly harm the normal homeostasis, known as metabolic syndrome (MetS) [8].

MetS is a set of symptoms that encompasses deep changes in a healthy metabolic status of an individual and make them prone to develop other cardiovascular diseases (CVD) besides increasing the risk of cancer, non-alcoholic fatty liver disease (NAFLD) and other health complications [9]. Obesity and the usually subsequent MetS have driven interest for a precise definition, over the concern of obesity's spread in western society [10], as a necessary way to standardize international clinical procedures. A patient is considered to have developed MetS when at least 3 of the 5 symptoms shown in **table 1** are present [9]. A symptom is also considered present when the patient is already under

drug treatment for its correction. MetS has been defined as a set of parameters and associated values, as a tool for easy measurement and therefore, easy prediction of health status of patients and their progression, thus, allowing foreseeable outcomes from treatment management and prognosis of recommended life changes.

Table 1. Symptoms of Metabolic syndrome.

	Men	**Woman**
Elevated waist circumference	≥ 102 cm	≥ 88 cm
Lowered high density lipoprotein cholesterol	< 40 mg/dl	< 50 mg/dl
	No gender differences	
Elevated triglycerides	≥ 150 mg/dl	
High blood glucose	≥ 100 mg/dl	
Elevated systolic blood pressure	≥ 130 mm Hg (Systolic)	≥ 85 mm Hg (Diastolic)

Obese individuals tend to accumulate fat ectopically, in places that is not usually present, in the waist, muscle and liver. Such accumulation is particularly harmful in liver, as it induces NAFLD, which could progress towards non-alcoholic steatohepatitis (NASH) when fibrosis and inflammation is developed [11]. The problem that leads to this accumulation of fat in liver comes from several sources as there could be an increase in the *de novo* production of fat in the liver itself (lipogenesis), an increase in the uptake of fatty acids (FA) from the bloodstream, a decrease of fatty acid oxidation or secretion or a combination of them [12]. This dysregulation of the normal lipid homeostasis in the liver is not well known but is related with a decrease in hepatic sensitivity to insulin and leptin, a common problem associated with obesity, which will be discussed further [12]. While fatty liver is the most health detrimental of the ectopic fat accumulation, is not easily ascertainable. However, waist accumulation is measurable, therefore, it is usually regarded when assessing the health status of patients and is considered one of the symptoms of the MetS. This accumulation of waist fat mass is mainly in the form of triglycerides, as that is the main form the body uses for the storage of energy. This excessive storage is also easily assessed in the bloodstream, as triglycerides levels are usually found elevated in patients with obesity and is considered another symptom of the MetS [13].

In addition, obesity is associated with an increase in other lipids such as cholesterol. Contemporarily, elevated cholesterol levels are considered a health risk, increased chances of coronary thrombosis and the onset of other cardiovascular complications, plus an indication of a dysregulation of lipid metabolism [14]. This molecule is a core component in the production of hormones and lack of it is detrimental in the normal regulation of their metabolism, implying that certain concentration levels and food supplementation must be maintained [15]. It is because of its importance that its regulation is key for a normal metabolism and it is indeed tightly regulated, therefore raising alarms as elevated concentration means either an excessive intake or the body having problems to regulate its levels. However, cholesterol is present in many different molecules depending on where it is transported to and from.

Cholesterol absorbed from food intake is packed in chylomicrons particles in the gut to be absorbed and processed in the liver. Low-density lipoprotein cholesterol (LDL-C) particles are responsible for the transporting of cholesterol from liver to the peripheral tissues. Initially the liver forms very low-density lipoprotein (VLDL-C) particles, rich in triglycerides and cholesterol. Triglycerides will later be hydrolysed, concentrating cholesterol, and evolving the VLDL-C particles into intermediate-density lipoproteins (IDL-C) and, subsequently, to LDL-C that will bind cells from peripheral tissues and unload cholesterol [16]. High-density lipoprotein cholesterol (HDL-C) particles, on the other hand, are responsible for the transportation of excess cholesterol from peripheral tissues to the liver for the processing and removal of cholesterol in the form of bile salts [17], into what is known as reverse cholesterol transport. The particles conformation and synthesis are a combined effort between the intestines and the liver, as HDL-C particles are conformed by apolipoproteins, mainly apolipoprotein A-I (APOA-I) and APOA-II. APOA-I is synthesized by both liver and intestines, adding to up to 70% of total lipoproteins of HDL-C while APOA-II is exclusively synthesized by liver, conforming 20% of HDL-C [18]. The rest of the non-lipidic portion of the HDL-C is conformed by other proteins carrying task of cellular communication, regulation of interactions with HDL-C and modulation of lipid metabolism [19].

Low levels of HDL-C have been linked to CVD and are considered one of the symptoms of the MetS. Studies linking HDL-C and CVD have found that they follow an inverse correlation, showing that increases in HDL-C reduces the risks of developing CVD [20]. Higher levels of HDL-C help prevent CVD as these particles show antioxidative properties by providing protection against free radicals and the subsequent formation of pro-inflammatory oxidized lipids [21]. HDL-C also provides beneficial effects over hypertension as it has been found to increase the expression of endothelial nitric oxide synthase (*eNOS*), thus generating an increase in nitric oxide (NO), a known atheroprotective compound [22].

In this regard, hypertension is another possible outcome from obesity and it is considered one more of the symptoms of the MetS [23]. While the progression of blood pressure (BP) has been found to increase with age [24], regardless of diet, obese patients tend to have a characteristically high BP. In addition, obese patients have a high disposition to develop insulin resistance, a key factor that it is believed to have major effects over BP [25]. Commonly obese patients develop high concentrations of insulin in blood, a hormone that will promote activation of the sympathetic nucleus system, inducing mechanisms to counteract the excess of energy reservoirs, among them, a rise in BP. This adaptive mechanism regulates the energy homeostasis but becomes an issue if the incidence of hyperinsulinemia persists for long periods of time [26]. A similar process is induced with hyperleptinemia, another common occurrence among obese people [27]. Deeper studies of insulin in regard to hypertension have shed light towards supplementary mechanisms for the development of hypertension as insulin promotes an increase of angiotensin II receptors, activating angiotensin's role of vasoconstriction [28].

Similarly to triglycerides, a common outcome from unhealthy feeding habits are persistent elevated levels of glucose in blood once a certain state of obesity has been developed [29]. Under normal circumstances, blood glucose levels remain fairly stable throughout the day, thanks to the control by insulin and the release of glucose from liver and adipose tissue. Under postprandial state, glucose intake from food will raise its concentration in blood, which will be dealt with by increasing levels of insulin, promoting lipogenesis [30] and glycogenesis in liver [31]. Under fasting conditions, insulin levels will diminish, causing the liver to release and produce glucose and ketone bodies to supply mainly the brain and other peripheral tissues [29]. Obese individuals who have developed type II diabetes are not capable of a fine adjust their glucose levels and they remain high. After prolonged times of excessive calories intake, insulin resistance might arise in peripheral organs, particularly liver and adipose tissue, being liver the main culprit of glucose impairment as it is the major provider. Due to this, parallelly to high levels of glucose, hyperinsulinemia is developed as the pancreatic β-cells release more insulin, trying to cope with the low insulin sensibility. In addition to the process, these cells, once accurate in measuring circulating glucose, lose the capacity for proper perception, hence, aggravating the control of glucose with impaired insulin secretion [29,32].

2. Physiopathology of metabolic syndrome

The onset of the MetS is easily preventable and can be averted without health repercussions. However, its study has shown that once developed, the patient's metabolism undergoes fundamental changes that alters it in a permanent/semi-permanent way. This induces a propension to re-develop obesity and harming the functionality of some of the more important pathways of the metabolism, thus, generating other diseases [33,34]. Some of the symptoms of these inner changes are summarized as the symptoms of the MetS (**Table 1**) which provide an easily measurable way of assessing how the development of these other illnesses is going.

2.1 Obesity and overweight

The study of obesity and its development has quickly concluded that it is a multifactorial dysregulation of the homeostasis of a healthy individual. In fact, excessive fat accumulation alters the functionality of liver and white and brown adipose tissues, induces pathologies and dysregulation of hormone production and leads to hypertension.

2.1.1 Dysregulation of body weight and food intake. Hyperleptinemia

A hormone tightly related with obesity and fats mass homeostasis is leptin. When first discovered, leptin was found to control appetite, being directly but inversely correlating the lack of appetite with high levels of leptin [35]. In addition, later studies found that leptin's binding to its receptor over nucleus of the hypothalamus not only triggered behavioural responses but induced metabolic changes as well, related to an overall increase in fatty acid burning and a reduction in its storage and production [36]. This hormone is expressed by the adipose tissue, graduating its excretion depending on its fat reservoirs, as the bigger the fat mass, the more it gets secreted. Its secretion is also related to food intake as soon after a meal leptin is secreted as adipocytes increases their reserves [37]. Leptin secretion is also activated by insulin, which relates with the previous, as insulin concentration rises after a meal.

Its relationship with obesity is paradoxical as obese individuals are characterized by high levels of leptin. Given that its role is to reduce food intake, leptin do not seem to be reducing the hyperphagia classically associated with obese individuals, although it is being over-secreted as corresponded with large fat mass [38]. Adding to lack of decreased appetite, studies show that the homeostasis of the obese individual is not as strongly shifted towards the burning of fatty acids and falls more into its storage, than expected for the amount of fat mass [39].

This phenomenon is known as leptin resistance and happens after obesity has been developed for long enough as to affect the homeostasis of the individual. Both peripheral organs and brain nucleus respond lightly to the same concentration of leptin and, as with insulin resistance, higher levels of leptin are required to achieve the same response [40].

Regarding leptin's activity in the nucleus system, the main effector in the homeostasis of leptin is the hypothalamic arcuate nucleus, in particular, two populations of neurons sensitive to leptin of opposing functions: the neuropeptide Y (NPY), agouti-related protein (AGrP) and pro-opiomelanocortin (POMC) neurons. NPY and AgrP neurons are named after their orexigenic peptides production which role is to induce appetite, thus, increasing food intake and accumulation of fat reservoirs [41]. On the other hand, POMC cells' role is to produce the anorexigenic protein of the same name and induce hypophagia [42]. Their activity is of crucial importance as effectors of leptin functionality and are major systems for the control of energy homeostasis and fat reservoirs. At the molecular level, leptin promotes the STAT/JAK (Signal transducer and activator of transcription / Janus kinase) pathway activation in both POMC and NPY/AGrP neuronal groups, working in a similar way [43]. In addition to leptin activation, the activity of these neurons might be also activated with insulin, showing certain overlapping of their correspondent pathways [44].

These populations of leptin-sensitive neurons, once leptin resistance has developed, seem to be less responsive to leptin, from both external-artificial or internal-natural sources, needing higher concentrations of circulating leptin and hardly ever quite achieving the same effect, regardless of concentration [45]. Several mechanisms involved with leptin have been presented as putative sources and aggravating factors of this problem. Leptin is imported from blood at the blood-brain barrier via isoforms of the leptin receptor and studies with high fat diets (HFD) mice have found a suboptimal transportation through the barrier, although how substantial is its real impact remains unknown [46]. NPY, AgRP and POMC, leptin-sensitive neurons have also been found to endocyte parts of their membranes, reducing the presence of leptin receptors and, consequently, the leptin pathway activation [47]. Besides it, concordantly with obesity, a chronic state of inflammation is developed, affecting among others, the brain and hypothalamus. It has been found that fatty acids binding to toll-like receptor 4 (TLR4) in macrophages promote inflammation and food intake [48].

A responsive activation of leptin sensitive neurons in the nervous system is of major importance as many organs normal function is directly modulated and, regarding liver, the sympathetic nervous system stimulates glucose production and release of fuel sources into the bloodstream for the uptake and utilization of other peripheral organs, in times of exercise, whilst the parasympathetic nervous system reverses this effect, promoting the storage of fats.

2.1.1.1 Leptin resistance from a molecular perspective

The underlying process behind leptin resistance, being similar to insulin resistance it is not completely understood, and it happens in both the nervous system and peripheral organs [49]. Leptin's receptors are present in several organs including liver, nervous system, skeletal muscle or adipose tissue [50] and activates an important pathway, the STAT3 phosphorylation pathway. Circulating leptin binds to its receptor, the obese gene receptor b (OBRb), inducing the activation via autophosphorylation of JAK-2, which, in turn, phosphorylates tyrosine residues of the OBRb receptor. This leads to the activation of STAT-3 by its phosphorylation into STAT3-P which acts as a transcription factor that promotes expression of anorexigenic genes, changing the energy homeostasis. In addition, the activation of STAT3-P activates as well the transcription of *SOC-3* which, in turn, inhibits the action of OBRb, creating a negative feedback that attenuates and modulates the activity of leptin in liver [51,52]. The leptin receptor downstream signalling is shown in **figure 1**.

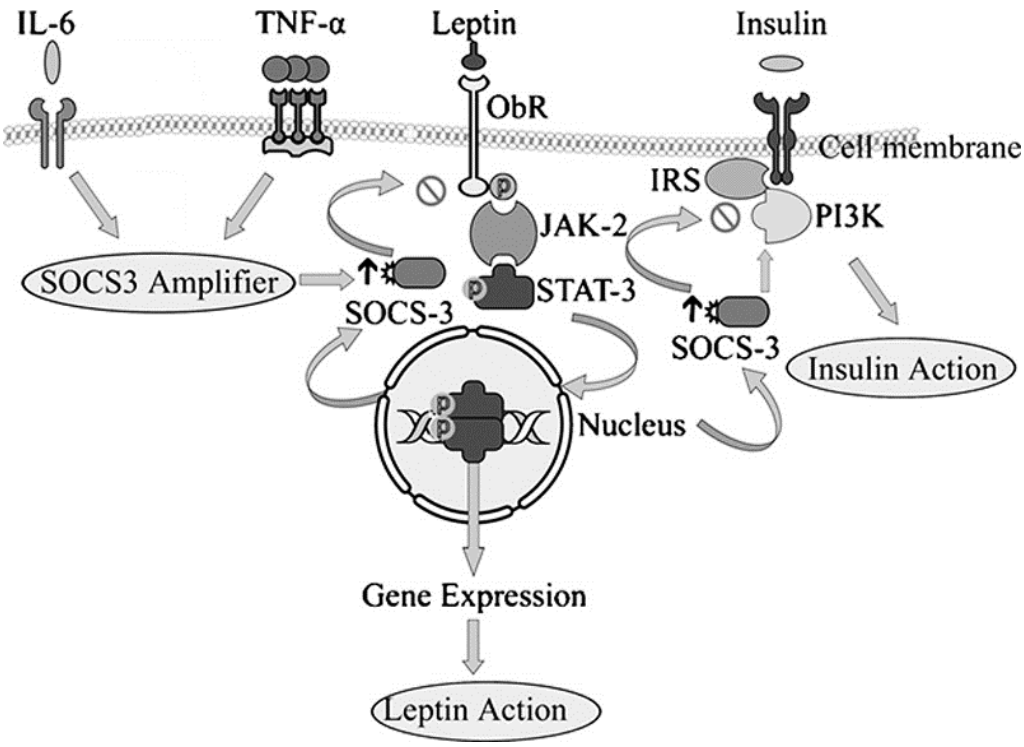


Figure 1. Leptin receptor's (OBR) pathway activation leads to changes in gene expression and induces different reactions depending on the tissue as the receptor is present in several organs. Activation of OBR activates JAK-2 (Janus kinase 2) which phosphorylates STAT3 (Signal transducer and activator of transcription 3), resulting in alteration of gene expression. This pathway is negatively regulated by SOC-3 (Suppressors of cytokine signalling 3) as it inhibits the receptor. The figure also explains the relationship between leptin activity and inflammatory cytokines, such as Il-6 (Interleukin 6) and TNF-α (Tumor necrosis factor α) as they over-activate SOC-3, reducing the sensitivity of the cell to leptin. The insulin and leptin signalling pathway is associated via SOC-3, also plays a role in modulating insulin response, as it alters the functionality of IRS (Insulin receptor substrate) and PI3K (Phosphatidylinositol 3-kinase). Figure adapted from [50].

SOC-3 is considered a major figure in the development of leptin resistance as its action is highly modulated but other factors besides leptin. Adipocytokines such as resistin and inflammatory factors like IL-6 and TNF-α are capable of binding into leptin receptors and induce an amplification of the activity of SOC-3, leading to an inhibition of leptin signalling [53]. Insulin has also been linked to increased activation of SOC-3 [54]. The accumulation of these factors in a tissue leads to what is known as leptin resistance, defined as the low activation of leptin pathways and its inability to reduce appetite and promote energy expenditure. Obese individuals are characterized by having high levels of glucose and triglycerides in blood, promoting over secretion of leptin and insulin. Once secondary problems like inflammation have appeared, the efficiency of leptin gets reduced, generating a negative loop that fails to promote weight loss.

2.1.2 Liver overview, role and association with MetS

The liver plays a major role in the body's homeostasis and the control of lipids and glucose metabolism. It is a central component of the flow of energy and it is in constant communication with other peripheral organs, such as skeletal muscle and adipose tissue, to better assess the needs of fuel according to the state of fasting or exercise [55]. The liver is also a major producer of glucose or fatty acids as an energy source. The liver's prime role in glucose metabolism is the release of glucose from gluconeogenesis, thanks to the skeletal muscle providing substrates such as lactate or pyruvate for this process [56]. Besides, the liver will supply the body with fatty acids, packed into VLDL-C particles and ketone bodies after β – oxidation of stored fatty acids, adding support to the release of non-esterified fatty acids (NEFA) from the white adipose tissue by lipolysis of triglycerides [57]. Given the importance of the liver's role in metabolism, a dysregulation such as obesity, has major harmful consequences over its function and health state.

2.1.2.1 Liver disease

A predisposition of people with MetS to develop NAFLD has been extensively documented and seems to have originated in a combination of multiple factors [58,59]. NAFLD is a loose term to define a wide spectrum of phenotypic changes in the liver, from slight asymptomatic accumulation of lipids in the hepatocytes that could develop into higher preponderance of fats, necrosis, cirrhosis and carcinoma [60]. NAFLD refers to non-alcoholic fatty liver (NAFL) and NASH, being NAFL a less harmful state of development with more than 5% of steatosis but no signs of hepatocyte damage, nor fibrosis that could potentially derivate to worse scenarios but has not yet taken a large impact in the functionality of the organ [61]. On one hand, NASH has been associated with the development of

cancer, shows major signs of apoptosis and cirrhosis and has greatly compromised regular functionality, which hints to a deep metabolic aberration and imbalance in the homeostasis of carbohydrates, lipids and energy [62]. In summary, NASH is considered a worse progression of NAFL when apoptosis, cellular infiltrations and inflammation are present, regardless of the presence of fibrosis [63,64]. The progression between the two has not been yet completely clarified (tissue examples shown in **figure 2**) and neither have a clear defined line between them, although the start of cellular damage establishes the onset of NASH, but prognosis might still be favourable and the disease reversible.

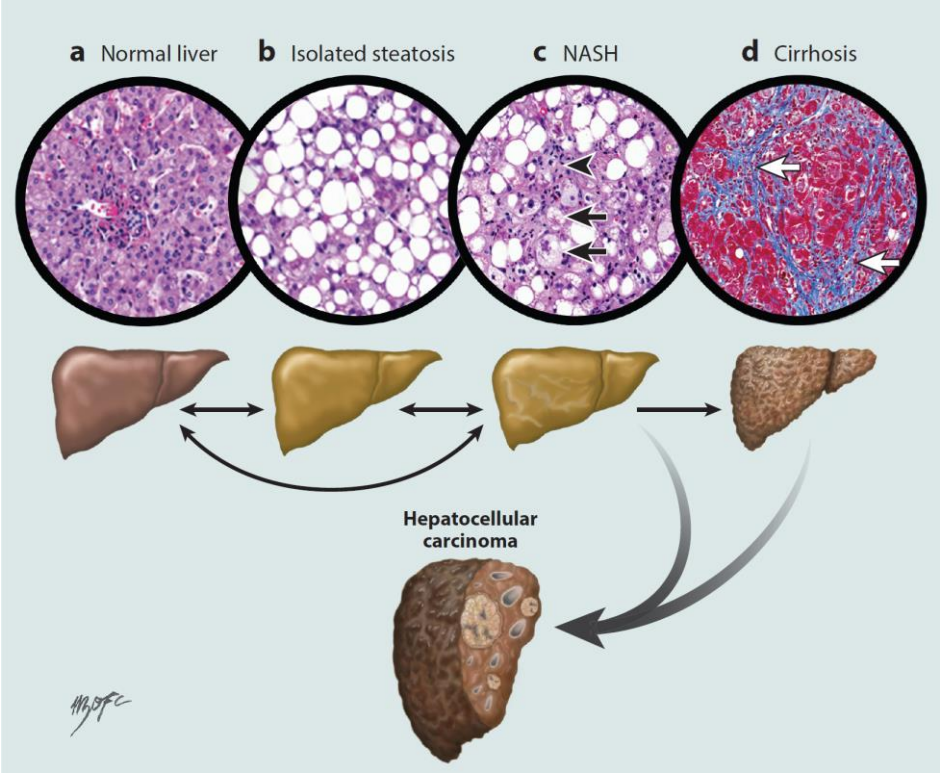


Figure 2. Progression of liver steatosis and tissue damage over increasing accumulation of lipids. Arrows show typical histopathological events that the liver undergoes as the disease progresses. Black arrows indicate hepatocyte cell ballooning (a form of apoptosis [65]) and white arrows show the collagen bands of fibrosis. NASH: Non-alcoholic steatohepatitis Figure obtained from [66].

2.1.2.2 Mechanisms of NAFLD

NAFLD is an excessive accumulation of lipids in the hepatocytes and is considered one of the main harmful outcomes from excess fat content in food intake. Factors leading to hepatic lipid accumulation are multifactorial, involving increased fatty acid influx, increased FA synthesis, altered FA oxidation and insufficient triglyceride secretion to prevent lipid accumulation [67–70]. These mechanisms could be co-ordinating to precipitate the development and progression of NAFLD. It has also been linked with obesity, being a predominant disease among obese patients, with a high probability of developing

among type II diabetes patients, whereas the development of both obesity and type II diabetes almost guarantees the subsequent development of NAFLD [63].

White adipose tissue is the main reservoir of lipids. It also greatly contributes to mobilization of lipids in the bloodstream by converting stored triglycerides into NEFA by lipolysis. NEFA are used in other organs as an energy source. However, the liver is one of the main regulators of them and they might become responsible for up to 60% of the lipidic content in the development of NAFLD [71]. Imported or synthetized lipids are stored in droplets in the form of triglycerides throughout the whole volume of the hepatocyte, also containing cholesterol esters. Their size and form may vary, being considered as macrovesicular steatosis whenever droplets are large, rounded and encompass a large volume of the hepatocyte, whereas, microvesicular steatosis is considered when the number of droplets greatly increases while their size decreases [72].

In fact, studies have linked the adipose tissue and overweight more directly responsible for the development of NAFLD. After long periods of excessive food intake, white adipose tissue expands to accumulate extra circulating lipids coming from food absorption, lowering their concentration in blood. While adipose tissues expand, they are infiltrated by macrophages, secreting pro-inflammatory cytokines that reduces insulin sensitivity of the adipose tissue, thus, blocking insulin-mediated suppression of lipolysis and promoting the breakdown of triglycerides and release of NEFA that will be mainly absorbed by the liver [73]. Among inflammatory cytokines, tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) play major roles in this process and suppress the secretion of adiponectin, which has been strongly correlated with fatty liver [74].

The liver itself is another producer of inflammatory cytokines and it has been postulated that excessive hepatic fat accumulation induces a state of local inflammation, similarly to the adipose tissue. This originates in oxidative stress of endoplasmic reticulum and could further worsen the NAFLD [75]. In fact, obese mice are prone to develop endoplasmic reticulum stress [76]. Furthermore, accumulation of fatty acids inside the hepatocyte results in an increase in β–oxidation, leading to the production of reactive oxygen species, further worsening the oxidative stress [77].

Several diagnostic tools are being used to analyse NAFLD. Proton Nuclear magnetic resonance (^{1}H-NMR) is being used to quantify the abundance of fatty acids, considering an accumulation of fat of 5.56% as the onset NAFLD [78]. Histological analyses are also performed as a cheaper and easier tool as NAFLD livers show macro- and micro-vesicular lipid accumulations. Histological studies also allow the assessment of whether NAFLD is developing into NASH and the presence and differentiation between micro- or macro- vesicular steatosis. This technique is of particular usefulness, as it easily shows the clear profile of macro-vesicles and the fuzzy presence of micro-vesicles. Quantification of

glutamic oxaloacetic transaminase (GOT/AST) and glutamic pyruvic transaminase (GPT/ALT) enzymes in serum are also been used to assess liver damage, which tends to correlate with fatty liver [79].

2.1.3 White adipose tissue and dysregulation under MetS

White adipose tissue (WAT) has always been considered as a fat reservoir as well as a heat insulator. In addition, other functions have been discovered, such as appetite modulation by secretion of the adipokine hormone leptin [80], inflammation regulation and control of the energy homeostasis [80,81]. WAT is a multifunctional organ whose distribution varies from other organs as it is spread throughout the whole body, implying a tight hormonal control of its fat accumulation and functionality. While WAT deposits are capable of quick expansion and reaches to wide localizations in the whole body, its main distribution divides it between visceral and subcutaneous compartments [82,83]. These two compartments are also divided: visceral WAT encompasses tissue deposits such as mesenteric (mWAT), retroperitoneal (rWAT) and epididymal (eWAT), depending on their localization; while subcutaneous WAT is usually divided into the anterior or posterior [82].

WAT is a diversely complex tissue as it englobes its main cell type, the adipocyte, plus others, such as macrophages, fibroblasts, mesenchymal and vascular cells. Adipocytes characteristic morphology is easily recognised as 80-90% of its cytoplasm is occupied by a big droplet of fat, mainly composed of triglycerides, displacing the nucleus to the side [84]. This droplet may vary greatly depending on physiological needs and overall fat reservoir, as WAT's role is finding a healthy equilibrium between the accumulation and release of fat due to energy needs. WAT may undergo hyperplasia (increase on adipocyte recount) or hypertrophy (increase of droplet size and, therefore, triglycerides content). Hypertrophy is usually associated with an increase in diet-induced fat accumulation.

The reservoir of triglycerides in the adipocytes are obtained by two ways: by *de novo* synthesis (process known as lipogenesis) or imported from the bloodstream. Triglycerides in the bloodstream usually come from diet, bound to proteins in lipoproteins, but mainly in the form of chylomicrons, secreted by the intestine after their absorption in the lumen, although they can also be absorbed from VLDL-C, produced by the liver or as free fatty acids bound to albumin (NEFA) [84]. Once these triglycerides-enriched particles reach the capillaries irrigating the WAT, their endothelial cells hydrolase the triglycerides, breaking them into glycerol and free fatty acids by action of the lipoprotein lipase (LPL). Fatty acids are then carried into the cells by transporters like cluster of differentiation 36 (CD36) and bound to fatty-acid-binding proteins (FABPs) so that they can be taken to the acyl-CoA synthase and esterified with coenzyme A (CoA) into acyl-CoA [84]. Regarding synthesis *de novo*,

glucose acts as a source, undergoing a series or reactions to ultimately form acyl-CoA, ground molecule for the production of triglycerides that are stored in the lipid droplet of the adipocyte.

WAT also acts as a distributor of energy whenever is needed. Since fat is in itself a reservoir of energy, WAT has a major role on energy homeostasis. This leads WAT to have a tight control of lipids released into the bloodstream. Lipids are exported in the form of NEFA, hydrolysed sequentially from triglycerides, first by adipose triglyceride lipase (ATGL) into diglycerides, second by hormone-sensitive lipase (HSL) into monoglycerides and, finally, into free fatty acids and glycerol by monoglyceride lipase (MGL) [85]. The release of fat from WAT occurs under fasting conditions as adipocytes are hormonally controlled by activation via β-adrenergic receptors (activated by norepinephrine) and glucagon receptors, both of them inducing the activation of the lipolysis pathway described. On the other hand, adipocytes are also inhibited from releasing lipids by insulin [86].

WAT's hormonal control goes both ways, being highly influenced by several hormones, whereas WAT is itself a major hormonal producer. WAT's other main function is the release of the adipokine hormone leptin into the bloodstream, whose production correlates positively with the fat body reserves [87]. Leptin levels are also influenced by other factors: insulin levels are increased after feeding triggering leptin production [88], sex hormones influence leptin secretion [89] and circulating fatty acids inhibit leptin production [90]. Leptin's relationship with insulin illustrates leptin's role as a modulator of energy reserves in regard to food intake, and, in fact, leptin works by modulating appetite [91]. Food intake increases insulin concentration, promoting leptin production; leptin reaches the hypothalamus where it triggers several responses, two of the main ones being a promotion of body energy expenditure and behavioural changes evidenced as a reduction of appetite [92].

However, leptin's tight control is dysregulated in obese individuals, diminishing leptin sensitivity, which means that leptin, after binding to its receptor, does not activate its pathway as strongly, generating a weaker response. The predominant consequences are an energy expenditure decrease and hyperphagia. This presents a clear positive feedback that further promotes obesity. Due to this, obese individuals are characterised by high levels of leptin [93]. Unfortunately, leptin sensitive changes have not yet been fully explained and many pharmacological drugs and scientific effort is directed towards inducing an amelioration of the sensitivity or fixing its dysregulation.

2.1.3.1 Inflammation

Concomitant with obesity, a low chronic level of inflammations tends to be developed [94], laying on the relationship between macrophages and adipocytes, as an inflammation feedback is developed. While macrophage's usual role is of debris cleaning and dead cells and pathogens elimination, it has

been shown that WAT keeps a stable population of macrophages, even in a healthy state. Healthy individual's WAT promotes an anti-inflammatory environment through a particular secretory pattern, pattern that is altered under diet-induced obesity [95]. However, additional macrophages could be recruited after immunological stimulus. These two types of macrophages population have been described and are easily labelled and differentiated by analyses of several markers and cytokine production. Infiltrated M2-macrophages are associated with lean-individuals, inducing an anti-inflammatory environment by the production of interleukin 10 (IL-10) and are recognised by an expression of cluster of differentiation 163 (*CD163*). On the other hand, M1-macrophages, marked by expression of integrin, alpha X (*CD11C*), get more predominant after diet-induction [96,97]. These macrophages are considered an "activated" form, associated with diet-induced obesity and elevated inflammation levels [98].

As adipocytes undergo hypertrophy, they release less anti-inflammatory (adiponectin) and more proinflammatory adipokines (such as TNF-α or monocyte chemoattractant protein 1 (MCP1)), promoting the recruitment and activation of M1-macrophages, which, in turn, further develops the inflammatory response. This process ends up in a low level, long-term, state of diet-induced systemic inflammation, WAT being its origin [99,100]. In fact, TNF-α or MCP1 are considered markers of inflammation and state an unhealthy metabolic state [101]. The described process is shown in **figure 3**.

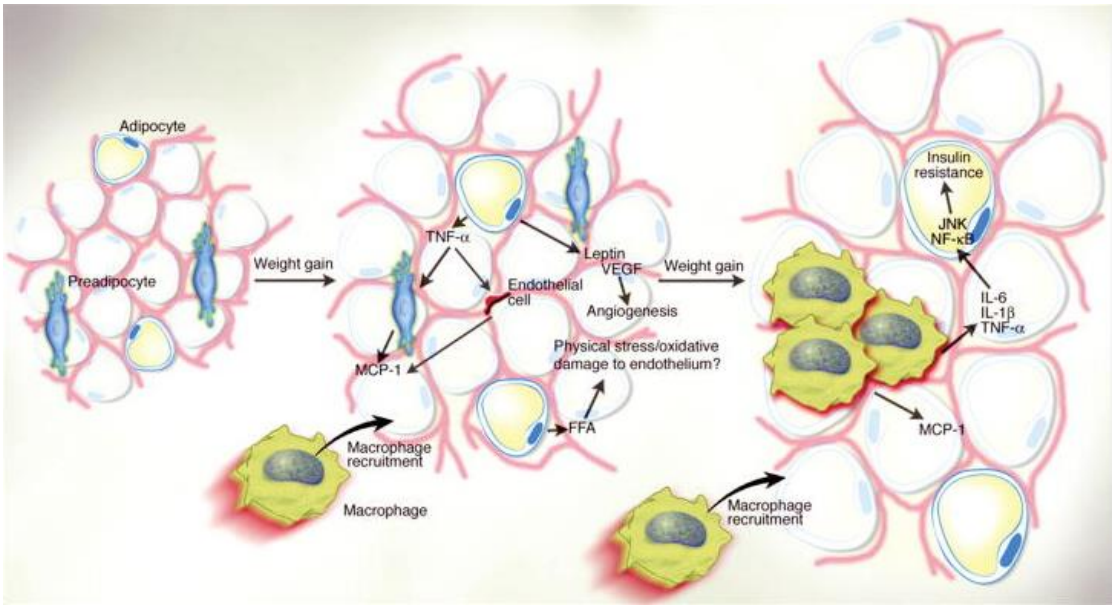


Figure 3. Aggravation of the inflammatory status on the adipose tissue after its hypertrophy. The recruitment of macrophages by the release of inflammatory cytokines such as TNF-α (Tumor necrosis factor alpha), IL-6 (interleukin 6) and MCP1 (Monocyte chemoattractant protein-1) leads to further release of cytokines and recruitment of macrophages. Hypertrophy also promotes angiogenesis via VEGF (Vascular Endothelial Growth Factor). This feedback might ultimately induce insulin resistance, which have been linked with c-Jun N-terminal kinase (JNK) and NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) activation. Figure obtained from [32].

The formation and its lasting persistent state follows a different mechanism of classic inflammation as there is no tissue damage, no reported infection and the intensity of the inflammatory response is much lower than the standard infection response [99,102]. Correlation between MetS and inflammation is not yet fully understood. With the abnormal increase in adipose tissue, a proportional increase of cytokines is released from the tissue, promoting the production of C-reactive protein (CRP) from liver into the bloodstream [103]. High levels of circulatory CRP have been associated with aggravation of coronary vessels and endothelial function [104] and is, in fact, used as predictive marker for NAFLD and CVD [105]. Another major marker is TNF-α, which is overexpressed in obese people [106], and has been associated with worsened insulin resistance in the adipose tissue [107].

The increase of circulatory cytokines might be an effect of the increased size of the tissue, the insulin resistance [108,109] or the local inflammatory state, promoted by the infiltration of macrophages [110], which develops when the adipose tissue increases its size quickly due to diet. It has also been postulated that with the hypertrophy, and the hypoxia that comes with lack of proper angiogenesis and cell failure leads to apoptosis which elevates the recruitment of macrophages, promoting local inflammation [111]. Under a healthy and low growth of the adipose tissue, additional vessels are formed. However, impaired adipocyte growth leads to the under-formation of surrounding vascular tissue, which induces hypoxia, release of cytokines and inflammation. The fast growth of the tissue has been directly linked to the development of insulin resistance [112].

2.1.4 Brown adipose tissue, energy expenditure and functionality

Brown adipose tissue's (BAT) role on the overall control of the energy homeostasis has recently been given a more prominent place of importance. Until a few years ago, its existence was not acknowledged in adults, as it was believed that remnant patches of BAT in new-borns were reabsorbed after the first few months. BAT's presence in new-borns allows a fine tuning of the infant's body temperature, this being its main role, achieved through the release of energy in the form of heat in the mitochondria. It is in the more recent years that the presence of BAT has been found in human adults, although it degenerates with age [113].

BAT is a specialised tissue that, similarly to WAT, stores lipids but whose morphology is greatly different. BAT's accumulation of lipids evidences a different functionality as their fatty acids are easier to access and mobilize, thanks to a conformation of smaller and more numerous lipid droplets. Moreover, BAT is characterised by a high number of mitochondria and vascularization, responsible for its brown colour [114,115].

High irrigation providing elevated oxygen concentration, plus the mitochondrial recount, points to an elevated metabolic activity and the need of energy consumption. However, given a similar context that of muscle, BAT does not store as much energy in the form of adenosine triphosphate (ATP). The energy homeostasis of BAT is geared towards the generation of a mitochondrial transmembrane potential that gets released, uncoupled form ATP production, in the form of heat [116]. Because of the mentioned high vascularization of the tissue, this heat is quickly distributed throughout the whole body, contributing to the maintenance of body temperature, as an alternative source of heat production, being shivering thermoregulation the main and more effective one against low temperature exposures [117].

The function of BAT is mainly performed by the uncoupling protein 1 (UCP1). This protein is located in the inner membrane of the mitochondria in contact with the highly concentrated proton accumulation of the interior of the organelle. The release of the electrochemical potential through the inner membrane into the mitochondrial matrix, by UCP1, dissociates the mitochondrial respiration from the ATP production, resulting in the dissipation of energy, exclusively in the form of heat [118]. The mitochondrial gradient is achieved by burning fatty acids and glucose and the process of burning them to produce heat is known as thermogenesis.

The discovery of UCP1 has been of great importance in scientific research as increased RNA expression or protein concentration correlates with increased thermogenesis, being used as a marker of the process [119]. As energy comes from the burn of fatty acids and glucose, their carriers are deeply studied and tightly regulated [120]. Glucose is imported by glucose transporters (GLUT family, specifically GLUT4 in BAT), which are moved from intracellular locations into the cell surfaces by stimulation with insulin [121] and, also, activated by norepinephrine secretion from the sympathetic nervous system after cold exposure [122,123]. Free fatty acids are absorbed by the CD36 carrier [124], by to the hydrolyzation of triglycerides from lipoproteins by LPL [121]. Once fatty acids have been imported, they are used as fuel and lipolysis starts. Genes such as *ATGL*, *HSL* or *MGL* are involved in this process [125].

The main activator of thermogenesis is the sympathetic nervous system via release of catecholamines that will bind to adrenergic receptors. This will lead to the activation of the adenylyl cyclase (AC) which will raise levels of cyclic adenosine monophosphate (cAMP), activating PKA (protein kinase A). PKA will phosphorylate HSL, inducing lipolysis, cleaving triglycerides and releasing free fatty acids, which are used as a primary source for the production of ATP, thus, providing energy for the electrochemical potential in the mitochondria [126]. Other regulators and activators of thermogenesis are fibroblast growth factor 21 (FGF21), an adipokine whose production is induced after cold-exposure

[127,128] and thyroid hormones [129]. A summary of the mentioned pathways and fuel sources is shown in **figure 4**.

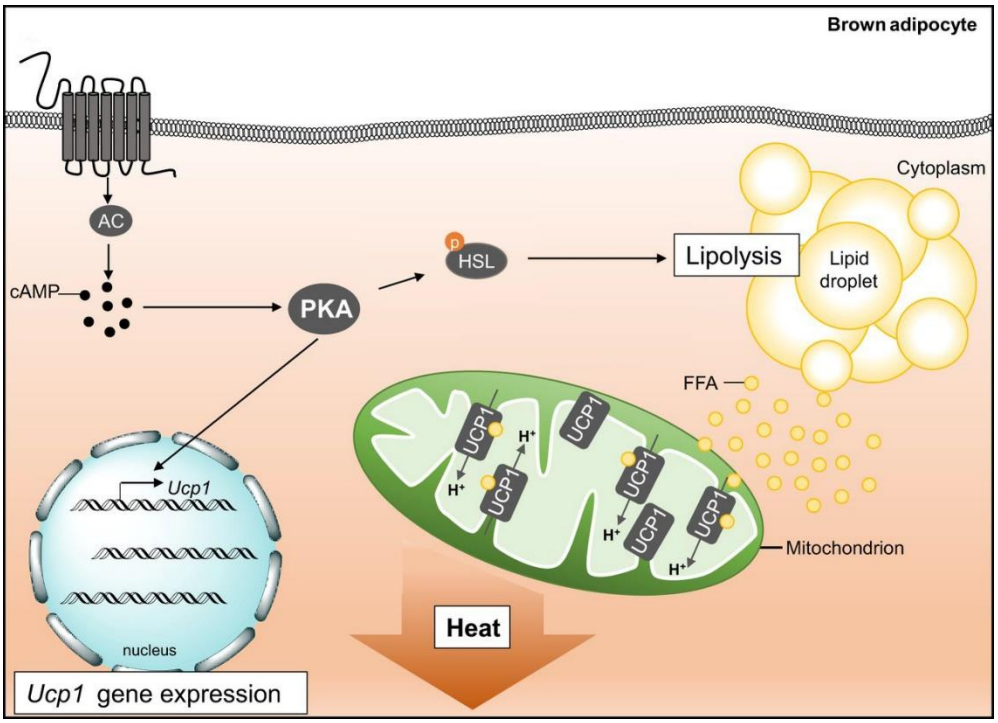


Figure 4. A schematic representation of a cell from the brown adipose tissue showing the activation of AC, by an adrenergic receptor, which would in turn activate PKA via the production of cAMP. PKA increases the expression of UCP1 and activates HSL via phosphorylation. This enzyme activates lipolysis, providing fuel for UCP1 and, thus, inducing thermogenesis. AC: Adenylyl cyclase; cAMP: Cyclic adenosine monophosphate; PKA: Protein kinase A; HSL: Hormone sensitive lipase; FFA: free fatty acids; UCP1: Uncoupling protein 1. Image adapted from [126].

The described mechanism of thermogenesis has a secondary importance, which has grown in interest as a drug target. BAT has an indirect control over fat accumulation given that fatty acids are extensively burned to produce heat and BAT is a reservoir of them. It has been reported that BAT is related to obesity and its control. BAT has been shown to help remove triglycerides content from the bloodstream after cold exposure [124], body fat has been diminished after BAT activation [130] and its deficiency has led to obesity [131]. Besides, BAT has been shown to contribute to metabolic efficiency and energy expenditure [132] and BAT mass increases after loss of fat mass [133], all of which indicates a relationship between BAT and obesity.

From another point of view, BAT, therefore, is in direct control of part of the body's energy expenditure, as a controlled but wasteful burning of fatty acids reduces the overall quantity of energy reservoirs. While, when needed, heat production is not wasteful, it has been proved that obese rodents have an increased activated thermogenesis as a tool for excess fat disposal [134]. Its discovery

has shifted part of the scientific effort in obesity control towards the search for BAT activating drugs that could activate thermogenesis. Increased thermogenesis leads to increase the energy output and acceleration of fat mass loss [135].

2.2 Glucose homeostasis and insulin dysregulation

Food intake supplies the metabolism with energy sources. On the other hand, fasting conditions prompts the body to release glucose to maintain the energy needs. The liver is the main organ that releases glucose in fasting state. The body's main source of glucose will be glycogen, a polymeric molecule used as storage of glucose. However, eventually glycogen reserves are depleted, only to be replenished by food intake, and alternative sources are used to maintain the flow of glucose from the liver.

Likewise, the liver is also responsible for the uptake of glucose from blood to lower is content. Glucose is primarily subtracted from the bloodstream in the liver by the channel glucose transporter 2 (GLUT2), which also mediates its release from the liver [136]. The absorption is regulated by the content of glucose itself inside the hepatocyte. Once glucose passes into the cytoplasm it is phosphorylated into glucose 6-phosphate (G6P) by the glucokinase (GCK), reducing the level of glucose, which when perceived, promotes further uptake of glucose [137]. G6P remains inside the hepatocyte, as there are no channels for its release, and will ultimately be converted into pyruvate via glycolysis, after which, pyruvate will enter the tricarboxylic acid (TCA) cycle (**Figure 5**), serving for the production of ATP in the mitochondria. G6P will also be metabolized in the pentose phosphate pathway to produce NADPH (Nicotinamide adenine dinucleotide phosphate). G6P is reverted into glucose, in fasted state, in the endoplasmic reticulum for its release from liver [138].

2.2.1 Gluconeogenesis

Due to the muscle-exported metabolites such as lactate and amino acids, and liver's reservoirs of glycerol or pyruvate, liver is capable of glucose production via gluconeogenesis. Lactate is converted into pyruvate by the lactate dehydrogenase. Pyruvate is then converted into oxaloacetate with the pyruvate carboxylase (PC), exported into the cytoplasm and converted into phosphoenolpyruvate with the cytoplasmic phosphoenolpyruvate carboxylase (PEPCK-C) [139]. Pyruvate formation is also achieved from amino acids [140]. Phosphoenolpyruvate undergoes several enzymatic conversions until the formation of fructose-6-phosphate (F6P), which is converted into G6P and transported into

the endoplasmic reticulum where it is dephosphorylated with G6Pase, generating free glucose to be exported from the hepatocyte into the bloodstream [141].

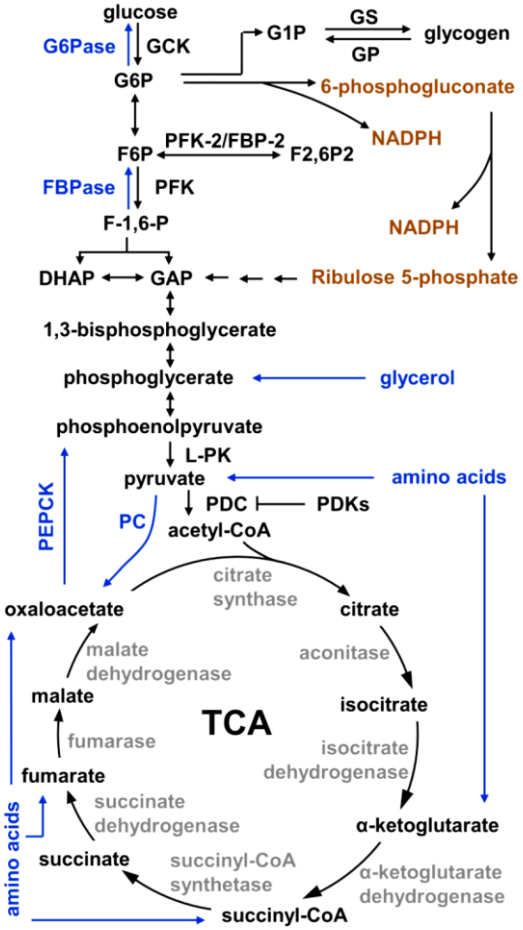


Figure 5. The citric acid cycle (TCA) and glycolysis, including relevant enzymes and metabolites. GCK: glucokinase; GAP: glyceraldehyde 3-phosphate; GP: glycogen phosphorylase; G6Pase: glucose-6-phosphatase; L-PK: liver pyruvate kinase; G6P: glucose 1-phosphate; PC: pyruvate carboxylase; GS: glycogen synthase; PFK: 6-phosphofructo-1 kinase; FBPase: fructose 1,6 bisphosphatase; F-1,6-P: Fructose 1,6-bisphosphate; G1P: glucose 1-phosphate; DHAP: dihydroxyacetone phosphate; PDC: pyruvate dehydrogenase complex; PDKs: pyruvate dehydrogenase kinases. Figure obtained from [138].

Due to the importance of glucose homeostasis, gluconeogenesis is a tightly controlled process. Pyruvate from skeletal muscle is converted with the pyruvate dehydrogenase complex (PDC) into acetyl-CoA and used in the TCA cycle to obtain energy but it can also be converted into lactate, exported and used by the liver for gluconeogenesis [142]. The activity of the PDC can be inhibited via phosphorylation by the pyruvate Dehydrogenase Kinase 4 (PDK4), in fasting conditions or exercise, forcing the conversion of pyruvate into acetyl-CoA and, thus, retaining energy sources for skeletal muscle. This restricts the liver from a major source material for gluconeogenesis, gravely diminishing gluconeogenesis [143]. Glycerol is an indirect regulator as well. Obtained from β – oxidation and

exported from the white adipose tissue, is key in the production of ATP, needed for the gluconeogenesis.

Insulin is another key regulator and potent suppressor of gluconeogenesis in the liver. Insulin resistance hinders its efficiency, inducing a permanent activation of gluconeogenesis and release of glucose into the bloodstream, which also increases the adiposity of the hepatic tissue and the appearance of NAFLD [144]. Glucagon, secreted by the pancreatic α cells, is mostly present in fasting state or exercise, promoting the release of glucose from the liver to provide fuel for the skeletal muscle [145]. Glucagon exerts its functionality via a G-protein receptor, which activates the Gα-cAMP/PKA pathway, phosphorylating and activating cAMP response element-binding (CREB) which promotes gluconeogenesis [146].

2.2.2 Glycolysis

The process of glucose breakdown, known as glycolysis, is mainly used during feeding (fed) states to obtain ATP and materials for amino acid production and lipids formation. Several enzymes are involved in the process and remain less active under fasting conditions [147]. Phosphatidylcholine molecules activate GCK, which under low levels of glucose remains inactivated by binding to glucokinase regulatory protein (GKRP). GCK unbinds with glucose, which converts glucose into G6P [137]. As mentioned, insulin also plays a role in suppressing protein kinase 4 (PK4) which allows the activation of PDC and the use of the glucose glycolysis product, pyruvate, for its conversion into acetyl-CoA and its integration in the TCA cycle to obtain ATP [142].

2.2.3 Glycogen genesis and its regulation

G6P is the precursor of glycogen. Two enzymes are involved in the elongation or hydrolysis of glycogen: glycogen synthase and glycogen phosphorylase, respectively. G6P by itself is, a promoter of glycogen formation as it acts as an activator of the glycogen synthase and inhibitor of glycogen phosphorylase, creating a positive loop to deplete and store the glucose inside the hepatocyte. However, the functionality of both enzymes is regulated by phosphorylation via glycogen synthase kinase 3 (GSK-3), inhibiting glycogen synthase and increasing the activity of glycogen phosphorylase. The opposite effect is achieved through dephosphorylation by protein phosphatase 1 [137].

The whole process is influenced by insulin, allowing the hormone to regulate the reserves and mobility of glucose. Glycogenolysis is suppressed with insulin as the signalling that generates upon binding to its receptor in the hepatocyte promotes the dephosphorylation of glycogen phosphorylase

with protein phosphatase 1 [148]. Insulin also promotes uptake of glucose as it upregulates the expression of *GCK*, converting glucose to G6P, thus, also promoting glucose uptake.

Glucagon, the opposing insulin hormone, and the adrenergic innervation of liver by the nervous system via release of catecholamines, promote the opposing effect on liver. Catecholamines bind with their correspondent receptor activates PKA which phosphorylates glycogen phosphorylase, increasing its activity, while inhibiting the acetylation, and thus, recruitment, of glycogen synthase [148,149].

2.2.4 Branched amino acid metabolism

Leucine, isoleucine and valine are known as branched amino acids (BCAA), essential amino acids that must be obtained via food intake [150]. BCAA have been associated with multiple roles such as glucose metabolism, lipid metabolism, immunity or intestinal barrier function, among others [151–153]. BCCA supplementation in diet has shown reductions in fat mass, correlating with increases of uncoupling protein 3 (UCP3) in muscle and higher levels of CD36 in liver [152]. This effect has been further corroborate by studies showing increments of lipolysis in white adipose tissue and suppression of lipogenesis in liver [154,155]. BCAA by themselves directly increase levels of acetyl-CoA which promotes the burning of fatty acids [156]. Furthermore, glucose metabolism is also affected by BCAA, as leucine upregulates the activity if GLUT4 in muscle [157], which lowers glucose levels [158].

BCAA are absorbed via the "L" transporter system [159] for their catabolism, shown in **Figure 6**. BCAA enter the mitochondria via the mitochondrial carrier family (MCF) where the branched-chain amino acid transferase 2 (BCAT-2) converts them into branched-chain α-keto acid (BCKA), reverted in the cytosol by the BCAT-1. BCKA are later decarboxylated with the branched-chain α-keto acid dehydrogenase (BCKD). The final product of these reactions is further metabolised into acetyl-CoA and succinyl-CoA, which will enter the TCA cycle [156].

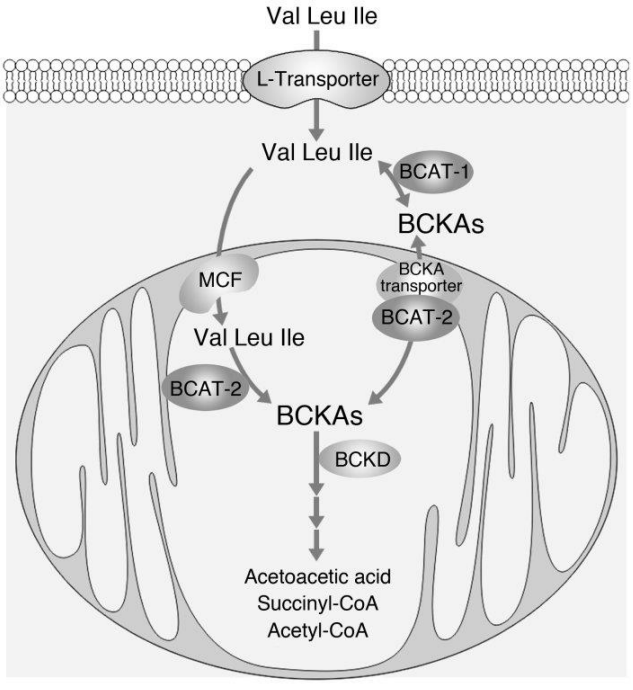


Figure 6. Pathways for the catabolism of BCAA (Branched amino acids) into the TCA (Tricarboxylic acid cycle) cycle. MCF: Mitochondrial carrier family; BCAT: Branched-chain amino acid transferase; BCKA: Branched-chain α-keto acid; BCKD: Branched-chain α-keto acid dehydrogenase Image adapted from [456].

2.2.5 Type II diabetes and insulin resistance

As mentioned, associated with obesity is usually an increase in glucose blood level. This parameter is of importance as elevated levels are the first step and onsets type II diabetes. This disease has been strongly correlated with obesity and both get aggravated when correlated with each other [160], while type II diabetes improves after body weight loss [161]. The relationship between the two has been established and the development of obesity usually promotes type II diabetes.

The core of the disease is the lack of production of enough insulin to perform its function, as tissues have developed resistance to the hormone, primarily in liver, adipose tissue and muscle. Insulin normally binds to its receptor and restrains the tissue from the excretion of glucose into the bloodstream, whereas when the tissue has some degree of insulin resistance, insulin does not avoid this release and the organ liberates glucose abnormally, at higher levels than required. Several factors might be involved in this problem such as low efficiency of insulin secretion by the β-cells, low glucose sensitivity of those cells and insulin resistance in peripheral organs like adipose tissue, liver or skeletal muscle [162,163]. A detailed depiction of the dysregulations developed under type II diabetes is shown in **figure 7**.

As glucose levels are strictly restricted, pancreatic beta cells release more insulin, trying to cope with the high levels of glucose This is known as hyperinsulinemia as insulin concentration in the bloodstream rises outside of normal levels. High levels of insulin and fasting glucose are used to assess the development of insulin resistance in obese individuals. Besides, glucose tolerance tests and insulin resistance tests are performed to check whether an individual is capable of managing glucose intake and has developed insulin resistance, respectively, and it has been shown that obese individuals tend to develop both symptoms.

Insulin resistance and type II diabetes, both usually concomitant with obesity, are partly consequences of liver malfunction. Regarding insulin resistance, it is also considered to play a major role in the development of MetS [164], leading to a reduced uptake from organs such as liver or WAT and over production and release of glucose from liver, promoting the accumulation of glucose in blood, known as hyperglycaemia. One of the factors leading to this is the inhibition of the activity of key enzymes in the glycolysis such us GCK, that occur in obese rats [165], which is usually followed by elevated circulatory concentration of insulin in an attempt to counteract it. Further development of inflammation and NAFLD exacerbates insulin resistance in liver, as inflammatory cytokines promote gluconeogenesis via glucagon, thus increasing a positive feedback and leading to more aggravated states of type II diabetes [166]. Molecular studies about insulin resistance [167] point towards dysregulations in the functionality of pathway's enzymes such as insulin receptor substrate-1 (IRS-1)

[168], suppressors of cytokine signalling (SOC) family [169] and protein tyrosine phosphatase 1B (PTP1B) [170].

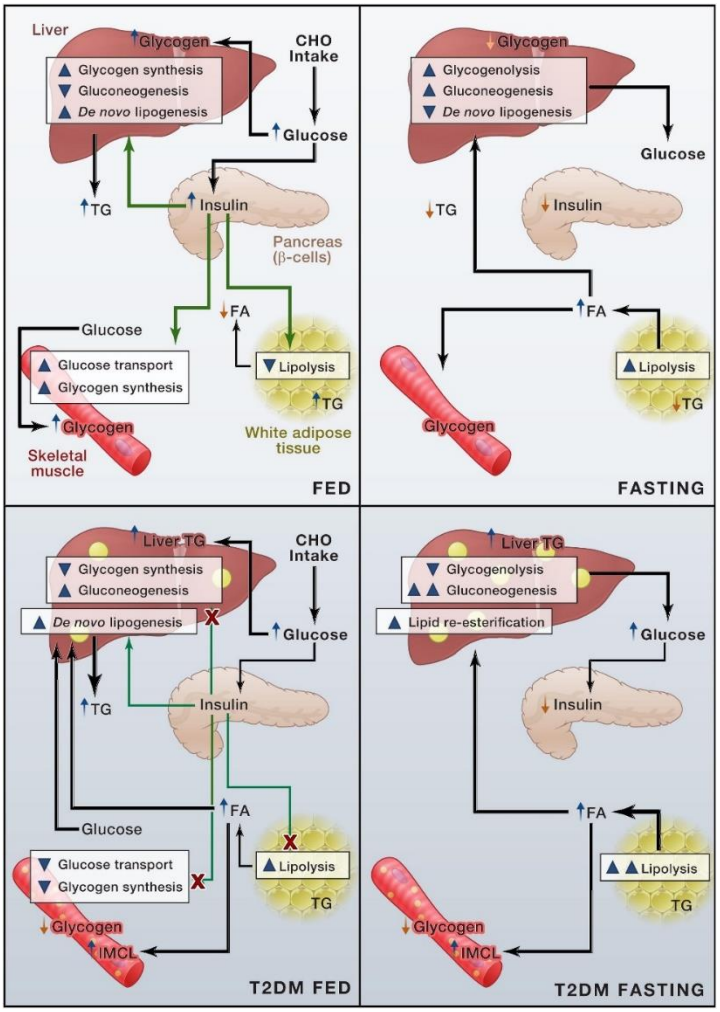


Figure 7. Figure shows the difference in metabolism under fed and fasting conditions. Food intake triggers the release of insulin from pancreas which promotes the increase in the reservoirs of energy by the liver, via lipogenesis and glycogen synthesis, the WAT, via reduction of lipolysis, and the skeletal muscle, which also increases its glycogen content. In fasting conditions, the WAT will release triglycerides from its reservoirs and the liver will produce glucose from its glycogen. In type II diabetes, the insulin release does not promote the same effect as tissues have grown resistant to the hormone which leads to accumulation of fat in liver as the organ does not reduce its lipogenesis and gets overloaded with fatty acids released from the WAT. CHO: carbohydrates; TG: triglycerides; FA: fatty acids; T2DM: type II diabetes mellitus; ICML: Intramyocellular lipids. Figure obtained from [457].

The origin of insulin resistance has been pinpointed to the abdominal white adipose tissue, thus, allowing some ability to predict the development of insulin resistance by the waist circumference [171]. Several molecular processes have been associated with the development of insulin resistance. For instance, the excessive release of NEFA by the adipose tissue, in an attempt to deliver them for their burning, will reach the liver, promoting the formation of triglycerides and diglycerides that will disrupt insulin's receptor pathway activation, thus, lowering the organ sensitivity to insulin [172–174]. Patients with ectopic abdominal fat accumulation tend to have an excess of diglycerides as pathways for their catabolization into triglycerides cannot cope with their elevated concentration, thus altering the regular signalling in adipose tissue [175]. Other lipids like ceramides, important components of the cellular membrane, have also been linked with insulin resistance. Experiments with rats have found a correlation with insulin resistance and increments in ceramides and diglycerides in liver and skeletal muscle [176]. Adding to this, the low chronic inflammatory state of obese individuals and

lowered adiponectin release from the adipose tissue, adds suppression to the insulin functionality in the liver [171].

2.3 Lipid homeostasis

In addition to liver's role in glucose metabolism, it is also an important regulator of lipids. The liver is an active figure in its storage and regulator of the lipid content of the bloodstream. It is considered the main organ to produce the conversion of glucose into fatty acids, and as such, it is also the first one to store and distribute them to other peripheral organs. Distribution of fatty acids occurs via packing into VLDL-C particles and release them into the bloodstream.

2.3.1 Lipid transport into liver

Following the natural flow of energy in the body, the first role of liver regarding lipids is their uptake from the bloodstream after food consumption. Fats from food are digested and broken down into free fatty acids that will be re-assembled into triglycerides in the enterocytes of the small intestine. Triglycerides will later reach the liver after the enterocytes pack them in chylomicrons that are released and reach the liver via the bloodstream. Once the chylomicrons reach the liver, triglycerides will be removed by the lipoprotein lipase (LPL), which breaks them down into single fatty acids, NEFA, to be absorbed into the liver, thus, lowering the lipid content of the bloodstream. NEFA are uptaked by specialized transporter proteins, such as CD36 or fatty acid transport protein 2, 4 and 5 (FATP2, 4, 5). This process is tightly controlled, and it has been found that dysregulations led to drastic changes in the lipid content of the liver. Over expression of CD36 increases fatty acid uptake resulting in hepatic steatosis [177] and depletions of *FATP5* led to improvements in fatty liver as less NEFA are absorbed [178].

2.3.2 Lipid catabolism

The liver is a prime modulator of lipid metabolism while WAT represents the body's reservoirs of lipids. Once fatty acids reach the organ, either coming from food intake or released by the WAT, depending of liver lipid content and fasting state they might be oxidised by β – oxidation, stored or released from the organ for storage and future use in adipose tissues. Lipid catabolism, β – oxidation, of stored lipids is highly dependent on the fasting state, being promoted in fasting state and lowered in fed state. Thus, one of the main purposes of β – oxidation is supplying energy for other peripheral organs in the form of ketone bodies (β-hydroxybutyrate, acetone and acetoacetate). These molecules

are exported into the bloodstream as a substitute and additional fuel when glucose levels are lowered after many hours without food intake.

Long chain fatty acids are transported into the mitochondria to undergo β – oxidation. The main limiting factor in the pathway of β – oxidation is the enzyme carnitine palmitoyltransferase 1, tasked with their translocation. The enzyme's activity is modulated by local increases in the concentration of malonyl-CoA (induced by acetyl-CoA carboxylase 2 (ACC2) in the mitochondria) which inhibits its activity [179]. Another important regulator is peroxisome proliferator-activated receptors α (*PPARα*), a key gene, promoter of β – oxidation in fasted state [180]. This nuclear receptor is activated by long chain fatty acids, phosphatidylcholines and several other coactivators like peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [181]. The depletion of *PPARα* greatly impacts β–oxidation, reducing circulating ketonic bodies, glycaemia and promoting hepatic steatosis [182].

PPARα is tightly regulated according to fasting state. Due to this, glucagon plays a major role in its activation. Glucagon increases expression and secretion of FGF21, stimulating in turn the expression of PGC-1α, therefore activating PPARα and β – oxidation [183]. The increment in circulating glucagon during exercise has been linked to a decrease in hepatic steatosis as it promotes burning of fats in liver [184].

2.3.3 Lipogenesis

De novo lipogenesis is the process of synthesizing fatty acids. The main initial molecule required is pyruvate, which origin comes from glucose but can be synthesized from amino acids, lactate or other molecules. Pyruvate will enter the TCA cycle to be metabolized into acetyl-CoA by the PDC, which is combined with oxaloacetate by the citrate synthase, forming citrate. Citrate is exported into the cytoplasm and split back into oxaloacetate and acetyl-CoA by the ATP-citrate lyase (ACL) [138].

Acetyl-CoA becomes the first step in a series of molecules to form a fatty acid. First, Acetyl-CoA is carboxylated into malonyl-CoA by the ACC (Acetyl-CoA carboxylase). Two versions of the enzyme, encoded by the genes *ACC1* and *ACC2*, one cytoplasmic and the other situated in the outer layer of the mitochondria, respectively, are key in the formation of fatty acids [185] and their malfunction is usually deleterious, although their down-regulation proves helpful to reduce hepatic steatosis, as the synthesis of lipids gets seriously hindered [186]. Malonyl-CoA and nicotinamide adenine dinucleotide phosphate (NADPH) are later converted into palmitic acid by fatty acid synthase (FAS).

Palmitic acid is the main molecular structure for the formation of fatty acids. Palmitic acid will be transported into the endoplasmic reticulum to be elongated into long-chain fatty acids (LCFAs) by enzymes like elongation of very long chain fatty acids protein 1 (ELOV1) [187]. LCFAs could be

desaturated by stearoyl-CoA desaturases (SCDs) into unsaturated fatty acids [188]. Saturated and unsaturated acids can be converted into triglycerides. The mentioned pathways are summarized in **figure 8**.

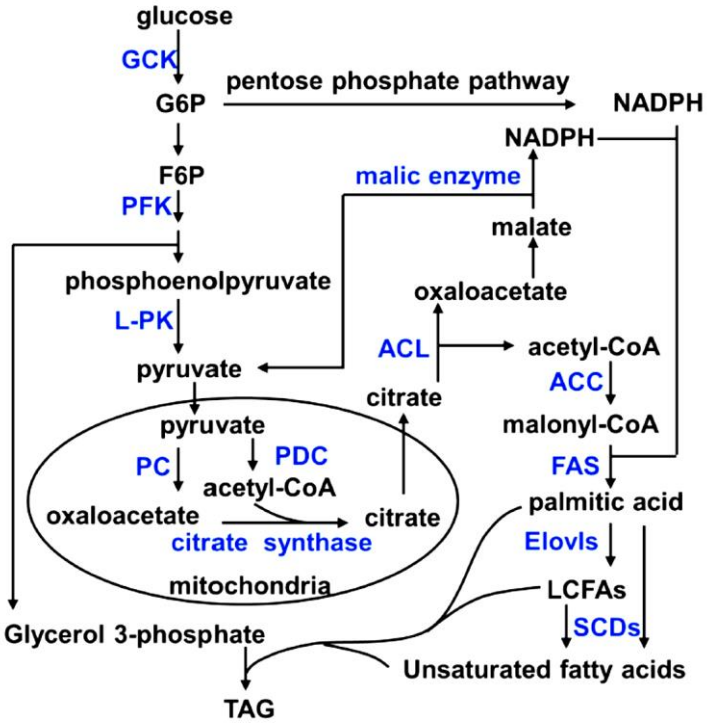


Figure 8. Summary of enzymes and metabolites involved in the synthesis of fatty acids. LCFAs: Long-chain fatty acids; ACC: acetyl-CoA carboxylase; ACL: ATP-citrate lyase; Elovls: fatty acyl-CoA elongases; TAG: triacylglycerol; FAS: fatty acid synthase; SCDs: stearoyl-CoA desaturases; NADPH: Nicotinamide adenine dinucleotide phosphate; GCK: Glucokinase; PDC: Pyruvate dehydrogenase complex; PC: Pyruvate carboxylase; PFK: 6-phosphofructo-1 kinase; L-PK: ; G6P: Glucose 6-phosphate; F6P: Fructose-6-phosphate. Figure obtained from [138].

As with other metabolic processes, lipogenesis is influenced by many factors. Limitations on the key components set up the rate at which fatty acids are generated. Relative amounts of pyruvate and the activity of PDC, PDKs and GCK define whether there is going to be a prevalence of lipogenesis or glycolysis [189]. The activity of the malic enzyme that forms NADPH from malate, the rate of formation of NADPH from the pentose phosphate pathway and the general availability of NADPH, will limit the amount of fatty acids that can be generated. Fasting state is also a modulator. Fed state inhibits lipogenesis by activating sirtuin-1 (SIRT-1) which will deacetylate and inhibit sterol regulatory element-binding protein-1c (SREBP-1C) [190]. SREBP-1C is the master key to activate genes involved in the control of triglycerides and fatty acid synthesis [191]. Finally, oxidative stress in the endoplasmic reticulum also influences the activation of lipogenesis and induces hepatic steatosis [192].

2.3.4 Cholesterol metabolism in liver

Cholesterol undergoes in liver both catabolic and anabolic processes, being the organ charged with its release to other organs, its secretion in the form of bile salts for its elimination and its synthesis. 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) is the key enzyme limiting step on the cholesterol catabolism pathway and its usually considered that its increment on gene expression promotes the synthesis of cholesterol and its suppression limits it [193]. SREBP-2 is another key factor that activates the set of genes involved in the synthesis of cholesterol [191].

Liver also uptakes cholesterol from other peripheral organs via HDL-C particles for its metabolization and releases it via LDL-C particles to be used in those same organs [194]. Releasing of cholesterol has the LDL-receptor as the main key step [195] and is usually analysed to assess the rate of cholesterol release into the bloodstream. In turn, excess cholesterol inside the liver might be excreted in the form of bile acids. Cholesterol's conversion relies on the cholesterol 7α-hydroxylase (CYP7A1) as the rate limiting enzyme [196]. Excretion of bile acids is mediated by apical sodium-dependent bile acid transporter (ASBT) carriers [197].

Both, lacking or excessive cholesterol in a cell is cytotoxic, as cholesterol plays a role in the structure and adequate fluidity of the membranes [198]. Moreover, excess cholesterol in peripheral organs will imply its prolonged state in the bloodstream, which has been associated with CVD [199]. Due to this, cholesterol concentration is tightly controlled. Uptake of cholesterol tends to repress its synthesis to prevent over-load [200]. Free cholesterol absorbed is quickly esterified by the acyl-CoA:cholesterol acyltransferase (ACAT), to prevent toxicity as free cholesterol would bind to any cell membrane, altering its functionality [201]. The esterification is reversed by the neutral cholesteryl esterase (NCEH) [200].

Several mechanisms have been found responsible for the accumulation of free cholesterol inside a cell. Release of cholesterol is a vesicle-mediated process, reliant on energy expenditure by the cell. ATP depletion could hinder the efflux, leading to free cholesterol accumulation [202]. Sphingomyelin binds cholesterol, modulation its distribution [203,204]. Under pathological circumstances, the sphingomyelinase, responsible for the sphingomyelin breakdown into ceramide and phosphocholine [205,206], might not function properly, leading to sphingomyelin accumulation and blocking of cholesterol mobilization [207]. Furthermore, accumulation of free cholesterol might be due to an excessive intake ACAT cannot compensate and convert into esterified-cholesterol, thus accumulating it [208].

2.3.5 Lipidic metabolism dysregulation in obesity and MetS

As previously mentioned, two of the symptoms of the MetS are directly related with management and mobilization of lipids, particularly the elevated triglycerides and lowered HDL-C particles in blood. What is otherwise a controlled regulatory mechanism, under MetS several key factors and genes involved in this process do not work as intended.

WAT shows reduced activity of LPL [209] and the skeletal muscle sees a reduction on LPL activity as well [210], although *LPL* expression in WAT might be increased [211,212]. The malfunction of this enzyme leads to an overload of fatty acids in liver, as they are hydrolysed and absorbed there for their metabolization, and an over-translocation of triglycerides from VLDL-C particles creating highly concentrated and dense LDL-C particles that are slowly metabolized [213], increasing the chances of atheroma development [214]. Besides it, the binding of LDL-C by the LDL-C receptor is reduced in adiposity, further hindering the clearance of this particles [215].

The increase in circulating triglycerides is partly due to an elevated release of fatty acids from the WAT and a lack of proper uptake by other peripheral organs [216]. High fatty acids in blood, NEFA, have been extensively proved to lead towards insulin resistance, which will further exacerbate the problem as a less insulin sensitive WAT will keep secreting more lipids and be less likely to uptake them [217]. However, this is yet to be clarified because, even though extensive studies have found a relationship between high NEFA and obesity, more recent analyses show that the metabolic state should be analysed individually for each patient. Obese patients sometimes show normalization of NEFA levels and gravely insulin resistant patients might also have low NEFA release [218]. The WAT also shows reduced expression of *ATGL* and *HSL*. ATGL reduced activity leads to less responsiveness of the tissue over adrenergic stimulation, leading to reduced hydrolysis of triglycerides from the fat droplets [219,220]. HSL dysfunction implies less release of fatty acids into the bloodstream, leading to a reduction of circulatory NEFA. However, the overall result tends to be the opposite as this is not enough to compensate for the insensibility to insulin or the over-stimulation by the nervous system for the increment in lipolysis [221]. In addition, channels in the WAT like FATPs that would otherwise be activated with insulin, functions poorly in obesity, reducing the uptake of fatty acids from the bloodstream [222]. Finally, a key factor in the regulation of lipogenesis in WAT, that shows diminished expression in obesity, are *SREBPs*. Normally over-expressed with insulin and down-regulated with excessive adiposity, under dysregulating obesity, reduces its expression and, thus, lipogenesis, promoting an over-secretion of fatty acids into the bloodstream [223].

As it has been hinted, the dysregulation of WAT is highly dependent on the tissue responsiveness to insulin, which forces careful evaluation of the patient's metabolic state for the treatment of obesity.

It is of notice in this regard the existence of metabolically healthy obese people [224], characterized by better insulin sensitivity and reduced accumulation of fats in liver. It is possible that these individuals will not report an appearance of the mentioned dysregulations and have lower risk of developing CVD [225].

2.4 Hypertension

Hypertension (HTN), commonly concomitant with obesity, and one of the symptoms of the MetS, creates a high risk of developing CVD. HTN is characterized by a high and chronic BP due to a dysfunction in the endothelium-dependent dilatation [226]. Several factors have been associated with HTN and could be considered responsible for its development, such as elevated inflammation secreted by an over-grown WAT [227] or oxidative stress [228], although the origins of the disease are not yet fully understood. HTN is usually associated with obesity but it might also be present in lean patients.

The control and modulation of BP relies on the compression and relaxation of blood vessels by muscle cells in the walls of the vessel [229]. The activity of these cells is directly controlled by the sympathetic nervous system, vasodilator molecules like NO or vasoconstrictor like endothelin-1 (ET-1) or angiotensin II [230].

An important factor involved in the modulation of BP is the renin-angiotensin-aldosterone system (RAAS), which directly controls vascular contraction and renal reabsorption [231]. Renin is secreted into the bloodstream, by the juxtaglomerular cells in the kidneys, after stimulation by the sympathetic nervous system or changes in circulatory sodium [232]. The N-terminal portion of angiotensin is cleavage by renin, forming angiotensin I, the rate-limiting step in the RAAS, which in turn is hydrolysed by the angiotensin-converting enzyme (ACE). ACE will convert angiotensin I into angiotensin II, the key step in the process, as angiotensin II is now active and a powerful vasoconstrictor [231]. ACE is mainly located in lungs but has also been found in kidneys, heart and adipose tissue [233]. Modulating this conversion relies on angiotensin-converting enzyme 2 (ACE 2), which converts angiotensin II back into angiotensin, down regulating its production and levels in blood [234]. Angiotensin II regulates contraction of the muscle cells in the blood vessels and indirectly modulates BP by inducing the production of aldosterone in the adrenal glands. Aldosterone regulates the amount of sodium and its reabsorption in the kidneys [235].

Another important vasoconstrictor, ET-1, is produced by the endothelin converting enzyme in the endothelial cells after stimulation with TNF-α or interleukins, or decreased after release of NO [236]. ET-1 will bind to channels receptors in the muscle cells of blood vessels and induce the entrance of extracellular calcium which forces constriction of the myofibrils and vasoconstriction [237].

NO is a vasodilator, that upon release into the bloodstream will activate a signalling pathway, resulting in the dephosphorylation of myosin in the muscle cells of blood vessels, promoting relaxation, and, thus, dilatation. NO is generated by the NO synthase, localized in brain and endothelium (eNOS). eNOS can be activated by increases in circulatory calcium, stress or NO agonists such as ATP or acetylcholine [238].

High food intake promotes the activity of the RAAS, increasing the production of Angiotensin II, which will promote the reabsorption of salts in the kidneys and enhance sympathetic activity, among other functions [239]. The sympathetic nervous system tends to be more activated as a compensation for the excessive adiposity, promoting is burning and use [240]. However, as mentioned, the sympathetic nervous system is also activated by the renin-angiotensin-aldosterone system, in a positive feedback that further promotes the elevation of BP and worsening of hypertension [241].

Several other factors could also lead to hypertension such as inflammatory cytokines like TNF-α or IL-6 which directly influence and increase the activity of ET-1 [242], which itself, is a promoter for the release of these cytokines [243,244]. As mentioned, oxidative stress is strongly linked to MetS [245] and renal sodium reabsorption, which directly increases BP [246]. HTN might also be developed as a consequence of insulin resistance in the endothelium which reduces the functionality of phosphatidylinositol 3-kinase (PI3K), altering the ratio of NO production and ET-1 [247], by reducing NO, which leads to a raise in BP [248]. Related to this, hyperinsulinemia has been associated with an elevated release of ET-1, as insulin induces its production and release, promoting vasoconstriction [249]. In addition, associated with obesity, elevated NEFA content have also been associated with elevated BP [250] and endothelial tissue damage [251].

3. Study of metabolic syndrome with metabolomics

The metabolism is a complex network of inter-related metabolites that are usually compartmentalized into separated pathways. However, most of the metabolites are present in multiple of these pathways, rendering them not very useful when trying to study metabolism from a holistic point of view. However, the study of individual metabolic pathways is particularly useful to understand the role of enzyme-coding genes and the ramifications that a particular enzymatic process and the abundance or lack of a metabolite, might generate over the whole metabolism, as their interactions with every other metabolite are easy to follow and there has been extensive research, allowing the creation of detailed metabolic maps and providing extensive bibliography.

Different techniques have been developed for the quantification of metabolites and analysis of metabolic pathways. The main tool for the analysis of metabolism used in this thesis was ^{1}H-NMR. The ^{1}H-NMR functionality is based on the magnetic properties of certain nuclei of molecules [252] and allows precise measurements of concentration of molecules. In scientific research with *in vivo* procedures, organs or blood from the animals are extracted, from which their content is divided between lipophilic and hydrophilic metabolites. These two extracts are later analysed by ^{1}H-NMR, obtaining a list of metabolites and their corresponding concentrations. Results obtained may vary greatly depending on how concentrated samples are and how overlapping certain metabolites are, which might be concealing others from being measured [253].

Analysis of the large amounts of data that ^{1}H-NMR provides are analysed by individualistic comparison of metabolites or from a holistic approach. Initial study of metabolites in experiments such as those performed in this thesis, require the use of statistical tools to compare differences among treatment groups, via ANOVAs or Student's t-test as ^{1}H-NMR provides dozens of metabolites. Univariate analyses help to build metabolic pathways and to define changes in the relative amounts of metabolites among experimental groups. They also allow an easy comparison among the highlighted metabolites that could be interesting for the particular effect to which treatment is aiming for, like triglycerides, glucose and pyruvate, among other key metabolites. On the other hand, holistic analysis allows the study of changes in the metabolic profile, as a whole. Multivariate statistical analysis such as PCAs, PLS-DAs, comparison of spectral patterns or heat maps give information on whether treatments are inducing overall changes in the metabolism of animals, instead of over particular pathways. Furthermore, this approach grants the ability to study the totality of the metabolites in a single result, simplifying the postulation of conclusions [254].

The analysis of the metabolome in obesity has found profound changes in several metabolic routes. The metabolism of branched amino acids is altered in obesity, increasing the concentration of

valine, isoleucine and leucine and their downstream metabolites in their catabolism [255]. The effect of obesity also induces an effect on other amino acids, such as cysteine, glutamate, phenylalanine or tyrosine, among others, increasing their concentration [256–258]. On the other hand, amino acids such as glycine and its derivative, dimethylglycine, have been found lowered in obesity [259]. Glutamine [260] and methionine [261] are found reduced in obesity as well.

Besides, as expected, obesity induces major changes in the lipid profile and metabolism. Fatty acids are incremented in serum, as the adipose tissue sends them for their burn in liver and muscle [262]. Carnitine, metabolite responsible for the transportation of fatty acids into the mitochondria for β-oxidation [263], has also been found incremented in tissues, as a compensatory mechanism for the elevated concentration of lipids inside the cell [264]. Other lipids such as oleic acid and palmitic acid are increased in obesity [256]. However, several lipid metabolism-related metabolites, such as ceramides, sphingomyelins or phosphatidylcholines are more controversial as some authors found them incremented, whereas others did not [264]. Obesity has also been linked with increased thermogenesis, which upon metabolomic study has revealed alterations of the energy homeostasis, as several metabolites of the TCA cycle are affected, increasing their concentration after cold exposure; metabolites such as fumarate, succinate and malate [265].

The study of diabetes, insulin resistance and the general dysregulation of the glucose homeostasis in MetS, with metabolomic techniques has also been linked with changes in the amino acids profile. Cysteine, creatine or acetyl carnitine are increased [266,267], whereas glycine, arginine, betaine and methionine are lowered in diabetes [256,268]. Despite diabetes being predominantly an alteration of the glucose metabolism, dysregulations in the homeostasis of lipids have also been found. This is partly due to the alteration of insulin concentration that in turn, alter the production of lipoproteins and LPL and directly alters the internal modulation of the adipose tissue [269]. Several lipid metabolites in diabetes increase their concentration, correlating with findings in obesity, such as ceramides, linoleic acid, oleic acid or palmitic acid, among others [270,271]. Diglycerides tend to increase as well, correlating positively with insulin resistance [272]. In addition, both diabetes and obesity are directly responsible for the increase of metabolites such as glucose, insulin, fumarate, fructose, isopropanol, lactate or glycerol [257,258,273,274]. On the other hand, pyruvate is decreased, as it is mainly converted into lactate, instead of entering the TCA cycle [264].

4. Experimental models of metabolic syndrome in rats

Obesity and the associated pathologies that fall within the scope of the MetS are currently being extensively studied in humans. However, human studies involve major challenges as they pose technical and ethical difficulties, imply the need for patient volunteers, lack the possibility of study molecular mechanisms and are severely restricted in the extent of the amount of data that can be extracted with non-invasive procedures [275].

On the other hand, rodents present the opposite side of the spectrum of the mentioned traits. They have faster life cycles, faster metabolism that accelerates metabolic changes and is similar to human's [276] and working with them in terms of equipment and techniques is cheaper and easier and require less space [277]. Their metabolic pathways have been studied even more than human's which provides a more desirable platform from where to study the effect of compounds, treatments and diet, studies that are more constrained and limited in humans [278].

Rodent models have been designed, through many generational inbreds, to be as genetically similar as possible, thus, reducing the effect of differential genetic background. Going one step further, many gene knock-outs, overexpression or ectopic expression of genes have been designed into models, mimicking a particular illness. Among them, several have been designed to model obesity and symptoms of the MetS. The ob/ob mouse strain with deficiency of the leptin receptor is a classic model that develops hyperphagia, insulin resistance and obesity as leptin is not able to develop its function [279]. Zucker rats mimic the previous model in this rodent, with a mutation in the leptin receptor leading to dysregulated glucose homeostasis and obesity [280]. Closely related, the Zucker Diabetic Fatty Rats is a strain that besides the described symptomatology of the Zucker rats, has an early development of impaired glucose metabolism and diabetes due to an altered expression of *Glut4* [281]. In addition, the spontaneously hypertensive rat (SHR) rat model has been largely used to mimic hypertension as these rats develop the illness without need for diets [282]. Despite the advantages of genetic models and being widely used in scientific research, they also have considerable problems when trying to study obesity. They do not precisely represent the illness in the way it develops in humans as genetically originated symptoms of the MetS in humans are less pominent than diet-induced [283].

Due to this, another approach has focused on producing phenotypes for the study of obesity and CVD, rather than particular genotypes. These models have tendencies to develop diet-induced obesity [284]. An example of this, the main model in this thesis, the Wistar rats, animals prone to develop diet-induced obesity or the Long-Evans and Sprague-Dawley rat strains [276,284]. In this regard, research of obesity and MetS is developed via supplementation of compounds, designed diets or

environmental changes. Regarding diet-induced obesity, many approaches are being used to resemble it in rodents. High-caloric diets are provided to fatten obesity prone rats, such as HFD or high-sucrose diet (HSD), which consist in foods saturated in fats or carbohydrates, respectively, or a combination of both. However, considerable variability is found in the precise description of diets in scientific publications as many different food formulas are labelled as HFD or HSD, which, in turn, leads to variability of results as well [285]. HFD diets are considered as harmful for the metabolic status when they are based on saturated fats, which better induces obesity [286]. On the other hand, HSD are also used to develop type II diabetes and obesity, although it is unclear if this diet-induced insulin resistance is directly associated with obesity [287].

The work developed in this thesis follows a different approach by feeding rats with what is known as the cafeteria diet (CAF. This model, the combination of CAF diet in Wistar rats, provides a close modelling of obesity in humans, as it not only fattens them, but also induced the development of MetS, a circumstance that better resembles the health status of these individuals [288–293].

The CAF diet is a combination of typical fat-rich high-caloric food that are classically accessible in every CAF, hence, the name. As humans in western society tend to develop obesity by eating highly processed, calories-enriches foods, these obesogenic diets have a similar effect in rodents. The exact composition of the CAF diet might vary between studies but the reasoning behind is always to have a diverse selection of food enriched in fat and sugar, so that the animals can eat a variety of foods and choose as they desire. The reason behind it is that diversification of the food intake, and, hence, of source of nutrients and calories, varies daily and poses a bigger metabolic challenge that accelerates the development of obesity. CAF diet is characterised by being highly-palatable foods enriched in unsaturated fats and high content of sugar, like sausages, condensed milk, cheese and cookies, among others. It has been proved in previous studies that CAF diet indeed induces obesity and hyperphagia; plus several of the symptoms of the MetS such as hypertension and high waist circumference; and alters the metabolism of glucose and fatty acids [288,294].

5. Treatment and management of metabolic syndrome

Early treatment and diagnosis of the metabolic syndrome is highly recommended to reduce the chances of further developing other diseases like type II diabetes or NAFLD [295]. Different treatments have been tested and are recommended depending on the gravity of the disease or the symptom to be treated.

5.1 Lifestyle modifications and pharmacological interventions

Obesity and the abnormal, excessive abdominal accumulation of fat are usually the main target when treating MetS, as it tends to be the first symptom to appear and the most exacerbated. Initial recommendations come from lifestyle changes to reducing sedentary life and increasing exercise [296]. These lifestyle modifications and lowering of body weight have been found to ameliorate glucose impairment and decrease the chances of developing type II diabetes [297–299]. However, lifestyle modifications are meant to be a steady and regular approach to improve overweight in patients who are still in the early stages of the disease [300]. These changes must be maintained over long term periods as, otherwise, a relapse and increase in body weight is likely [301].

In addition, diet modifications represent the other important change required to ameliorate symptoms of the MetS [302]. Lowered caloric intake, added to exercise, might be enough to reduce the energy balance to negative numbers, thus reducing fat reservoirs [303]. Special diets have been designed to optimise the food intake for body weight loss, as a decline in obesity tends to improve the other symptoms of the MetS [300]. Low-carbohydrates diets have been used to reduce glucose intake [304], forcing the metabolism to use fat reservoirs as a source of energy. However, detrimental long term results have been found, as the elevated content of proteins and fat on the diet promote damage on kidneys and coronary atherosclerosis [305]. Other diets focus on lowering the content of fat [306,307], but studies have found that the effectiveness of low-fat diets might not be significantly different than high-fat diets for lowering body weight [308]. On the other hand, the Mediterranean diet, a pattern of food consisting of mostly vegetable, fruit, nuts, moderate and low quantities of fish and red meat, respectively, has shown positive effects on the MetS [309]. Regardless of the specific diet, the importance of long term consistency tends to be overlooked and studies have found little difference when comparing both diets after one year, as they were not properly followed [310,311].

As mentioned, diets are usually designed to reduce obesity and improve glucose levels. In addition, drugs have also been used to improve the rest of the symptoms of the MetS (High triglycerides and low HDL-C concentration in blood and hypertension), although treatments are usually recommended to be combined with lifestyle modifications.

Statins have been able to slightly reduce triglycerides and increase HDL-C [312]. These drugs have a direct impact on the synthesis of cholesterol, inhibiting HMGCR, thus reducing cholesterol in liver [313]. This in turn, promotes the expression of LDL-C receptors in liver, which removes cholesterol from blood and slightly reduces triglycerides and increases HDL-C [314]. Fibrates are also showing amelioration on the levels of triglycerides and HDL-C [315]. Several fibrate-base compounds are currently being marketed, and are being primarily used to raise HDL-C and lower triglycerides, with minor improvements over LDL-C [314,316]. However, some authors have reported opposite results where fibrates reduce HDL-C [317]. Nicotinic acid is a controversial drug that has shown major improvements in the lipidic profile, lowering triglycerides and LDL-C and increasing HDL-C, although detrimental side effects have been reported [314].

Regarding hypertension, several pharmaceutical drugs have been tested such as ACE inhibitors, beta-blockers or diuretics [318]. Diuretic drugs reduce the absorption of sodium in the kidneys, lowering blood volume and vascular resistance [319]. ACE inhibitors, such as captopril or enalapril, block the activity of ACE, reducing the concentration and the vasoconstrictor activity of angiotensin II [320,321]. Regarding beta-blockers, these competitive agonists of the catecholamines receptors would inhibit the sympathetic nervous system from increasing the BP [322]. Unfortunately, dosage and correct administration of antihypertensive drugs is an important, but difficult to achieve, key factor for normalizing BP, which failing hinders their efficacy [323]. In addition, administration might induce detrimental effects on glucose homeostasis, as certain drugs have also been linked with higher risks of developing diabetes [324].

5.2 Dietary approaches to prevent metabolic syndrome

Considering the predominance of obesity in western society, both scientists and the general public are interested in finding approaches for its treatment. Among the many alternatives being discussed, nutritional supplements are considered for the treatment of obesity and MetS.

Nutritional supplements are bioactive compounds, usually considered as soft treatment and developed from natural sources such as food or extracts from plants [325]. Their aim is to ameliorate symptoms and to be safely supplement as part of the normal diet [326].

5.2.1 Conjugated linoleic acid

Conjugated linoleic acid (CLA) is a fatty acid produced by a fermentative bacteria, *Butyrivibrio fibrisolvens*, commonly found in the rumen of ruminant animals, which isomerizes linoleic acid into conjugated linoleic acid [327]. Due to this, the main natural source of dietary CLA comes from ruminant meat like lamb or beef, or milk [328].

Linoleic acid (**Figure 9**), of *c9,c12* conformation, according to the configuration (cis: *c* or trans: *t*) of its ninth and twelfth carbon, can be isomerized into several conformations, being *c9,t11* and *t10,c12* the most commonly studied as they are the most bioactive [329]. However, is the *c9,t11* the most common to be naturally found in diet [327]. The isomer *c9,t11* tends to accumulate in milk and the animal's tissue, reaching a presence of up to 75-80% [330] of all present CLA, whereas *t10,c12* only counts to a 1% of CLA in milk [161].

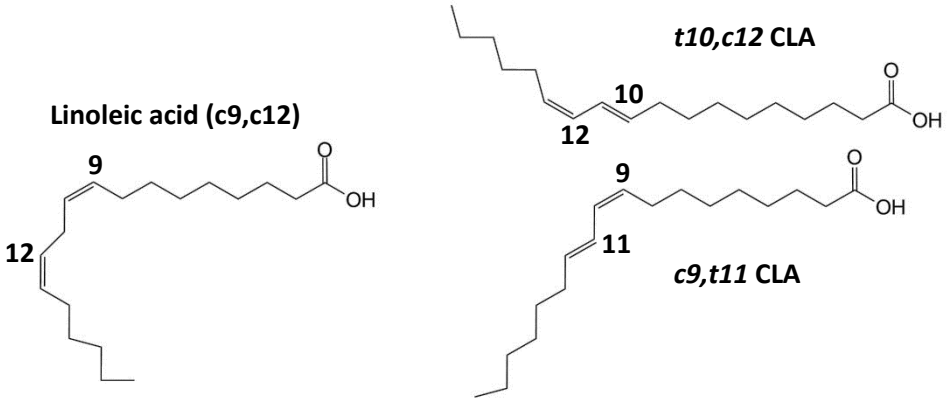


Figure 9. Linoleic acid and the *c9-t11* and *t10-c12* conjugated conformations (CLA). Image obtained from [331].

CLA has been associated for some time with reductions of fat mass, which has driven the interest of the pharmacological industry and research efforts to develop CLA as a food supplement that could help obese individuals to lose body weight. The isomer *t10,c12* has been the one mostly associated with changes in body weight due to a loss of fat mass [332]. This has been reported in several animal species with doses ranging from 0.5 to 1.5% of CLA in diet [333–336]. In addition, associated with this, studies performed in rodents with the isomer *t10,c12* have found reductions in body weight in mice [337] and rats [338], although results in rats are not as consistent as with mice [339]. These reductions in body weight with *t10,c12* CLA have been linked with loss of body fat [340], which has shown *t10,c12* as a promising dietary supplement to combat obesity. On the other hand, *c9,t11* have not been associated with changes in body composition, showing striking differences between the two isomers in their bioactivity in regards to body weight [341].

These results have been replicated in humans, although with less consistency. Some reports with *t10,c12* CLA supplementation in humans have been favourable and show reductions of fat mass [342–344], although others suggest that CLA might not have an effect on body composition [345,346]. Overall, CLA is still considered for human supplementation as some meta-analysis indeed prove a beneficial effect on obesity and fat loss with doses of 3.2 g per day [347]. Regarding *c9,t11*, it has not been reported to have major effects on adiposity [341], as with rodents.

Anti-obesity properties of the isomer *t10,c12* have been studied, aiming to understand the molecular mechanism that promotes fat mass loss. One of the proposed mechanisms of CLA to reduce adiposity relies on the activation of thermogenesis [348,349]. Increased basal metabolism has been reported in mice [350,351]. The increase in energy expenditure has been mostly associated with changes in the lipid metabolism as *t10-c12* seems to activate lipolysis and β–oxidation, which disrupts the storage of triglycerides in the adipose tissue [352]. Besides promoting the adipose tissue to release and burn its reservoirs of fat, *t10-c12* also inhibits the tissue from further replenishing its storage of energy as this isomer has also been found to reduce the uptake of glucose, by reducing the expression of *GLUT4*, and triglycerides by also reducing the expression of *LPL* [353,354]. The effect of CLA on body fat results in the expected decline in leptin. CLA has been found to decrease its concentration in the bloodstream [344,355], which is suggested to be a consequence of reductions in fat mass, although this statement is controversial because CLA could also be inhibiting leptin production as cultivation of *t10-c12* CLA with 3T3-L1 adipocytes have proved [356].

Besides these mechanisms, *t10-c12* also impacts the adiposity by reducing late adipogenesis (the process of differentiation of adipocytes from pre-adipocytes) in mature cultures adipocytes, whereas both *t10-c12* and *t9-c11* promote adipogenesis in early-stage immature adipocytes [357]. Other authors have also proposed an induction of apoptosis as a way to diminish WAT [358].

However, the isomer *t10-c12* has also been associated with detrimental effects. Studies in cultured rat adipocytes have found a decrease in the production of adiponectin, which usually onsets the development of type II diabetes and hyperinsulinemia [328]. This has been also corroborated with studies in humans that found *t10-c12* to be promoting insulin resistance [359]. On the other hand, reports with *t9-c11* in rats indicate an amelioration in insulin sensitivity, along with markers of inflammation [360]. Paradoxically, supplementation with equal doses of both isomers have shown improvements in glucose tolerance, but not with just *t9-c11* [361], which suggested that the detrimental effects of *t10-c12* could be counteracted with *t9-c11* supplementation.

Under certain circumstances such as dose or species, the problematic with *t10-c12* supplementation also affects the lipid metabolism in liver. Some authors have shown that *t10-c12*

induces inflammation and insulin resistance in human adipocytes [362]. However, more concern is raised with the association between *t10-c12* and hepatic lipid accumulation [363]. In particular, *t10-c12* has been shown to promote the uptake of triglycerides by the liver in mice, via activation of PPAR-γ. Besides, channels like *Cd36*, whose role is the absorption of NEFA, are overexpressed, further increasing the lipid content and hepatic steatosis [364]. Furthermore, other studies with *t10-c12* in mice have found the isomer to induce *de novo* lipogenesis [67] by promoting the expression of the transcription factor *Srebp-1c* and its regulated genes [69,365].

The link between fatty liver and *t10-c12* has been studied in human cell cultures as well. It has been reported that the secretion of fatty acids via VLDL-C is diminished in HepG2 cells, hindering improvements in NAFLD [366], although results are not consistent with studies on mice [367]. On the other hand *t10-c12* mostly enhances β–oxidation [68,368], although results are still controversial as some authors found from no effect [369] to actual reduction in fatty acid burning [370].

Despite some ameliorating effects on β–oxidation with the *t10-c12* isomer, the effect does not seem to compensate the high lipogenesis and increased influx of fatty acids into the liver, plus the insulin resistance, further worsening the energy metabolism, as shown in **figure 10**, hence, some studies on CLA report an aggravation on NAFLD [363].

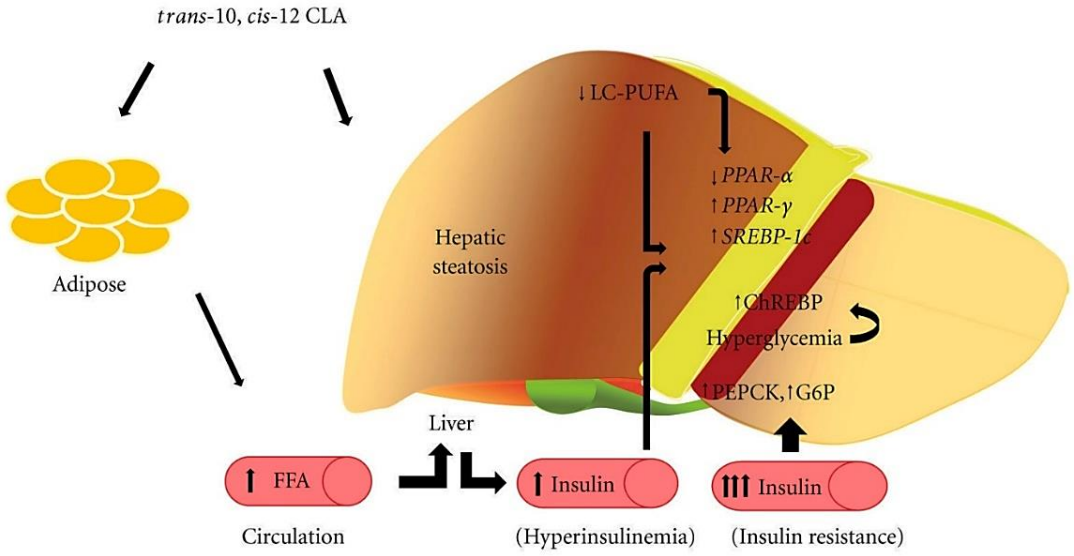


Figure 10. Effect of conjugated linoleic acid (CLA) on liver and adipose tissue. Some studies have reported that CLA promotes a release of fatty acids (FFA) in blood which will be absorbed by the liver. Besides the increase of fatty acids uptake, CLA has also been found to be able to regulates lipid metabolism-related gene in liver, altering the hepatic metabolism and inducing hepatic steatosis, which might be exacerbated by insulin resistance that also elevates glucose. PPAR: Peroxisome proliferator-activated receptors family; SREBP: Sterol regulatory element-binding protein; ChREBP: Carbohydrate-responsive element-binding protein; PEPCK: Cytoplasmic phosphoenolpyruvate carboxylase; G6P: Glucose 6-phosphate; LC-PUFA: Long-chain polyunsaturated fatty acids Image adapted from [363].

However, the adverse effects seem to vary greatly depending on the dose [363] and species, as rats do not seem to upregulate *Srebp-1c* under the effect of CLA but due to hyperinsulinemia [371]. Besides, detrimental results in the administration of CLA in animal models might not be easily comparable with humans as human trials have been performed with higher doses than those used in animal studies, finding no hepatic damage or aggravation of NAFLD, with supplementation of equal amounts of both isomers [372].

In addition, CLA effect has striking differences depending on metabolic status, sex of the animals, species and isomer of administration. Metabolic status is of major importance. For instance, Zucker rats (model of type II diabetes) fed with both isomers improved their glucose metabolism by ameliorating insulin sensitivity and reducing levels of circulating insulin and glucose [373], while non-obese mice, also treated with both isomers promoted insulin resistance [374]. Species are also of capital importance. Most experiments with CLA have been performed with mice, but rats seem to be more resilient to its detrimental effects [363]. CLA's effect, besides the previously described conditions, might be highly dependent on dose. Most studies in mice are designed using from 0,5% of CLA in diet [349,375], to 1% [376], or higher.

Finally, it is important to consider that the undesired effects of *t10-c12* supplementation have been reported to be palliated by the anti-inflammatory properties of *t9-c11* supplementation. Studies in mice have found a reduction in the release of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) by *c9-t11* [377], an important improvement in the development of MetS. Other studies have also reported reductions of inflammatory cytokines and NAFLD [378] and oxidative stress [379].

5.2.2 Polyphenols

Polyphenol compounds are secondary metabolites from plants. They are not required for the plant growth and essential life functions but are important for other functions such as ultraviolet protection, chemical-signalling for the correct growth of root nodes and attractors for pollination animals, among others [380,381]. The name encompasses a wide variety of different molecules, all of them having at least one aromatic ring and one or more hydroxyl groups attached. Combinations of attached groups and different conformations create more than 8000 unique compounds, spread throughout the plant kingdom [382].

These compounds have driven scientific interest as they have been linked with health improvements [380] such as cardiovascular risk, BP [383] or as treatments for obesity [384]. Their exact mechanism of action depends on the compound as each molecule might interact differently with each part of the metabolism [385]. Besides, it should also be noticed that the source of the

polyphenols and their bioavailability, which tends to be low, also play a role in their health effects [386]. However, upon their absorption in the enterocytes, they will undergo several enzymatic modifications [380,387], that will increase their life expectancy [388] allowing them to reach and effect their action in a diversity of organs such as liver, brain, skeletal muscle or adipose tissue [389,390]. In addition, some polyphenols that the enterocytes will not directly absorb, will receive structural modifications by the microbiota in the gut, which would allow their additional distribution in the body [391].

Polyphenols are classically divided into flavonoids and non-flavonoids. Among the flavonoids (**Figure 11**), several other classifications are made according to slight differences in the main molecular conformation of the flavonoid: flavonol, flavone, isoflavone, anthocyanidin, flavanone and flavan-3-ol (or flavanols). Flavonoids are the most common polyphenols to be found in diet and their study has shown amelioration in risks of CVD, inflammation, cancer [392,393] and diabetes [394].

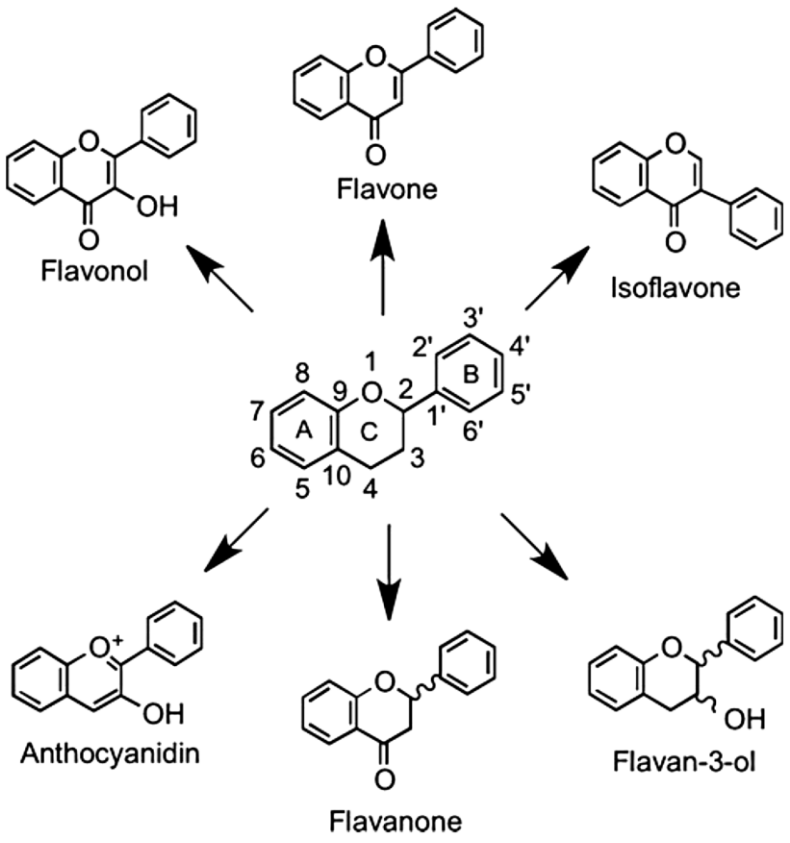


Figure 11. Flavonoids main molecular structure (centre) and derived subclasses. Figure obtained from [380].

5.2.2.1 Grape seed proanthocyanidins extract

Proanthocyanidins (PA) are conformed by oligomers and polymers of flavanols [395].They are abundant in fruits, cereals and legumes and prominent in diet via teas, wine and other beverages derived from fruits like grapes [396]. These bioactive compounds have been associated with improvements in a wide variety of health conditions and some of the main factors involved in obesity and MetS, which make them interesting candidates for nutritional supplementation. For instance, PA have been found to ameliorate oxidative stress and inflammation [397] by lowering the circulatory concentration of inflammatory cytokines such as interleukins, TNF-α or NF-κB [398].

Many studies have been performed using extracts from tea and grape seeds, which have further elucidated their beneficial effects on lipid homeostasis [399], in particular, against enzymes like the LPL [400]. PA are also capable of partially disrupting the absorption of lipids in the gut [399] by inhibiting digestive enzymes [401]. However, results with PA in lipid metabolism and dyslipidaemia amelioration have been inconsistent as some meta-analysis have found no differences in HDL-C or triglycerides [402] while other studies did found improvements in cholesterol [403]. They have also been associated with improvements in the endothelial functions, BP and insulin sensitivity [404] and high levels of glucose [405].

Grape seed proanthocyanidins extract (GSPE) is a product rich in polyphenols, particularly PA. PA have been found to have a wide variety of health effects, but particularly GSPE have been mostly studied as a nutritional supplement against dyslipidaemia, glucose homeostasis and hypertension.

The study of lipid metabolism with GSPE has found ameliorating effects in rodents, improving MetS-dyslipidaemia. GSPE administration has an effect against cholesterol dysregulation, as it increases HDL-C, while reducing LDL-C [406]. This is achieved by increasing the expression of the ATP-binding cassette transporter 1 gene (*Abca1*), in liver via a repression of miR-33, which ultimately increases the flux of cholesterol towards the formation of HDL-C particles [407].

Other studies in rats have also found a decrease in circulatory triglycerides after an acute dose of GSPE [408] and an amelioration in their mobility and modulation in a lipid tolerance test [409]. This reduction in triglycerides have also been found in livers of HFD diet fed rats, after chronic administration, improving hepatic steatosis [410]. The reduction of both, hepatic and serum triglycerides have been associated with activation of the expression of *Sirt-1*, promotion of nicotinamide adenine dinucleotide (NAD+) production and a repression of *Srebp1* in liver, which would in turn activate fatty acid oxidation and reduce lipogenesis, shifting hepatic lipid metabolism towards lowering lipid content [411–413]. Similar results are obtained in WAT as GSPE improves mobility and

oxidation of lipids in obese rats [414], adiposity in obese hamsters [415] and activates lipolysis in cultured adipocytes [416].

In addition, GSPE is also involved in the homeostasis of glucose. GSPE have been found to directly reduce circulatory glucose in rat models of diabetes with acute doses of 250 mg/kg [417]. This has been associated with studies where GSPE administration in obese rats was found to increase the expression of *Glut4* and *Irs-1* in adipose tissue, which also ameliorates the insulin resistance of the tissue [418]. However, the role of GSPE in glucose homeostasis is still controversial as some studies have found no improvements in animal models [419] while others have reported body symptoms associated with diabetes like body weight gain [420].

PA have also been largely associated with the amelioration of hypertension, not only from grape seed sources but extracted from several others such as cocoa or green teas, in both short [421–423] and long-term [424,425] effects upon administration. The anti-hypertensive effect of GSPE on BP has been corroborated in SHR models [426] and CAF diet-fed rats [410]. Concretely, GSPE at low physiological doses of just 25 mg/kg is capable of long-term reduction of BP in CAF diet-fed rats, showing an anti-hypertensive effect in models of MetS [290].

Finally, GSPE has also been related with modulation of leptin functionality. GSPE has been found to improve leptin sensitivity in leptin resistant hypothalamus of CAF diet-fed rats as levels of STA3-P and the expression of *Soc3* and *Ptp1b* were normalized with long-term supplementation with GSPE [427]. These results have also been associated with amelioration on hypothalamic inflammation, which could be partly mediating the modulation of leptin resistance [427]. This has been corroborated in studies showing GSPE to reduce food intake [428].

5.2.2.2 Anthocyanidins extract

As mentioned, anthocyanidins is a sub-group of flavonoids, which includes several molecules, the most common in diet being peonidin, petunidin, malvidin, pelargonidin, delphinidin and cyanidin, usually found in fruits like berries [429]. Similar to proanthocyanidins, anthocyanidins have been associated with amelioration of symptoms of the MetS. Supplementation of a dose 200 mg/kg of anthocyanidins in mice have been shown to reduce obesity and adipocyte size [430]. Other studies with anthocyanidins have corroborated a reduction in obesity and improvements in triglycerides, glucose and cholesterol [431].

Medox is an extract of bilberries and blackcurrant, optimized for the extraction of certain anthocyanins. The product was developed as a nutritional supplement that could combat symptoms of the MetS via a highly concentrated dose of anthocyanins that would have a high representation of

Cyanidin-3G and Delphinidin-3G. Medox have been studied extensively studied in humans and reported to improve inflammation and oxidative stress [432,433]. In particular, a decrease of NF-κB and other circulatory inflammatory cytokines like TNF-α [432,434] and CRP [435]. Related to this, Medox has also been found to ameliorate NAFLD in patients that have already developed the illness: levels of GOT/GPT were decreased and improvements of glucose homeostasis and insulin resistance were found [436]. Other authors have reported similar findings as diabetic patients also showed improvements in insulin resistance and fasting glucose, besides improvements of the lipid metabolism by increasing HDL-C and decreasing triglycerides and LDL-C [437].

Medox has also been reported to ameliorate dyslipidaemia. Administration of Medox was found to increase HDL-C and reduce LDL-C concentration while also promoting the release of cholesterol from peripheral organs, possibly to be metabolized by the liver [438,439]. In a similar approach, another study with hypercholesterolemic patients has found similar increments on circulatory HDL-C and amelioration in vascular dilatation [440]. Medox's amelioration of cholesterol management and endothelial function have also been found in mice and linked with a reduction of the ATP-binding cassette sub-family G member 1 (ABCG1) pathway. This study also reported a correlation with a reduction of NO and oxidative stress after promoting eNOS activity [441].

5.2.3 Anti-hypertensive hydrolysate

The Chicken foot protein hydrolysate (Hpp11) is a hydrolysate of chicken foot, a patented product developed in our laboratory [442], with the aim of obtaining a nutritional supplement that could lower BP. Hpp11 is a mix of bioactive peptides with antihypertensive properties [443]. Bioactive peptides are considered as such when the peptides, besides their natural nutritional value, have additional health properties and alter body functions [444]. However, for a peptide to be considered bioactive, it must be able to produce an effect in physiological doses, meaning, a dose that is not considered too high or unrealistic. In addition, the effect they exert on the metabolism must be beneficial [445].

Bioactive peptides are obtained as fragments from original proteins. They are sequences of amino acids of variable length and sequence (137) that are easily metabolized, limiting their effect, compared with pharmacological drugs, while also ensuring that they are less likely to accumulate in tissues, which reduces the chances of developing side effects (136). Among the use of bioactive peptides as health ameliorating supplements, many reports have found improvements in a wide variety of health issues and they are being used as anti-oxidative, osteoprotective or anti-lipidemic supplements, among others [325,446,447].

Besides the mentioned properties, many bioactive peptides have been found to have anti-hypertensive properties [448]. An initial report found peptides with ACE inhibitory properties after protein hydrolysis with casein [449]. This started the study of the potential anti-hypertensive effect that bioactive peptides could perform and showed that these peptides could be obtained from many food sources [450,451]. Of particular interest have been the development of bioactive peptides from by-products of the food industry, which grants cheap source material [452]. In particular, chicken feet are a waste product that would be otherwise discarded and have been issued as the protein source for their hydrolysation in the development of Hpp11 [442].

Several studies have already been performed involving Hpp11. Initial studies *in vivo* have found the hydrolysate to have anti-hypertensive properties, reducing BP after an acute administration of 55 mg/kg in hypertensive rats [443]. In addition, Hpp11 has been tested in long-term administration, showing that it also promotes long-term lowering of BP in diet-induced hypertensive rats [453], particularly interesting as HTN requires chronic treatment [454].

The molecular mechanisms by which Hpp11 reduces BP are starting to be elucidated. The hydrolysate has ACE inhibitory activity, which prevents the cleavage of angiotensin I into angiotensin II and the release of this vasoconstrictor [442]. Moreover, Hpp11 also promotes an effect on the release of NO by enhancing the expression of the aortic genes *Sirt-1* and NADPH oxidase (*Nox-4*) and lowering of *Et-1*, which would induce a higher release of the vasodilator NO [453]. In addition to its properties to reduce HTN, Hpp11 has been associated with reductions of oxidative stress as it promotes an increase in the production hepatic reduced glutathione [453], a known antioxidant that removes reactive oxygen species [455].

References

1. Chandon, P.; Wansink, B. Does food marketing need to make us fat? A review and solutions. *Nutr. Rev.* **2012**, *70*, 571–593.
2. Agha, M.; Agha, R. The rising prevalence of obesity. *Int. J. Surg. Oncol.* **2017**, *2*, e19.
3. Whitaker, R.C.; Wright, J.A.; Pepe, M.S.; Seidel, K.D.; Dietz, W.H. Predicting Obesity in Young Adulthood from Childhood and Parental Obesity. *N. Engl. J. Med.* **1997**, *337*, 869–873.
4. Saunders, J.; Smith, T. Malnutrition: Causes and consequences. *Clin. Med. J. R. Coll. Physicians London* **2010**, *10*, 624–627.
5. Hruby, A.; Manson, J.A.E.; Qi, L.; Malik, V.S.; Rimm, E.B.; Sun, Q.; Willett, W.C.; Hu, F.B. Determinants and consequences of obesity. *Am. J. Public Health* **2016**, *106*, 1656–1662.
6. Reddon, H.; Gerstein, H.C.; Engert, J.C.; Mohan, V.; Bosch, J.; Desai, D.; Bailey, S.D.; Diaz, R.; Yusuf, S.; Anand, S.S.; et al. Physical activity and genetic predisposition to obesity in a multiethnic longitudinal study. *Sci. Rep.* **2016**, *6*, 18672.
7. James, W.P.T. WHO recognition of the global obesity epidemic. *Int. J. Obes.* **2008**, *32*, S120–S126.
8. Cornier, M.-A.; Dabelea, D.; Hernandez, T.L.; Lindstrom, R.C.; Steig, A.J.; Stob, N.R.; Van Pelt, R.E.; Wang, H.; Eckel, R.H. The Metabolic Syndrome. *Endocr. Rev.* **2008**, *29*, 777–822.
9. American, A.; Association, H.; Heart, N.; Grundy, S.M.; Cleeman, J.I.; Daniels, S.R.; Donato, K.A.; Eckel, R.H.; Franklin, B.A.; Gordon, D.J.; et al. Diagnosis and managment of the metabolic syndrome AHA / NHLBI Scientific Statement. **2006**, 2735–2752.
10. Ng, M.; Fleming, T.; Robinson, M. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: A systematic analysis. *Lancet* **2014**, *384*, 746.
11. Hashimoto, E.; Taniai, M.; Tokushige, K. Characteristics and diagnosis of NAFLD/NASH. *J. Gastroenterol. Hepatol.* **2013**, *28*, 64–70.
12. Kawano, Y.; Cohen, D.E. Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. *J. Gastroenterol.* **2013**, *48*, 434–441.
13. Koyama, K.; Chen, G.; Lee, Y.; Unger, R.H. Tissue triglycerides, insulin resistance, and insulin production: implications for hyperinsulinemia of obesity. *Am. J. Physiol. Metab.* **1997**, *273*, E708–E713.
14. Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55 000 vascular deaths. *Lancet* **2007**, *370*, 1829–1839.
15. Manolio, T.A.; Ettinger, W.H.; Tracy, R.P.; Kuller, L.H.; Borhani, N.O.; Lynch, J.C.; Fried, L.P. Epidemiology of low cholesterol levels in older adults. The Cardiovascular Health Study. *Circulation* **1993**, *87*, 728–737.
16. Chen, S.Y.; Li, N.; Jin, T.L.; Gou, L.; Hao, D.X.; Tian, Z.Q.; Zhang, S.L.; Zhang, L. Lipoprotein in cholesterol transport: Highlights and recent insights into its structural basis and functional mechanism. *Chinese Phys. B* **2018**, *27*.
17. Toth, P.P. Reverse cholesterol transport: High-density lipoprotein's magnificent mile. *Curr. Atheroscler. Rep.* **2003**, *5*, 386–393.
18. Rader, D.J.; Rader, D.J. Molecular regulation of HDL metabolism and function : implications for novel therapies Find the latest version : Science in medicine Molecular regulation of HDL metabolism and function : implications for novel therapies. **2006**, *116*, 3090–3100.
19. Vaisar, T.; Pennathur, S.; Green, P.S.; Gharib, S.A.; Hoofnagle, A.N.; Cheung, M.C.; Byun, J.; Vuletic, S.; Kassim, S.; Singh, P.; et al. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. *J. Clin. Invest.* **2007**, *117*, 746–756.
20. Gordon, D.J.; Probstfield, J.L.; Garrison, R.J.; Neaton, J.D.; Castelli, W.P.; Knoke, J.D.; Jacobs, D.R.; Bangdiwala, S.; Tyroler, H.A. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation* **1989**, *79*, 8–15.
21. Kontush, A.; Chapman, M.J. Antiatherogenic function of HDL particle subpopulations: Focus on

antioxidative activities. *Curr. Opin. Lipidol.* **2010**, *21*, 312–318.

22. Mineo, C.; Deguchi, H.; Griffin, J.H.; Shaul, P.W. Endothelial and antithrombotic actions of HDL. *Circ. Res.* **2006**, *98*, 1352–1364.
23. Mulè, G. Metabolic syndrome in hypertensive patients: An unholy alliance. *World J. Cardiol.* **2014**, *6*, 890.
24. Mule, G.; Cottone, S.; Nardi, E.; Andronico, G.; Cerasola, G. Metabolic syndrome in subjects with essential hypertension: relationships with subclinical cardiovascular and renal damage. *Minerva Cardioangiol.* **2006**, *54*, 173–194.
25. Ferrannini, E.; Buzzigoli, G.; Bonadonna, R.; Giorico, M.A.; Oleggini, M.; Graziadei, L.; Pedrinelli, R.; Brandi, L.; Bevilacqua, S. Insulin Resistance in Essential Hypertension. *N. Engl. J. Med.* **1987**, *317*, 350–357.
26. Reaven, G.M.; Lithell, H.; Landsberg, L. Hypertension and Associated Metabolic Abnormalities — The Role of Insulin Resistance and the Sympathoadrenal System. *N. Engl. J. Med.* **1996**, *334*, 374–382.
27. Hall, J. Obesity hypertension: role of leptin and sympathetic nervous system. *Am. J. Hypertens.* **2001**, *14*, S103–S115.
28. Nickenig, G.; Röling, J.; Strehlow, K.; Schnabel, P.; Böhm, M. Insulin induces upregulation of vascular receptor gene expression by posttranscriptional mechanisms. *Circulation* **1998**, *98*, 2453–2460.
29. Porte, D. Mechanisms for Hyperglycemia in the Metabolic Syndrome. The Key Role of β-Cell Dysfunction. 73–83.
30. Lee, S.J.; Heinrich, G.; Fedorova, L.; Al-Share, Q.Y.; Ledford, K.J.; Fernstrom, M.A.; McInerney, M.F.; Erickson, S.K.; Gatto-Weis, C.; Najjar, S.M. Development of nonalcoholic steatohepatitis in insulin-resistant liver-specific S503A carcinoembryonic antigen-related cell adhesion molecule 1 mutant mice. *Gastroenterology* **2008**, *135*, 2084–2095.
31. Xu, A.; Wang, Y.; Keshaw, H.; Xu, L.Y.; Lam, K.S.L.; Cooper, G.J.S. The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. *J. Clin. Invest.* **2003**, *112*, 91–100.
32. Roberts, C.K.; Hevener, A.L.; Barnard, R.J. Metabolic syndrome and insulin resistance: underlying causes and modification by exercise training. *Compr. Physiol.* **2013**, *3*, 1–58.
33. Hruby, A.; Hu, F.B. The Epidemiology of Obesity: A Big Picture. *Pharmacoeconomics* **2015**, *33*, 673–689.
34. Blomain, E.S.; Dirhan, D.A.; Valentino, M.A.; Kim, G.W.; Waldman, S.A. Mechanisms of Weight Regain following Weight Loss. *ISRN Obes.* **2013**, *2013*, 210524.
35. Harris, R.B.S.; Kasser, T.R.; Martin, R.J. Dynamics of recovery of body composition after overfeeding, food restriction or starvation of mature female rats. *J. Nutr.* **1986**, *116*, 2536–2546.
36. Campfield, L.A.; Smith, F.J.; Guisez, Y.; Devos, R.; Burn, P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science (80-.).* **1995**, *269*, 546–549.
37. Sims, E.A.H.; Danforth, E. Expenditure and storage of energy in man. *J. Clin. Invest.* **1987**, *79*, 1019–1025.
38. Considine, R. V.; Sinha, M.K.; Heiman, M.L.; Kriauciunas, A.; Stephens, T.W.; Nyce, M.R.; Ohannesian, J.P.; Marco, C.C.; Mckee, L.J.; Bauer, T.L.; et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N. Engl. J. Med.* **1996**, *334*, 292–295.
39. Ostlund Jr, R.E.; Yang, J.W.; Klein, S.; Gingerich, R. Relation between plasma leptin concentration and body fat, gender, diet, age, and metabolic covariates. *J. Clin. Endocrinol. Metab.* **1996**, *81*, 3909–3913.
40. Morrison, C.D. Leptin signaling in brain: A link between nutrition and cognition? *Biochim. Biophys. Acta - Mol. Basis Dis.* **2009**, *1792*, 401–408.
41. Luquet, S.; Perez, F.A.; Hnasko, T.S.; Palmiter, R.D. NPY/AgRP Neurons Are Essential for Feeding in Adult Mice but Can Be Ablated in Neonates. *Science (80-.).* **2005**, *310*, 683–685.
42. Balthasar, N.; Coppari, R.; McMinn, J.; Liu, S.M.; Lee, C.E.; Tang, V.; Kenny, C.D.; McGovern, R.A.; Chua, S.C.; Elmquist, J.K.; et al. Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. *Neuron* **2004**, *42*, 983–991.

43. Håkansson, M.L.; Meister, B. Transcription factor STAT3 in leptin target neurons of the rat hypothalamus. *Neuroendocrinology* **1998**, *68*, 420–427.

44. Plum, L.; Belgardt, B.F.; Brüning, J.C. Central insulin action in energy and glucose homeostasis. *J. Clin. Invest.* **2006**, *116*, 1761–1766.

45. El-Haschimi, K.; Pierroz, D.D.; Hileman, S.M.; Bjørbæk, C.; Flier, J.S. Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. *J. Clin. Invest.* **2000**, *105*, 1827–1832.

46. Banks, W.A.; DiPalma, C.R.; Farrell, C.L. Impaired transport of leptin across the blood-brain barrier in obesity. *Peptides* **1999**, *20*, 1341–1345.

47. Belouzard, S.; Delcroix, D.; Rouillé, Y. Low levels of expression of leptin receptor at the cell surface result from constitutive endocytosis and intracellular retention in the biosynthetic pathway. *J. Biol. Chem.* **2004**, *279*, 28499–28508.

48. Shi, H.; Kokoeva, M. V; Inouye, K.; Tzameli, I.; Yin, H.; Flier, J.S. TLR4 links innate immunity and fatty acid–induced insulin resistance. *J. Clin. Invest.* **2006**, *116*, 3015–3025.

49. Buettner, C.; Pocai, A.; Muse, E.D.; Etgen, A.M.; Myers, M.G.; Rossetti, L. Critical role of STAT3 in leptin's metabolic actions. *Cell Metab.* **2006**, *4*, 49–60.

50. Polyzos, S.A.; Kountouras, J.; Zavos, C.; Deretzi, G. The potential adverse role of leptin resistance in nonalcoholic fatty liver disease: A hypothesis based on critical review of the literature. *J. Clin. Gastroenterol.* **2011**, *45*, 50–54.

51. Howard, J.K.; Flier, J.S. Attenuation of leptin and insulin signaling by SOCS proteins. *Trends Endocrinol. Metab.* **2006**, *17*, 365–371.

52. Robertson, S.A.; Leinninger, G.M.; Myers, M.G. Molecular and neural mediators of leptin action. *Physiol. Behav.* **2008**, *94*, 637–642.

53. Myers, M.G.; Cowley, M.A.; Münzberg, H. Mechanisms of Leptin Action and Leptin Resistance. *Annu. Rev. Physiol.* **2008**, *70*, 537–556.

54. Emanuelli, B.; Peraldi, P.; Filloux, C.; Sawka-Verhelle, D.; Hilton, D.; Van Obberghen, E. SOCS-3 is an insulin-induced negative regulator of insulin signaling. *J. Biol. Chem.* **2000**, *275*, 15985–15991.

55. Han, H.-S.; Kang, G.; Kim, J.S.; Choi, B.H.; Koo, S.-H. Regulation of glucose metabolism from a liver-centric perspective. *Exp. Mol. Med.* **2016**, *48*, e218–e218.

56. Hu, C.; Hoene, M.; Plomgaard, P.; Hansen, J.S.; Zhao, X.; Li, J.; Wang, X.; Clemmesen, J.O.; Secher, N.H.; Häring, H.U.; et al. Muscle-liver substrate fluxes in exercising humans and potential effects on hepatic metabolism. *J. Clin. Endocrinol. Metab.* **2019**.

57. Towle, H.C.; Kaytor, E.N.; Shih, H.-M. Regulation of the expression of lipogenic enzyme genes by carbohydrate. *Annu. Rev. Nutr.* **1997**, *17*, 405–433.

58. Benedict, M.; Zhang, X. Non-alcoholic fatty liver disease: An expanded review. *World J. Hepatol.* **2017**, *9*, 715–732.

59. Calzadilla Bertot, L.; Adams, L.A. The Natural Course of Non-Alcoholic Fatty Liver Disease. *Int. J. Mol. Sci.* **2016**, *17*.

60. Sayiner, M.; Koenig, A.; Henry, L.; Younossi, Z.M. Epidemiology of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis in the United States and the Rest of the World. *Clin. Liver Dis.* **2016**, *20*, 205–214.

61. Kanwar, P.; Kowdley, K. V The Metabolic Syndrome and Its Influence on Nonalcoholic Steatohepatitis. *Clin. Liver Dis.* **2016**, *20*, 225–243.

62. Caldwell, S.H.; Oelsner, D.H.; Iezzoni, J.C.; Hespenheide, E.E.; Battle, E.H.; Driscoll, C.J. Cryptogenic cirrhosis: Clinical characterization and risk factors for underlying disease. *Hepatology* **1999**, *29*, 664–669.

63. Stefan, N.; Kantartzis, K.; Häring, H.U. Causes and metabolic consequences of fatty liver. *Endocr. Rev.* **2008**, *29*, 939–960.

64. Bechmann, L.P.; Hannivoort, R.A.; Gerken, G.; Hotamisligil, G.S.; Trauner, M.; Canbay, A. The

interaction of hepatic lipid and glucose metabolism in liver diseases. *J. Hepatol.* **2012**, *56*, 952–964.

65. Yip, W.W.; Burt, A.D. Alcoholic liver disease. *Semin. Diagn. Pathol.* **2006**, *23*, 149–160.

66. Arab, J.P.; Arrese, M.; Trauner, M. Recent Insights into the Pathogenesis of Nonalcoholic Fatty Liver Disease. *Annu. Rev. Pathol. Mech. Dis.* **2018**, *13*, 321–350.

67. Clément, L.; Poirier, H.; Niot, I.; Bocher, V.; Guerre-Millo, M.; Krief, S.; Staels, B.; Besnard, P. Dietary trans-10,cis-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse . *J. Lipid Res.* **2002**, *43*, 1400–1409.

68. Ide, T. Interaction of fish oil and conjugated linoleic acid in affecting hepatic activity of lipogenic enzymes and gene expression in liver and adipose tissue. *Diabetes* **2005**, *54*, 412–423.

69. Takahashi, Y.; Kushiro, M.; Shinohara, K.; Ide, T. Activity and mRNA levels of enzymes involved in hepatic fatty acid synthesis and oxidation in mice fed conjugated linoleic acid. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **2003**, *1631*, 265–273.

70. Javadi, M.; Beynen, A.C.; Hovenier, R.; Lankhorst, Æ.; Lemmens, A.G.; Terpstra, A.H.M.; Geelen, M.J.H. Prolonged feeding of mice with conjugated linoleic acid increases hepatic fatty acid synthesis relative to oxidation. *J. Nutr. Biochem.* **2004**, *15*, 680–687.

71. Long, Y.C.; White, M.F. IRS-1 and IRS-2 Signaling Is Essential for Skeletal Muscle Growth and Protein Homeostasis. *Diabetes* **2009**, *58*, A392–A392.

72. Tandra, S.; Yeh, M.M.; Brunt, E.M.; Vuppalanchi, R.; Cummings, O.W.; Ünalp-Arida, A.; Wilson, L.A.; Chalasani, N. Presence and significance of microvesicular steatosis in nonalcoholic fatty liver disease. *J. Hepatol.* **2011**, *55*, 654–659.

73. Xu, H.; Barnes, G.T.; Yang, Q.; Tan, G.; Yang, D.; Chou, C.J.; Sole, J.; Nichols, A.; Ross, J.S.; Tartaglia, L.A.; et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* **2003**, *112*, 1821–1830.

74. Bugianesi, E.; Pagotto, U.; Manini, R.; Vanni, E.; Gastaldelli, A.; De Iasio, R.; Gentilcore, E.; Natale, S.; Cassader, M.; Rizzetto, M.; et al. Plasma Adiponectin in nonalcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity. *J. Clin. Endocrinol. Metab.* **2005**, *90*, 3498–3504.

75. Shoelson, S.E.; Lee, J.; Goldfine, A.B. Inflammation and insulin resistance. *Diabetes* **2006**, *116*, 1793–1801.

76. Özcan, U.; Cao, Q.; Yilmaz, E.; Lee, A.H.; Iwakoshi, N.N.; Özdelen, E.; Tuncman, G.; Görgün, C.; Glimcher, L.H.; Hotamisligil, G.S. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science (80-.).* **2004**, *306*, 457–461.

77. Yang, S.; Zhu, H.; Li, Y.; Lin, H.; Gabrielson, K.; Trush, M.A.; Diehl, A.M. Mitochondrial adaptations to obesity-related oxidant stress. *Arch. Biochem. Biophys.* **2000**, *378*, 259–268.

78. Szczepaniak, L.S.; Nurenberg, P.; Leonard, D.; Browning, J.D.; Reingold, J.S.; Grundy, S.; Hobbs, H.H.; Dobbins, R.L. Magnetic resonance spectroscopy to measure hepatic triglyceride content: Prevalence of hepatic steatosis in the general population. *Am. J. Physiol. - Endocrinol. Metab.* **2005**, *288*, 462–468.

79. Kotronen, A.; Westerbacka, J.; Bergholm, R.; Pietiläinen, K.H.; Yki-Järvinen, H. Liver fat in the metabolic syndrome. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 3490–3497.

80. Flier, J.S. Obesity Wars: Molecular Progress Confronts an Expanding Epidemic. *Cell* **2004**, *116*, 337–350.

81. Juge-Aubry, C.E.; Henrichot, E.; Meier, C.A. Adipose tissue: a regulator of inflammation. *Best Pract. Res. Clin. Endocrinol. Metab.* **2005**, *19*, 547–566.

82. Cinti, S. The Adipose Organ BT - Adipose Tissue and Adipokines in Health and Disease. In; Fantuzzi, G., Mazzone, T., Eds.; Humana Press: Totowa, NJ, 2007; pp. 3–19 ISBN 978-1-59745-370-7.

83. Sanchez-Gurmaches, J.; Guertin, D.A. Adipocyte lineages: Tracing back the origins of fat. *Biochim. Biophys. Acta - Mol. Basis Dis.* **2014**, *1842*, 340–351.

84. Proença, A.R.G.; Sertié, R.A.L.; Oliveira, A.C.; Campaña, A.B.; Caminhotto, R.O.; Chimin, P.; Lima, F.B. New concepts in white adipose tissue physiology. *Brazilian J. Med. Biol. Res. = Rev. Bras. Pesqui.*

medicas e Biol. **2014**, *47*, 192–205.

85. Vázquez-Vela, M.E.F.; Torres, N.; Tovar, A.R. White Adipose Tissue as Endocrine Organ and Its Role in Obesity. *Arch. Med. Res.* **2008**, *39*, 715–728.
86. Thompson, B.R.; Lobo, S.; Bernlohr, D.A. Fatty acid flux in adipocytes: The in's and out's of fat cell lipid trafficking. *Mol. Cell. Endocrinol.* **2010**, *318*, 24–33.
87. Cammisotto, P.G.; Bukowiecki, L.J. Mechanisms of leptin secretion from white adipocytes. *Am. J. Physiol. Physiol.* **2002**, *283*, C244–C250.
88. Tsai, M.; Asakawa, A.; Amitani, H.; Inui, A. Stimulation of leptin secretion by insulin. *Indian J. Endocrinol. Metab.* **2012**, *16*, S543–S548.
89. Haffner, S.M.; Miettinen, H.; Karhapää, P.; Mykkänen, L.; Laakso, M. Leptin Concentrations, Sex Hormones, and Cortisol in Nondiabetic Men1. *J. Clin. Endocrinol. Metab.* **1997**, *82*, 1807–1809.
90. Suga, A.; Hirano, T.; Inoue, S.; Tsuji, M.; Osaka, T.; Namba, Y.; Miura, M.; Adachi, M. Plasma leptin levels and triglyceride secretion rates in VMH-lesioned obese rats: a role of adiposity. *Am. J. Physiol. Metab.* **1999**, *276*, E650–E657.
91. Blundell, J.E.; Goodson, S.; Halford, J.C. Regulation of appetite: role of leptin in signalling systems for drive and satiety. *Int. J. Obes. Relat. Metab. Disord.* **2001**, *25 Suppl 1*, S29-34.
92. Jéquier, E. Leptin Signaling, Adiposity, and Energy Balance. *Ann. N. Y. Acad. Sci.* **2002**, *967*, 379–388.
93. Flier, J.S. What's in a Name? In Search of Leptin's Physiologic Role. *J. Clin. Endocrinol. Metab.* **1998**, *83*, 1407–1413.
94. Monteiro, R.; Azevedo, I. Chronic inflammation in obesity and the metabolic syndrome. *Mediators Inflamm.* **2010**, *2010*.
95. Surmi, B.K.; Hasty, A.H. Macrophage infiltration into adipose tissue: initiation, propagation and remodeling. *Future Lipidol.* **2008**, *3*, 545–556.
96. Liu, P.-S.; Lin, Y.-W.; Burton, F.H.; Wei, L.-N. M1-M2 balancing act in white adipose tissue browning - a new role for RIP140. *Adipocyte* **2015**, *4*, 146–148.
97. Thomas, D.; Apovian, C. Macrophage functions in lean and obese adipose tissue. *Metabolism.* **2017**, *72*, 120–143.
98. Chylikova, J.; Dvorackova, J.; Tauber, Z.; Kamarad, V. M1/M2 macrophage polarization in human obese adipose tissue. *Biomed. Pap.* **2018**, *162*, 79–82.
99. Hotamisligil, G.S. Inflammation and metabolic disorders. *Nature* **2006**, *444*, 860–867.
100. Wellen, K.E.; Hotamisligil, G.S. Inflammation, stress, and diabetes. *J. Clin. Invest.* **2005**, *115*, 1111–1119.
101. Cranford, T.L.; Enos, R.T.; Velázquez, K.T.; McClellan, J.L.; Davis, J.M.; Singh, U.P.; Nagarkatti, M.; Nagarkatti, P.S.; Robinson, C.M.; Murphy, E.A. Role of MCP-1 on inflammatory processes and metabolic dysfunction following high-fat feedings in the FVB/N strain. *Int. J. Obes. (Lond).* **2016**, *40*, 844–851.
102. Medzhitov, R. Origin and physiological roles of inflammation. *Nature* **2008**, *454*, 428–435.
103. Sproston, N.R.; Ashworth, J.J. Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front. Immunol.* **2018**, *9*, 754.
104. Grundy, S.M. Inflammation, metabolic syndrome, and diet responsiveness. *Circulation* **2003**, *108*, 126–128.
105. Foroughi, M.; Maghsoudi, Z.; Khayyatzadeh, S.; Ghiasvand, R.; Askari, G.; Iraj, B. Relationship between non-alcoholic fatty liver disease and inflammation in patients with non-alcoholic fatty liver. *Adv. Biomed. Res.* **2016**, *5*, 28.
106. Hotamisligil, G.S.; Shargill, N.S.; Spiegelman, B.M. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science (80-.).* **1993**, *259*, 87–91.
107. Hotamisligil, G.S.; Arner, P.; Caro, J.F.; Atkinson, R.L.; Spiegelman, B.M. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J. Clin. Invest.* **1995**, *95*, 2409–2415.

108. Bluher, M. Adipose tissue dysfunction in obesity. *Exp. Clin. Endocrinol. Diabetes* **2009**, *117*, 241–250.

109. Hammarstedt, A.; Graham, T.E.; Kahn, B.B. Adipose tissue dysregulation and reduced insulin sensitivity in non-obese individuals with enlarged abdominal adipose cells. *Diabetol. Metab. Syndr.* **2012**, *4*, 42.

110. Acosta, J.R.; Douagi, I.; Andersson, D.P.; Bäckdahl, J.; Rydén, M.; Arner, P.; Laurencikiene, J. Increased fat cell size: a major phenotype of subcutaneous white adipose tissue in non-obese individuals with type 2 diabetes. *Diabetologia* **2016**, *59*, 560–570.

111. Cinti, S.; Mitchell, G.; Barbatelli, G.; Murano, I.; Ceresi, E.; Faloia, E.; Wang, S.; Fortier, M.; Greenberg, A.S.; Obin, M.S. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J. Lipid Res.* **2005**, *46*, 2347–2355.

112. Sun, K.; Kusminski, C.M.; Scherer, P.E. Adipose tissue remodeling and obesity. *J. Clin. Invest.* **2011**, *121*, 2094–2101.

113. Ruiz, J.R.; Martinez-Tellez, B.; Sanchez-Delgado, G.; Osuna-Prieto, F.J.; Rensen, P.C.N.; Boon, M.R. Role of Human Brown Fat in Obesity, Metabolism and Cardiovascular Disease: Strategies to Turn Up the Heat. *Prog. Cardiovasc. Dis.* **2018**, *61*, 232–245.

114. Townsend, K.; Tseng, Y.-H. Brown adipose tissue: Recent insights into development, metabolic function and therapeutic potential. *Adipocyte* **2012**, *1*, 13–24.

115. Kolonin, M.G. How brown is brown fat that we can see? *Adipocyte* **2014**, *3*, 155–159.

116. Carpentier, A.C.; Blondin, D.P.; Virtanen, K.A.; Richard, D.; Haman, F.; Turcotte, É.E. Brown Adipose Tissue Energy Metabolism in Humans. *Front. Endocrinol. (Lausanne).* **2018**, *9*, 447.

117. Haman, F.; Blondin, D.P. Shivering thermogenesis in humans: Origin, contribution and metabolic requirement. *Temperature* **2017**, *4*, 217–226.

118. Kozak, L.P.; Anunciado-Koza, R. UCP1: its involvement and utility in obesity. *Int. J. Obes. (Lond).* **2008**, *32 Suppl 7*, S32–S38.

119. Nedergaard, J.; Golozoubova, V.; Matthias, A.; Asadi, A.; Jacobsson, A.; Cannon, B. UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. *Biochim. Biophys. Acta - Bioenerg.* **2001**, *1504*, 82–106.

120. Townsend, K.L.; Tseng, Y.-H. Brown fat fuel utilization and thermogenesis. *Trends Endocrinol. Metab.* **2014**, *25*, 168–177.

121. Cannon, B.; Nedergaard, J.A.N. Brown Adipose Tissue: Function and Physiological Significance. *Physiol. Rev.* **2004**, *84*, 277–359.

122. Shimizu, Y.; Kielar, D.; Minokoshi, Y.; Shimazu, T. Noradrenaline increases glucose transport into brown adipocytes in culture by a mechanism different from that of insulin. *Biochem. J.* **1996**, *314 (Pt 2*, 485–490.

123. Young, J.B.; Saville, E.; Rothwell, N.J.; Stock, M.J.; Landsberg, L. Effect of diet and cold exposure on norepinephrine turnover in brown adipose tissue of the rat. *J. Clin. Invest.* **1982**, *69*, 1061–1071.

124. Bartelt, A.; Bruns, O.T.; Reimer, R.; Hohenberg, H.; Ittrich, H.; Peldschus, K.; Kaul, M.G.; Tromsdorf, U.I.; Weller, H.; Waurisch, C.; et al. Brown adipose tissue activity controls triglyceride clearance. *Nat. Med.* **2011**, *17*, 200.

125. Grenier-Larouche, T.; Labbé, S.M.; Noll, C.; Richard, D.; Carpentier, A.C. Metabolic inflexibility of white and brown adipose tissues in abnormal fatty acid partitioning of type 2 diabetes. *Int. J. Obes. Suppl.* **2012**, *2*, S37–S42.

126. Braun, K.; Oeckl, J.; Westermeier, J.; Li, Y.; Klingenspor, M. Non-adrenergic control of lipolysis and thermogenesis in adipose tissues. *J. Exp. Biol.* **2018**, *221*, jeb165381.

127. Fisher, F.F.; Kleiner, S.; Douris, N.; Fox, E.C.; Mepani, R.J.; Verdeguer, F.; Wu, J.; Kharitonenkov, A.; Flier, J.S.; Maratos-Flier, E.; et al. FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev.* **2012**, *26*, 271–281.

128. Lee, P.; Linderman, J.D.; Smith, S.; Brychta, R.J.; Wang, J.; Idelson, C.; Perron, R.M.; Werner, C.D.; Phan, G.Q.; Kammula, U.S.; et al. Irisin and FGF21 are cold-induced endocrine activators of brown fat

function in humans. *Cell Metab.* **2014**, *19*, 302–309.

129. Cioffi, F.; Gentile, A.; Silvestri, E.; Goglia, F.; Lombardi, A. Effect of Iodothyronines on Thermogenesis: Focus on Brown Adipose Tissue. *Front. Endocrinol. (Lausanne).* **2018**, *9*, 254.

130. Yoneshiro, T.; Aita, S.; Matsushita, M.; Kayahara, T.; Kameya, T.; Kawai, Y.; Iwanaga, T.; Saito, M. Recruited brown adipose tissue as an antiobesity agent in humans. *J. Clin. Invest.* **2013**, *123*, 3404–3408.

131. Lowell, B.B.; S-Susulic, V.; Hamann, A.; Lawitts, J.A.; Himms-Hagen, J.; Boyer, B.B.; Kozak, L.P.; Flier, J.S. Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* **1993**, *366*, 740–742.

132. Rothwell, N.J.; Stock, M.J. A role for brown adipose tissue in diet-induced thermogenesis. *Nature* **1979**, *281*, 31–35.

133. Vijgen, G.H.E.J.; Bouvy, N.D.; Teule, G.J.J.; Brans, B.; Hoeks, J.; Schrauwen, P.; van Marken Lichtenbelt, W.D. Increase in Brown Adipose Tissue Activity after Weight Loss in Morbidly Obese Subjects. *J. Clin. Endocrinol. Metab.* **2012**, *97*, E1229–E1233.

134. Kozak, L.P.; Koza, R.A.; Anunciado-Koza, R. Brown fat thermogenesis and body weight regulation in mice: relevance to humans. *Int. J. Obes.* **2010**, *34*, S23–S27.

135. Arch, J.; Trayhurn, P. Detection of thermogenesis in rodents in response to anti-obesity drugs and genetic modification . *Front. Physiol.* 2013, *4*, 64.

136. Seyer, P.; Vallois, D.; Poitry-Yamate, C.; Schütz, F.; Metref, S.; Tarussio, D.; Maechler, P.; Staels, B.; Lanz, B.; Grueter, R.; et al. Hepatic glucose sensing is required to preserve β cell glucose competence. *J. Clin. Invest.* **2013**, *123*, 1662–1676.

137. Agius, L. Glucokinase and molecular aspects of liver glycogen metabolism. *Biochem. J.* **2008**, *414*, 1–18.

138. Rui, L. Energy metabolism in the liver. *Compr. Physiol.* **2014**, *4*, 177–197.

139. Méndez-Lucas, A.; Duarte, J.A.G.; Sunny, N.E.; Satapati, S.; He, T.; Fu, X.; Bermúdez, J.; Burgess, S.C.; Perales, J.C. PEPCK-M expression in mouse liver potentiates, not replaces, PEPCK-C mediated gluconeogenesis. *J. Hepatol.* **2013**, *59*, 105–113.

140. Burgess, S.C.; Hausler, N.; Merritt, M.; Jeffrey, F.M.H.; Storey, C.; Milde, A.; Koshy, S.; Lindner, J.; Magnuson, M.A.; Malloy, C.R.; et al. Impaired tricarboxylic acid cycle activity in mouse livers lacking cytosolic phosphoenolpyruvate carboxykinase. *J. Biol. Chem.* **2004**, *279*, 48941–48949.

141. Mutel, E.; Abdul-Wahed, A.; Ramamonjisoa, N.; Stefanutti, A.; Houberdon, I.; Cavassila, S.; Pilleul, F.; Beuf, O.; Gautier-Stein, A.; Penhoat, A.; et al. Targeted deletion of liver glucose-6 phosphatase mimics glycogen storage disease type 1a including development of multiple adenomas. *J. Hepatol.* **2011**, *54*, 529–537.

142. Jeong, J.Y.; Jeoung, N.H.; Park, K.-G.; Lee, I.-K. Transcriptional regulation of pyruvate dehydrogenase kinase. *Diabetes Metab. J.* **2012**, *36*, 328–335.

143. Jeoung, N.H.; Wu, P.; Joshi, M.A.; Jaskiewicz, J.; Bock, C.B.; Depaoli-Roach, A.A.; Harris, R.A. Role of pyruvate dehydrogenase kinase isoenzyme 4 (PDHK4) in glucose homoeostasis during starvation. *Biochem. J.* **2006**, *397*, 417–425.

144. Michael, M.D.; Kulkarni, R.N.; Postic, C.; Previs, S.F.; Shulman, G.I.; Magnuson, M.A.; Kahn, C.R. Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol. Cell* **2000**, *6*, 87–97.

145. Wasserman, D.H.; Spalding, J.A.; Lacy, D.B.; Colburn, C.A.; Goldstein, R.E.; Cherrington, A.D. Glucagon is a primary controller of hepatic glycogenolysis and gluconeogenesis during muscular work. *Am. J. Physiol. Metab.* **1989**, *257*, E108–E117.

146. Jiang, G.; Zhang, B.B. Glucagon and regulation of glucose metabolism. *Am. J. Physiol. Metab.* **2003**, *284*, E671–E678.

147. Kim, H.S.; Xiao, C.; Wang, R.H.; Lahusen, T.; Xu, X.; Vassilopoulos, A.; Vazquez-Ortiz, G.; Jeong, W. Il; Park, O.; Ki, S.H.; et al. Hepatic-specific disruption of SIRT6 in mice results in fatty liver formation due to enhanced glycolysis and triglyceride synthesis. *Cell Metab.* **2010**, *12*, 224–236.

148. Zhang, T.; Wang, S.; Lin, Y.; Xu, W.; Ye, D.; Xiong, Y.; Zhao, S.; Guan, K.L. Acetylation negatively regulates glycogen phosphorylase by recruiting protein phosphatase 1. *Cell Metab.* **2012**, *15*, 75–87.

149. Ren, D.; Li, M.; Duan, C.; Rui, L. Identification of SH2-B as a key regulator of leptin sensitivity, energy balance, and body weight in mice. *Cell Metab.* **2005**, *2*, 95–104.

150. Wu, G. Functional amino acids in nutrition and health. *Amino Acids* **2013**, *45*, 407–411.

151. Lei, J.; Feng, D.; Zhang, Y.; Dahanayaka, S.; Li, X.; Yao, K.; Wang, J.; Wu, Z.; Dai, Z.; Wu, G. Regulation of leucine catabolism by metabolic fuels in mammary epithelial cells. *Amino Acids* **2012**, *43*, 2179–2189.

152. Nishimura, J.; Masaki, T.; Arakawa, M.; Seike, M.; Yoshimatsu, H. Isoleucine Prevents the Accumulation of Tissue Triglycerides and Upregulates the Expression of PPARα and Uncoupling Protein in Diet-Induced Obese Mice. *J. Nutr.* **2010**, *140*, 496–500.

153. Negro, M.; Giardina, S.; Marzani, B.; Marzatico, F. Branched-chain amino acid supplementation does not enhance athletic performance but affects muscle recovery and the immune system. *J. Sports Med. Phys. Fitness* **2008**, *48*, 347–351.

154. Guo, F.; Cavener, D.R. The GCN2 eIF2α Kinase Regulates Fatty-Acid Homeostasis in the Liver during Deprivation of an Essential Amino Acid. *Cell Metab.* **2007**, *5*, 103–114.

155. Cheng, Y.; Meng, Q.; Wang, C.; Li, H.; Huang, Z.; Chen, S.; Xiao, F.; Guo, F. Leucine Deprivation Decreases Fat Mass by Stimulation of Lipolysis in White Adipose Tissue and Upregulation of Uncoupling Protein 1 (UCP1) in Brown Adipose Tissue. *Diabetes* **2010**, *59*, 17–25.

156. Zhang, S.; Zeng, X.; Ren, M.; Mao, X.; Qiao, S. Novel metabolic and physiological functions of branched chain amino acids: A review. *J. Anim. Sci. Biotechnol.* **2017**, *8*, 4–15.

157. Nishitani, S.; Takehana, K.; Fujitani, S.; Sonaka, I. Branched-chain amino acids improve glucose metabolism in rats with liver cirrhosis. *Am. J. Physiol. Liver Physiol.* **2005**, *288*, G1292–G1300.

158. Doi, M.; Yamaoka, I.; Fukunaga, T.; Nakayama, M. Isoleucine, a potent plasma glucose-lowering amino acid, stimulates glucose uptake in C2C12 myotubes. *Biochem. Biophys. Res. Commun.* **2003**, *312*, 1111–1117.

159. Le Cam, A.; Freychet, P. Neutral amino acid transport. Characterization of the A and L systems in isolated rat hepatocytes. *J. Biol. Chem.* **1977**, *252*, 148–156.

160. Leitner, D.R.; Frühbeck, G.; Yumuk, V.; Schindler, K.; Micic, D.; Woodward, E.; Toplak, H. Obesity and type 2 diabetes: Two diseases with a need for combined treatment strategies - EASO can lead the way. *Obes. Facts* **2017**, *10*, 483–492.

161. Buchwald, H.; Estok, R.; Fahrbach, K.; Banel, D.; Jensen, M.D.; Pories, W.J.; Bantle, J.P.; Sledge, I. Weight and Type 2 Diabetes after Bariatric Surgery: Systematic Review and Meta-analysis. *Am. J. Med.* **2009**, *122*, 248-256.e5.

162. Edelstein, S.L.; Knowler, W.C.; Bain, R.P.; Andres, R.; Barrett-Connor, E.L.; Dowse, G.K.; Haffher, S.M.; Pettitt, D.J.; Sorkin, J.D.; Muller, D.C.; et al. Predictors of Progression From Impaired Glucose Tolerance to NIDDM: An Analysis of Six Prospective Studies. *Diabetes* **1997**, *46*, 701–710.

163. Porte, D.J.; Kahn, S.E. The key role of islet dysfunction in type II diabetes mellitus. *Clin. Invest. Med.* **1995**, *18*, 247–254.

164. DeFronzo, R.A.; Ferrannini, E. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* **1991**, *14*, 173–194.

165. Torres, T.P.; Catlin, R.L.; Chan, R.; Fujimoto, Y.; Sasaki, N.; Printz, R.L.; Newgard, C.B.; Shiota, M. Restoration of hepatic glucokinase expression corrects hepatic glucose flux and normalizes plasma glucose in zucker diabetic fatty rats. *Diabetes* **2009**, *58*, 78–86.

166. Chen, Z.; Sheng, L.; Shen, H.; Zhao, Y.; Wang, S.; Brink, R.; Rui, L. Hepatic TRAF2 regulates glucose metabolism through enhancing glucagon responses. *Diabetes* **2012**, *61*, 566–573.

167. Meshkani, R.; Adeli, K. Hepatic insulin resistance, metabolic syndrome and cardiovascular disease. *Clin. Biochem.* **2009**, *42*, 1331–1346.

168. Rui, L.; Yuan, M.; Frantz, D.; Shoelson, S.; White, M.F. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. *J. Biol. Chem.* **2002**, *277*, 42394–42398.

169. Kile, B.T.; Schulman, B.A.; Alexander, W.S.; Nicola, N.A.; Martin, H.M.E.; Hilton, D.J. The SOCS box: A tale of destruction and degradation. *Trends Biochem. Sci.* **2002**, *27*, 235–241.

170. Haj, F.G.; Zabolotny, J.M.; Kim, Y.B.; Kahn, B.B.; Neel, B.G. Liver-specific protein-tyrosine phosphatase 1B (PTP1B) re-expression alters glucose homeostasis of PTP1B-/- mice. *J. Biol. Chem.* **2005**, *280*, 15038–15046.

171. Després, J.-P.; Lemieux, I. Abdominal obesity and metabolic syndrome. *Nature* **2006**, *444*, 881–887.

172. Randle, P.J.; Garland, P.B.; Hales, C.N.; Newsholme, E.A. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet (London, England)* **1963**, *1*, 785–789.

173. Reaven, G.M. Role of Insulin Resistance in Human Disease. *Diabetes* **1988**, *37*, 1595–1607.

174. Bjorntorp, P. “Portal” adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. *Arteriosclerosis* **1990**, *10*, 493–496.

175. Liu, L.; Zhang, Y.; Chen, N.; Shi, X.; Tsang, B.; Yu, Y.-H. Upregulation of myocellular DGAT1 augments triglyceride synthesis in skeletal muscle and protects against fat-induced insulin resistance. *J. Clin. Invest.* **2007**, *117*, 1679–1689.

176. Turinsky, J.; O’Sullivan, D.M.; Bayly, B.P. 1,2-Diacylglycerol and ceramide levels in insulin-resistant tissues of the rat in vivo. *J. Biol. Chem.* **1990**, *265*, 16880–16885.

177. Yoon, J.C.; Puigserver, P.; Chen, G.; Donovan, J.; Wu, Z.; Rhee, J.; Adelmant, G.; Stafford, J.; Kahn, C.R.; Granner, D.K.; et al. Control of hepatic gluconeogenesis through the transcriptional coaotivator PGC-1. *Nature* **2001**, *413*, 131–138.

178. Dentin, R.; Tomas-Cobos, L.; Foufelle, F.; Leopold, J.; Girard, J.; Postic, C.; Ferré, P. Glucose 6-phosphate, rather than xylulose 5-phosphate, is required for the activation of ChREBP in response to glucose in the liver. *J. Hepatol.* **2012**, *56*, 199–209.

179. Abu-Elheiga, L.; Brinkley, W.R.; Zhong, L.; Chirala, S.S.; Woldegiorgis, G.; Wakil, S.J. The subcellular localization of acetyl-CoA carboxylase 2. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 1444–1449.

180. Jornayvaz, F.R.; Shulman, G.I. Diacylglycerol activation of protein kinase Cε and hepatic insulin resistance. *Cell Metab.* **2012**, *15*, 574–584.

181. Su, Y.; Lam, T.K.T.; He, W.; Pocai, A.; Bryan, J.; Aguilar-Bryan, L.; Gutiérrez-Juárez, R. Hypothalamic leucine metabolism regulates liver glucose production. *Diabetes* **2012**, *61*, 85–93.

182. Lee, J.H.; Giannikopoulos, P.; Duncan, S.A.; Wang, J.; Johansen, C.T.; Brown, J.D.; Plutzky, J.; Hegele, R.A.; Glimcher, L.H.; Lee, A.H. The transcription factor cyclic AMP-responsive element-binding protein H regulates triglyceride metabolism. *Nat. Med.* **2011**, *17*, 812–815.

183. Liang, Y.; Osborne, M.C.; Monia, B.P.; Bhanot, S.; Gaarde, W.A.; Reed, C.; She, P.; Jetton, T.L.; Demarest, K.T. Reduction in Glucagon Receptor Expression by an Syndrome in db / db Mice. *Image (Rochester, N.Y.)* **2004**, *53*.

184. Berglund, E.D.; Lustig, D.G.; Baheza, R.A.; Hasenour, C.M.; Lee-Young, R.S.; Donahue, E.P.; Lynes, S.E.; Swift, L.L.; Charron, M.J.; Damon, B.M.; et al. Hepatic glucagon action is essential for exercise-induced reversal of mouse fatty liver. *Diabetes* **2011**, *60*, 2720–2729.

185. Harada, N.; Oda, Z.; Hara, Y.; Fujinami, K.; Okawa, M.; Ohbuchi, K.; Yonemoto, M.; Ikeda, Y.; Ohwaki, K.; Aragane, K.; et al. Hepatic De Novo Lipogenesis Is Present in Liver-Specific ACC1-Deficient Mice. *Mol. Cell. Biol.* **2007**, *27*, 1881–1888.

186. Savage, D.B.; Choi, C.S.; Samuel, V.T.; Liu, Z.-X.; Zhang, D.; Wang, A.; Zhang, X.-M.; Cline, G.W.; Yu, X.X.; Geisler, J.G.; et al. Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. *J. Clin. Invest.* **2006**, *116*, 817–824.

187. Matsuzaka, T.; Atsumi, A.; Matsumori, R.; Nie, T.; Shinozaki, H.; Suzuki-Kemuriyama, N.; Kuba, M.; Nakagawa, Y.; Ishii, K.; Shimada, M.; et al. Elovl6 promotes nonalcoholic steatohepatitis. *Hepatology* **2012**, *56*, 2199–2208.

188. Cohen, P.; Miyazaki, M.; Socci, N.D.; Hagge-Greenberg, A.; Liedtke, W.; Soukas, A.A.; Sharma, R.; Hudgins, L.C.; Ntambi, J.M.; Friedman, J.M. Role for Stearoyl-CoA Desaturase-1 in Leptin-Mediated Weight Loss. *Science (80-.).* **2002**, *297*, 240–243.

189. Oosterveer, M.H.; Mataki, C.; Yamamoto, H.; Harach, T.; Moullan, N.; van Dijk, T.H.; Ayuso, E.; Bosch, F.; Postic, C.; Groen, A.K.; et al. LRH-1–dependent glucose sensing determines intermediary metabolism in liver. *J. Clin. Invest.* **2012**, *122*, 2817–2826.

190. Kim, J.-E.; Chen, J.; Lou, Z. DBC1 is a negative regulator of SIRT1. *Nature* **2008**, *451*, 583–586.

191. Horton, J.D.; Goldstein, J.L.; Brown, M.S. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **2002**, *109*, 1125–1131.

192. Rutkowski, D.T.; Wu, J.; Back, S.H.; Callaghan, M.U.; Ferris, S.P.; Iqbal, J.; Clark, R.; Miao, H.; Hassler, J.R.; Fornek, J.; et al. UPR Pathways Combine to Prevent Hepatic Steatosis Caused by ER Stress-Mediated Suppression of Transcriptional Master Regulators. *Dev. Cell* **2008**, *15*, 829–840.

193. Gibbons, G. Regulation of HMG-CoA Reductase. *Biochem. Soc. Trans.* **1986**, *14*, 992.

194. Röhrl, C.; Stangl, H. Cholesterol metabolism—physiological regulation and pathophysiological deregulation by the endoplasmic reticulum. *Wiener Medizinische Wochenschrift* **2018**, *168*, 280–285.

195. Goldstein, J.L.; Brown, M.S. The LDL receptor. *Arterioscler. Thromb. Vasc. Biol.* **2009**, *29*, 431–438.

196. Chiang, J.Y. Regulation of bile acid synthesis. *Front. Biosci.* **1998**, *3*, d176-93.

197. Dawson, P.A.; Lan, T.; Rao, A. Thematic review series: Bile acids. Bile acid transporters. *J. Lipid Res.* **2009**, *50*, 2340–2357.

198. Cohen, D.E. Balancing cholesterol synthesis and absorption in the gastrointestinal tract. *J. Clin. Lipidol.* **2008**, *2*, S1–S3.

199. Goldstein, J.L.; Brown, M.S.; Anderson, R.G.W.; Russell, D.W.; Schneider, W.J. Receptor-Mediated Endocytosis: Concepts Emerging from the LDL Receptor System. *Annu. Rev. Cell Biol.* **1985**, *1*, 1–39.

200. Tabas, I. Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *J. Clin. Invest.* **2002**, *110*, 905–911.

201. Simons, K.; Ikonen, E. How cells handle cholesterol. *Science (80-.).* **2000**, *290*, 1721 LP – 1726.

202. Irit, M.; Hanna, M.; Michael, A. Macrophage Uptake of Oxidized LDL Inhibits Lysosomal Sphingomyelinase, Thus Causing the Accumulation of Unesterified Cholesterol–Sphingomyelin–Rich Particles in the Lysosomes . *Arterioscler. Thromb. Vasc. Biol.* **1995**, *15*, 1378–1387.

203. Slotte, J.P. Sphingomyelin–cholesterol interactions in biological and model membranes. *Chem. Phys. Lipids* **1999**, *102*, 13–27.

204. Ridgway, N.D. Interactions between metabolism and intracellular distribution of cholesterol and sphingomyelin. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **2000**, *1484*, 129–141.

205. Horinouchi, K.; Erlich, S.; Perl, D.P.; Ferlinz, K.; Bisgaier, C.L.; Sandhoff, K.; Desnick, R.J.; Stewart, C.L.; Schuchman, E.H. Acid sphingomyelinase deficient mice: a model of types A and B Niemann–Pick disease. *Nat. Genet.* **1995**, *10*, 288–293.

206. Kanfer, J.N.; Young, O.M.; Shapiro, D.; Brady, R.O. The metabolism of sphingomyelin. I. Purification and properties of a sphingomyelin-cleaving enzyme from rat liver tissue. *J. Biol. Chem.* **1966**, *241*, 1081–1084.

207. Jiang, X.-C.; Yeang, C.; Li, Z.; Chakraborty, M.; Liu, J.; Zhang, H.; Fan, Y. Sphingomyelin biosynthesis: its impact on lipid metabolism and atherosclerosis. *Clin. Lipidol.* **2009**, *4*, 595–609.

208. Akopian, D.; Medh, J.D. Genetics and molecular biology: macrophage ACAT depletion - mechanisms of atherogenesis. *Curr. Opin. Lipidol.* **2006**, *17*, 85–88.

209. Clemente-Postigo, M.; Queipo-Ortuño, M.I.; Fernandez-Garcia, D.; Gomez-Huelgas, R.; Tinahones, F.J.; Cardona, F. Adipose Tissue Gene Expression of Factors Related to Lipid Processing in Obesity. *PLoS One* **2011**, *6*, e24783.

210. Klop, B.; Wouter Jukema, J.; Rabelink, T.J.; Castro Cabezas, M. A physician's guide for the management of hypertriglyceridemia: the etiology of hypertriglyceridemia determines treatment strategy.

Panminerva Med. **2012**, *54*, 91–103.

211. Wang, H.; Eckel, R.H. Lipoprotein lipase: from gene to obesity. *Am. J. Physiol. Metab.* **2009**, *297*, E271–E288.

212. Bays, H.E.; Tighe, A.P.; Sadovsky, R.; Davidson, M.H. Prescription omega-3 fatty acids and their lipid effects: physiologic mechanisms of action and clinical implications. *Expert Rev. Cardiovasc. Ther.* **2008**, *6*, 391–409.

213. Klop, B.; Elte, J.W.F.; Cabezas, M.C. Dyslipidemia in obesity: mechanisms and potential targets. *Nutrients* **2013**, *5*, 1218–1240.

214. Packard, C.J. Triacylglycerol-rich lipoproteins and the generation of small, dense low-density lipoprotein. *Biochem. Soc. Trans.* **2003**, *31*, 1066–1069.

215. Mamo, J.C.L.; Watts, G.F.; Barrett, P.H.R.; Smith, D.; James, A.P.; Pal, S. Postprandial dyslipidemia in men with visceral obesity: an effect of reduced LDL receptor expression? *Am. J. Physiol. Metab.* **2001**, *281*, E626–E632.

216. Bjorntorp, P.; Bergman, H.; Varnauskas, E. Plasma free fatty acid turnover rate in obesity. *Acta Med. Scand.* **1969**, *185*, 351–356.

217. Eckel, R.H.; Grundy, S.M.; Zimmet, P.Z. The metabolic syndrome. *Lancet* **2005**, *365*, 1415–1428.

218. Karpe, F.; Dickmann, J.R.; Frayn, K.N. Fatty Acids, Obesity, and Insulin Resistance: Time for a Reevaluation. *Diabetes* **2011**, *60*, 2441 LP – 2449.

219. Steinberg, G.R.; Kemp, B.E.; Watt, M.J. Adipocyte triglyceride lipase expression in human obesity. *Am. J. Physiol. Metab.* **2007**, *293*, E958–E964.

220. Young, S.G.; Zechner, R. Biochemistry and pathophysiology of intravascular and intracellular lipolysis. *Genes Dev.* **2013**, *27*, 459–484.

221. Bays, H.E.; Toth, P.P.; Kris-Etherton, P.M.; Abate, N.; Aronne, L.J.; Brown, W.V.; Gonzalez-Campoy, J.M.; Jones, S.R.; Kumar, R.; La Forge, R.; et al. Obesity, adiposity, and dyslipidemia: A consensus statement from the National Lipid Association. *J. Clin. Lipidol.* **2013**, *7*, 304–383.

222. Black, M.D.; Varty, G.B.; Arad, M.; Barak, S.; De Levie, A.; Boulay, D.; Pichat, P.; Griebel, G.; Weiner, I. Procognitive and antipsychotic efficacy of glycine transport 1 inhibitors (GlyT1) in acute and neurodevelopmental models of schizophrenia: latent inhibition studies in the rat. *Psychopharmacology (Berl).* **2009**, *202*, 385–396.

223. Sewter, C.; Berger, D.; Considine, R. V; Medina, G.; Rochford, J.; Ciaraldi, T.; Henry, R.; Dohm, L.; Flier, J.S.; O'Rahilly, S.; et al. Human Obesity and Type 2 Diabetes Are Associated With Alterations in SREBP1 Isoform Expression That Are Reproduced Ex Vivo by Tumor Necrosis Factor-α. *Diabetes* **2002**, *51*, 1035 LP – 1041.

224. Primeau, V.; Coderre, L.; Karelis, A.D.; Brochu, M.; Lavoie, M.-E.; Messier, V.; Sladek, R.; Rabasa-Lhoret, R. Characterizing the profile of obese patients who are metabolically healthy. *Int. J. Obes.* **2011**, *35*, 971–981.

225. Martínez-Larrad, M.T.; Corbatón Anchuelo, A.; Del Prado, N.; Ibarra Rueda, J.M.; Gabriel, R.; Serrano-Ríos, M. Profile of Individuals Who Are Metabolically Healthy Obese Using Different Definition Criteria. A Population-Based Analysis in the Spanish Population. *PLoS One* **2014**, *9*, e106641.

226. Endemann, D.H.; Schiffrin, E.L. Endothelial Dysfunction. *J. Am. Soc. Nephrol.* **2004**, *15*, 1983 LP – 1992.

227. Hideki, K.; Tetsuya, Y.; Yoshitomo, O. Adiposity and Cardiovascular Disorders. *Circ. Res.* **2007**, *101*, 27–39.

228. Roberts, C.K.; Barnard, R.J.; Sindhu, R.K.; Jurczak, M.; Ehdaie, A.; Vaziri, N.D. A high-fat, refined-carbohydrate diet induces endothelial dysfunction and oxidant/antioxidant imbalance and depresses NOS protein expression. *J. Appl. Physiol.* **2005**, *98*, 203–210.

229. Galley, H.F.; Webster, N.R. Physiology of the endothelium. *Br. J. Anaesth.* **2004**, *93*, 105–113.

230. Sandoo, A.; van Zanten, J.J.C.S.V.; Metsios, G.S.; Carroll, D.; Kitas, G.D. The endothelium and its role in regulating vascular tone. *Open Cardiovasc. Med. J.* **2010**, *4*, 302–312.

231. Atlas, S.A. The Renin-Angiotensin Aldosterone System: Pathophysiological Role and Pharmacologic Inhibition. *J. Manag. Care Pharm.* **2007**, *13*, 9–20.

232. Persson, P.B. Renin: origin, secretion and synthesis. *J. Physiol.* **2003**, *552*, 667–671.

233. Ryan, U.S.; Ryan, J.W.; Whitaker, C.; Chiu, A. Localization of angiotensin converting enzyme (kininase II). II. Immunocytochemistry and immunofluorescence. *Tissue Cell* **1976**, *8*, 125–145.

234. Tikellis, C.; Thomas, M.C. Angiotensin-Converting Enzyme 2 (ACE2) Is a Key Modulator of the Renin Angiotensin System in Health and Disease. *Int. J. Pept.* **2012**, *2012*, 256294.

235. Briet, M.; Schiffrin, E.L. Aldosterone: effects on the kidney and cardiovascular system. *Nat. Rev. Nephrol.* **2010**, *6*, 261–273.

236. Yanagisawa, M.; Kurihara, H.; Kimura, S.; Tomobe, Y.; Kobayashi, M.; Mitsui, Y.; Yazaki, Y.; Goto, K.; Masaki, T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **1988**, *332*, 411–415.

237. Böhm, F.; Pernow, J. The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. *Cardiovasc. Res.* **2007**, *76*, 8–18.

238. Förstermann, U.; Sessa, W.C. Nitric oxide synthases: regulation and function. *Eur. Heart J.* **2012**, *33*, 829-837d.

239. Engeli, S.; Schling, P.; Gorzelniak, K.; Boschmann, M.; Janke, J.; Ailhaud, G.; Teboul, M.; Massiéra, F.; Sharma, A.M. The adipose-tissue renin–angiotensin–aldosterone system: role in the metabolic syndrome? *Int. J. Biochem. Cell Biol.* **2003**, *35*, 807–825.

240. Grassi, G.; Dell'Oro, R.; Facchini, A.; Quarti Trevano, F.; Bolla, G.B.; Mancia, G. Effect of central and peripheral body fat distribution on sympathetic and baroreflex function in obese normotensives. *J. Hypertens.* **2004**, *22*.

241. Grassi, G. Renin–angiotensin–sympathetic crosstalks in hypertension: reappraising the relevance of peripheral interactions. *J. Hypertens.* **2001**, *19*.

242. Molet, S.; Furukawa, K.; Maghazechi, A.; Hamid, Q.; Giaid, A. Chemokine- and cytokine-induced expression of endothelin 1 and endothelin-converting enzyme 1 in endothelial cells. *J. Allergy Clin. Immunol.* **2000**, *105*, 333–338.

243. Liang, L.; Yu, J.; Zhou, W.; Liu, N.; E, L.L.; Wang, D.S.; Liu, H. Endothelin-1 stimulates proinflammatory cytokine expression in human periodontal ligament cells via mitogen-activated protein kinase pathway. *J. Periodontol.* **2014**, *85*, 618–626.

244. Kowalczyk, A.; Kleniewska, P.; Kolodziejczyk, M.; Skibska, B.; Goraca, A. The role of endothelin-1 and endothelin receptor antagonists in inflammatory response and sepsis. *Arch. Immunol. Ther. Exp. (Warsz).* **2015**, *63*, 41–52.

245. Furukawa, S.; Fujita, T.; Shimabukuro, M.; Iwaki, M.; Yamada, Y.; Nakajima, Y.; Nakayama, O.; Makishima, M.; Matsuda, M.; Shimomura, I. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J. Clin. Invest.* **2017**, *114*, 1752–1761.

246. Sarafidis, P.A.; Bakris, G.L. The Antinatriuretic Effect of Insulin: An Unappreciated Mechanism for Hypertension Associated with Insulin Resistance? *Am. J. Nephrol.* **2007**, *27*, 44–54.

247. Jeong-a, K.; Monica, M.; Kon, K.K.; J., Q.M. Reciprocal Relationships Between Insulin Resistance and Endothelial Dysfunction. *Circulation* **2006**, *113*, 1888–1904.

248. Ignarro, L.J. Nitric oxide. A novel signal transduction mechanism for transcellular communication. *Hypertension* **1990**, *16*, 477–483.

249. Sarafidis, P.A.; Bakris, G.L. Insulin and Endothelin: An Interplay Contributing to Hypertension Development? *J. Clin. Endocrinol. Metab.* **2007**, *92*, 379–385.

250. Florian, J.P.; Pawelczyk, J.A. Non-esterified fatty acids increase arterial pressure via central sympathetic activation in humans. *Clin. Sci.* **2009**, *118*, 61–69.

251. Steinberg, H.O.; Paradisi, G.; Hook, G.; Crowder, K.; Cronin, J.; Baron, A.D. Free fatty acid elevation impairs insulin-mediated vasodilation and nitric oxide production. *Diabetes* **2000**, *49*, 1231 LP – 1238.

252. Tekely, P. Malcolm H. Levitt. Spin dynamics: basics of nuclear magnetic resonance. *Magn. Reson. Chem.* **2002**, *40*, 800.

253. Hatzakis, E. Nuclear Magnetic Resonance (NMR) Spectroscopy in Food Science: A Comprehensive Review. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18*, 189–220.

254. Cloarec, O.; Dumas, M.-E.; Craig, A.; Barton, R.H.; Trygg, J.; Hudson, J.; Blancher, C.; Gauguier, D.; Lindon, J.C.; Holmes, E.; et al. Statistical Total Correlation Spectroscopy: An Exploratory Approach for Latent Biomarker Identification from Metabolic 1H NMR Data Sets. *Anal. Chem.* **2005**, *77*, 1282–1289.

255. Butte, N.F.; Liu, Y.; Zakeri, I.F.; Mohney, R.P.; Mehta, N.; Voruganti, V.S.; Göring, H.; Cole, S.A.; Comuzzie, A.G. Global metabolomic profiling targeting childhood obesity in the Hispanic population. *Am. J. Clin. Nutr.* **2015**, *102*, 256–267.

256. Fiehn, O.; Garvey, W.T.; Newman, J.W.; Lok, K.H.; Hoppel, C.L.; Adams, S.H. Plasma Metabolomic Profiles Reflective of Glucose Homeostasis in Non-Diabetic and Type 2 Diabetic Obese African-American Women. *PLoS One* **2010**, *5*, e15234.

257. Gogna, N.; Krishna, M.; Oommen, A.M.; Dorai, K. Investigating correlations in the altered metabolic profiles of obese and diabetic subjects in a South Indian Asian population using an NMR-based metabolomic approach. *Mol. Biosyst.* **2015**, *11*, 595–606.

258. Moore, S.C.; Matthews, C.E.; Sampson, J.N.; Stolzenberg-Solomon, R.Z.; Zheng, W.; Cai, Q.; Tan, Y.T.; Chow, W.-H.; Ji, B.-T.; Liu, D.K.; et al. Human metabolic correlates of body mass index. *Metabolomics* **2014**, *10*, 259–269.

259. Newgard, C.B.; An, J.; Bain, J.R.; Muehlbauer, M.J.; Stevens, R.D.; Lien, L.F.; Haqq, A.M.; Shah, S.H.; Arlotto, M.; Slentz, C.A.; et al. A Branched-Chain Amino Acid-Related Metabolic Signature that Differentiates Obese and Lean Humans and Contributes to Insulin Resistance. *Cell Metab.* **2009**, *9*, 311–326.

260. Xie, B.; Waters, M.J.; Schirra, H.J. Investigating Potential Mechanisms of Obesity by Metabolomics. *J. Biomed. Biotechnol.* **2012**, *2012*, 805683.

261. Mihalik, S.J.; Michaliszyn, S.F.; de las Heras, J.; Bacha, F.; Lee, S.; Chace, D.H.; DeJesus, V.R.; Vockley, J.; Arslanian, S.A. Metabolomic Profiling of Fatty Acid and Amino Acid Metabolism in Youth With Obesity and Type 2 Diabetes. *Diabetes Care* **2012**, *35*, 605 LP – 611.

262. Golay, A.; Swislocki, A.L.M.; Chen, Y.D.I.; Jaspan, J.B.; Reaven, G.M. Effect of Obesity on Ambient Plasma Glucose, Free Fatty Acid, Insulin, Growth Hormone, and Glucagon Concentrations*. *J. Clin. Endocrinol. Metab.* **1986**, *63*, 481–484.

263. Cha, Y.-S. Effects of L-carnitine on obesity, diabetes, and as an ergogenic aid. *Asia Pac. J. Clin. Nutr.* **2008**, *17 Suppl 1*, 306–308.

264. Park, S.; Sadanala, K.C.; Kim, E.-K. A Metabolomic Approach to Understanding the Metabolic Link between Obesity and Diabetes. *Mol. Cells* **2015**, *38*, 587–596.

265. Hiroshima, Y.; Yamamoto, T.; Watanabe, M.; Baba, Y.; Shinohara, Y. Effects of cold exposure on metabolites in brown adipose tissue of rats. *Mol. Genet. Metab. Reports* **2018**, *15*, 36–42.

266. Bentley-Lewis, R.; Xiong, G.; Lee, H.; Yang, A.; Huynh, J.; Kim, C. Metabolomic analysis reveals amino-acid responses to an oral glucose tolerance test in women with prior history of gestational diabetes mellitus. *J. Clin. Transl. Endocrinol.* **2014**, *1*, 38–43.

267. Guan, M.; Xie, L.; Diao, C.; Wang, N.; Hu, W.; Zheng, Y.; Jin, L.; Yan, Z.; Gao, H. Systemic Perturbations of Key Metabolites in Diabetic Rats During the Evolution of Diabetes Studied by Urine Metabonomics. *PLoS One* **2013**, *8*, e60409.

268. Wang, T.J.; Larson, M.G.; Vasan, R.S.; Cheng, S.; Rhee, E.P.; McCabe, E.; Lewis, G.D.; Fox, C.S.; Jacques, P.F.; Fernandez, C.; et al. Metabolite profiles and the risk of developing diabetes. *Nat. Med.* **2011**, *17*, 448–453.

269. Mooradian, A.D.; Haas, M.J.; Wehmeier, K.R.; Wong, N.C.W. Obesity-related Changes in High-density Lipoprotein Metabolism. *Obesity* **2008**, *16*, 1152–1160.

270. Liu, L.; Wang, M.; Yang, X.; Bi, M.; Na, L.; Niu, Y.; Li, Y.; Sun, C. Fasting Serum Lipid and

Dehydroepiandrosterone Sulfate as Important Metabolites for Detecting Isolated Postchallenge Diabetes: Serum Metabolomics via Ultra-High-Performance LC-MS. *Clin. Chem.* **2020**, *59*, 1338–1348.

271. Xu, F.; Tavintharan, S.; Sum, C.F.; Woon, K.; Lim, S.C.; Ong, C.N. Metabolic Signature Shift in Type 2 Diabetes Mellitus Revealed by Mass Spectrometry-based Metabolomics. *J. Clin. Endocrinol. Metab.* **2013**, *98*, E1060–E1065.

272. Watt, M.; Steinberg, G. Pathways Involved In Lipid-Induced Insulin Resistance In Obesity. *Future Lipidol.* **2007**, *2*, 659–667.

273. Drogan, D.; Dunn, W.B.; Lin, W.; Buijsse, B.; Schulze, M.B.; Langenberg, C.; Brown, M.; Floegel, A.; Dietrich, S.; Rolandsson, O.; et al. Untargeted Metabolic Profiling Identifies Altered Serum Metabolites of Type 2 Diabetes Mellitus in a Prospective, Nested Case Control Study. *Clin. Chem.* **2020**, *61*, 487–497.

274. Suhre, K.; Meisinger, C.; Döring, A.; Altmaier, E.; Belcredi, P.; Gieger, C.; Chang, D.; Milburn, M. V; Gall, W.E.; Weinberger, K.M.; et al. Metabolic Footprint of Diabetes: A Multiplatform Metabolomics Study in an Epidemiological Setting. *PLoS One* **2010**, *5*, e13953.

275. Gordon Jr., R.S. Issues in Human Experimentation. *Ann. Intern. Med.* **1978**, *89*, 846–848.

276. Kasper, C.E.V.O.-31 Animal Models of Exercise and Obesity. *Annu Rev Nurs Res* 1–2013.

277. Bryda, E.C. The Mighty Mouse: the impact of rodents on advances in biomedical research. *Mo. Med.* **2013**, *110*, 207–211.

278. Vandamme, T.F. Use of rodents as models of human diseases. *J. Pharm. Bioallied Sci.* **2014**, *6*, 2–9.

279. Coleman, D.L. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. *Diabetologia* **1978**, *14*, 141–148.

280. Bray, G.A. The Zucker-fatty rat: a review. *Fed. Proc.* **1977**, *36*, 148–153.

281. Shiota, M.; Printz, R.L. Diabetes in Zucker Diabetic Fatty Rat BT - Animal Models in Diabetes Research. In; Joost, H.-G., Al-Hasani, H., Schürmann, A., Eds.; Humana Press: Totowa, NJ, 2012; pp. 103–123 ISBN 978-1-62703-068-7.

282. Pinto, Y.M.; Paul, M.; Ganten, D. Lessons from rat models of hypertension: from Goldblatt to genetic engineering. *Cardiovasc. Res.* **1998**, *39*, 77–88.

283. Wong, S.K.; Chin, K.-Y.; Suhaimi, F.H.; Fairus, A.; Ima-Nirwana, S. Animal models of metabolic syndrome: a review. *Nutr. Metab. (Lond).* **2016**, *13*, 65.

284. Lutz, T.A.; Woods, S.C. Overview of animal models of obesity. *Curr. Protoc. Pharmacol.* **2012**, *Chapter 5*, Unit5.61-Unit5.61.

285. Buettner, R.; Parhofer, K.G.; Woenckhaus, M.; Wrede, C.E.; Kunz-Schughart, L.A.; Schölmerich, J.; Bollheimer, L.C. Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. *J. Mol. Endocrinol. 36*, 485–501.

286. Storlien, L.H.; Baur, L.A.; Kriketos, A.D.; Pan, D.A.; Cooney, G.J.; Jenkins, A.B.; Calvert, G.D.; Campbell, L. V Dietary fats and insulin action. *Diabetologia* **1996**, *39*, 621–631.

287. Sumiyoshi, M.; Sakanaka, M.; Kimura, Y. Chronic Intake of High-Fat and High-Sucrose Diets Differentially Affects Glucose Intolerance in Mice. *J. Nutr.* **2006**, *136*, 582–587.

288. Sampey, B.P.; Vanhoose, A.M.; Winfield, H.M.; Freemerman, A.J.; Muehlbauer, M.J.; Fueger, P.T.; Newgard, C.B.; Makowski, L. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: Comparison to high-fat diet. *Obesity* **2011**, *19*, 1109–1117.

289. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Acute administration of single oral dose of grape seed polyphenols restores blood pressure in a rat model of metabolic syndrome: role of nitric oxide and prostacyclin. *Eur. J. Nutr.* **2016**, *55*, 749–758.

290. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Chronic administration of grape-seed polyphenols attenuates the development of hypertension and improves other cardiometabolic risk factors associated with the metabolic syndrome in cafeteria diet-fed rats. *Br. J. Nutr.* **2017**, *117*, 200–208.

291. Casanova, E.; Baselga-Escudero, L.; Ribas-Latre, A.; Cedó, L.; Arola-Arnal, A.; Pinent, M.; Bladé, C.; Arola, L.; Salvadó, M.J. Chronic intake of proanthocyanidins and docosahexaenoic acid improves skeletal muscle oxidative capacity in diet-obese rats. *J. Nutr. Biochem.* **2014**, *25*, 1003–1010.

292. Pajuelo, D.; Quesada, H.; Díaz, S.; Fernández-Iglesias, A.; Arola-Arnal, A.; Bladé, C.; Salvadó, J.; Arola, L. Chronic dietary supplementation of proanthocyanidins corrects the mitochondrial dysfunction of brown adipose tissue caused by diet-induced obesity in Wistar rats. *Br. J. Nutr.* **2012**, *107*, 170–178.

293. Ardid-Ruiz, A.; Ibars, M.; Mena, P.; Del Rio, D.; Muguerza, B.; Bladé, C.; Arola, L.; Aragonès, G.; Suárez, M. Potential Involvement of Peripheral Leptin/STAT3 Signaling in the Effects of Resveratrol and Its Metabolites on Reducing Body Fat Accumulation. *Nutrients* **2018**, *10*, 1757.

294. Gomez-Smith, M.; Karthikeyan, S.; Jeffers, M.S.; Janik, R.; Thomason, L.A.; Stefanovic, B.; Corbett, D. A physiological characterization of the Cafeteria diet model of metabolic syndrome in the rat. *Physiol. Behav.* **2016**, *167*, 382–391.

295. Naveed, S.; Allan, G.; Olga, S.; Ian, F.; St.J., O.D.; M., H.S.; Chris, I.; W., M.P.; J., P.C.; M., C.S.; et al. Metabolic Syndrome With and Without C-Reactive Protein as a Predictor of Coronary Heart Disease and Diabetes in the West of Scotland Coronary Prevention Study. *Circulation* **2003**, *108*, 414–419.

296. Warburton, D.E.R.; Nicol, C.W.; Bredin, S.S.D. Health benefits of physical activity: the evidence. *CMAJ* **2006**, *174*, 801–809.

297. Pan, X.R.; Li, G.W.; Hu, Y.H.; Wang, J.X.; Yang, W.Y.; An, Z.X.; Hu, Z.X.; Lin, J.; Xiao, J.Z.; Cao, H.B.; et al. Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance. The Da Qing IGT and Diabetes Study. *Diabetes Care* **1997**, *20*, 537–544.

298. Reduction in the Incidence of Type 2 Diabetes with Lifestyle Intervention or Metformin. *N. Engl. J. Med.* **2002**, *346*, 393–403.

299. Tuomilehto, J.; Lindström, J.; Eriksson, J.G.; Valle, T.T.; Hämäläinen, H.; Ilanne-Parikka, P.; Keinänen-Kiukaanniemi, S.; Laakso, M.; Louheranta, A.; Rastas, M.; et al. Prevention of Type 2 Diabetes Mellitus by Changes in Lifestyle among Subjects with Impaired Glucose Tolerance. *N. Engl. J. Med.* **2001**, *344*, 1343–1350.

300. Pérez-Martínez, P.; Mikhailidis, D.P.; Athyros, V.G.; Bullo, M.; Couture, P.; Covas, M.I.; de Koning, L.; Delgado-Lista, J.; Díaz-López, A.; Drevon, C.A.; et al. Lifestyle recommendations for the prevention and management of metabolic syndrome: an international panel recommendation. *Nutr. Rev.* **2017**, *75*, 307–326.

301. Expert Panel on the Identification and Treatment of Overweight and Obesity in Adults, E. Executive Summary of the Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults. *Arch. Intern. Med.* **1998**, *158*, 1855–1867.

302. Abete, I.; Astrup, A.; Martínez, J.A.; Thorsdottir, I.; Zulet, M.A. Obesity and the metabolic syndrome: role of different dietary macronutrient distribution patterns and specific nutritional components on weight loss and maintenance. *Nutr. Rev.* **2010**, *68*, 214–231.

303. Minich, D.M.; Bland, J.S. Dietary management of the metabolic syndrome beyond macronutrients. *Nutr. Rev.* **2008**, *66*, 429–444.

304. Bravata, D.M.; Sanders, L.; Huang, J.; Krumholz, H.M.; Olkin, I.; Gardner, C.D.; Bravata, D.M. Efficacy and Safety of Low-Carbohydrate DietsA Systematic Review. *JAMA* **2003**, *289*, 1837–1850.

305. Bonow, R.O.; Eckel, R.H. Diet, Obesity, and Cardiovascular Risk. *N. Engl. J. Med.* **2003**, *348*, 2057–2058.

306. Barnard, R.J. Effects of Life-style Modification on Serum Lipids. *Arch. Intern. Med.* **1991**, *151*, 1389–1394.

307. Ornish, D.; Brown, S.E.; Billings, J.H.; Scherwitz, L.W.; Armstrong, W.T.; Ports, T.A.; McLanahan, S.M.; Kirkeeide, R.L.; Gould, K.L.; Brand, R.J. Can lifestyle changes reverse coronary heart disease?: The Lifestyle Heart Trial. *Lancet* **1990**, *336*, 129–133.

308. Tobias, D.K.; Chen, M.; Manson, J.E.; Ludwig, D.S.; Willett, W.; Hu, F.B. Effect of low-fat diet interventions versus other diet interventions on long-term weight change in adults: a systematic review and meta-analysis. *lancet. Diabetes Endocrinol.* **2015**, *3*, 968–979.

309. Steffen, L.M.; Van Horn, L.; Daviglus, M.L.; Zhou, X.; Reis, J.P.; Loria, C.M.; Jacobs, D.R.; Duffey, K.J. A modified Mediterranean diet score is associated with a lower risk of incident metabolic syndrome over 25 years among young adults: the CARDIA (Coronary Artery Risk Development in Young Adults) study. *Br. J. Nutr.* **2014**, *112*, 1654–1661.

310. Samaha, F.F.; Iqbal, N.; Seshadri, P.; Chicano, K.L.; Daily, D.A.; McGrory, J.; Williams, T.; Williams, M.; Gracely, E.J.; Stern, L. A Low-Carbohydrate as Compared with a Low-Fat Diet in Severe Obesity. *N. Engl. J. Med.* **2003**, *348*, 2074–2081.

311. Foster, G.D.; Wyatt, H.R.; Hill, J.O.; McGuckin, B.G.; Brill, C.; Mohammed, B.S.; Szapary, P.O.; Rader, D.J.; Edman, J.S.; Klein, S. A Randomized Trial of a Low-Carbohydrate Diet for Obesity. *N. Engl. J. Med.* **2003**, *348*, 2082–2090.

312. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20 536 high-risk individuals: a randomised placebocontrolled trial. *Lancet* **2002**, *360*, 7–22.

313. Pinal-Fernandez, I.; Casal-Dominguez, M.; Mammen, A.L. Statins: pros and cons. *Med. Clin. (Barc).* **2018**, *150*, 398–402.

314. Thompson, G.R. Management of dyslipidaemia. *Heart* **2004**, *90*, 949–955.

315. Bart, S.; Jean, D.; Johan, A.; Kristina, S.; Eran, L.; Jean-Charles, F. Mechanism of Action of Fibrates on Lipid and Lipoprotein Metabolism. *Circulation* **1998**, *98*, 2088–2093.

316. Shipman, K.E.; Strange, R.C.; Ramachandran, S. Use of fibrates in the metabolic syndrome: A review. *World J. Diabetes* **2016**, *7*, 74–88.

317. Olukoga, A.O. Paradoxical severe decrease in serum HDL-cholesterol after treatment with a fibrate. *J. Clin. Pathol.* **2002**, *55*, 718.

318. Ettehad, D.; Emdin, C.A.; Kiran, A.; Anderson, S.G.; Callender, T.; Emberson, J.; Chalmers, J.; Rodgers, A.; Rahimi, K. Blood pressure lowering for prevention of cardiovascular disease and death: a systematic review and meta-analysis. *Lancet* **2016**, *387*, 957–967.

319. Silke, B. Haemodynamic Impact of Diuretic Therapy in Chronic Heart Failure. *Cardiology* **1994**, *84(suppl 2*, 115–123.

320. Sun, W.; Zhang, H.; Guo, J.; Zhang, X.; Zhang, L.; Li, C.; Zhang, L. Comparison of the Efficacy and Safety of Different ACE Inhibitors in Patients With Chronic Heart Failure: A PRISMA-Compliant Network Meta-Analysis. *Medicine (Baltimore).* **2016**, *95*, e2554–e2554.

321. Atkinson, A.B.; Robertson, J.I.S. Captopril in the treatment of clinical hypertension and cardiac failure. *Lancet* **1979**, *314*, 836–839.

322. Davies, J.C. A review of beta-blockers and their use in general practice. *J. R. Coll. Gen. Pract.* **1976**, *26*, 219–226.

323. Taddei, S.; Bruno, R.M.; Ghiadoni, L. The Correct Administration of Antihypertensive Drugs According to the Principles of Clinical Pharmacology. *Am. J. Cardiovasc. Drugs* **2011**, *11*, 13–20.

324. Karnes, J.H.; Cooper-DeHoff, R.M. Antihypertensive medications: benefits of blood pressure lowering and hazards of metabolic effects. *Expert Rev. Cardiovasc. Ther.* **2009**, *7*, 689–702.

325. Möller, N.P.; Scholz-Ahrens, K.E.; Roos, N.; Schrezenmeir, J. Bioactive peptides and proteins from foods: Indication for health effects. *Eur. J. Nutr.* 2008, *47*, 171–182.

326. Torres-Fuentes, C.; Schellekens, H.; Dinan, T.G.; Cryan, J.F. A natural solution for obesity: Bioactives for the prevention and treatment of weight gain. A review. *Nutr. Neurosci.* 2015, *18*, 49–65.

327. Kepler, C.R.; Hirons, K.P.; McNeill, J.J.; Tove, S.B. Intermediates and products of the biohydrogenation of linoleic acid by Butyrinvibrio fibrisolvens. *J. Biol. Chem.* **1966**, *241*, 1350–1354.

328. Pérez-Matute, P.; Marti, A.; Martínez, J.A.; Fernández-Otero, M.P.; Stanhope, K.L.; Havel, P.J.; Moreno-Aliaga, M.J. Conjugated linoleic acid inhibits glucose metabolism, leptin and adiponectin secretion in primary cultured rat adipocytes. *Mol. Cell. Endocrinol.* **2007**, *268*, 50–58.

329. Banni, S. Conjugated linoleic acid metabolism. *Curr. Opin. Lipidol.* **2002**, *13*.

330. Lee, Y. Isomer specificity of conjugated linoleic acid (CLA): 9E,11E-CLA. *Nutr. Res. Pract.* **2008**, *2*, 326–

330.

331. Nakamura, Y.K.; Flintoff-Dye, N.; Omaye, S.T. Conjugated linoleic acid modulation of risk factors associated with atherosclerosis. *Nutr. Metab. (Lond).* **2008**, *5*, 22.

332. Pariza, M.W.; Park, Y.; Cook, M.E. The biologically active isomers of conjugated linoleic acid. *Prog. Lipid Res.* **2001**, *40*, 283–298.

333. Kim, M.R.; Park, Y.; Albright, K.J.; Pariza, M.W. Differential responses of hamsters and rats fed high-fat or low-fat diets supplemented with conjugated linoleic acid. *Nutr. Res.* **2002**, *22*, 715–722.

334. Koba, K.; Akahoshi, A.; Yamasaki, M.; Tanaka, K.; Yamada, K.; Iwata, T.; Kamegai, T.; Tsutsumi, K.; Sugano, M. Dietary conjugated linolenic acid in relation to CLA differently modifies body fat mass and serum and liver lipid levels in rats. *Lipids* **2002**, *37*, 343–350.

335. Ostrowska, E.; Muralitharan, M.; Cross, R.F.; Bauman, D.E.; Dunshea, F.R. Dietary Conjugated Linoleic Acids Increase Lean Tissue and Decrease Fat Deposition in Growing Pigs. *J. Nutr.* **2018**, *129*, 2037–2042.

336. Szymczyk, B.; Pisulewski, P.M.; Szczurek, W.; Hanczakowski, P. Effects of conjugated linoleic acid on growth performance, feed conversion efficiency, and subsequent carcass quality in broiler chickens. *Br. J. Nutr.* **2001**, *85*, 465–473.

337. Takahashi, Y.; Kushiro, M.; Shinohara, K.; Ide, T. Dietary conjugated linoleic acid reduces body fat mass and affects gene expression of proteins regulating energy metabolism in mice. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* **2002**, *133*, 395–404.

338. Jobgen, W.; Meininger, C.J.; Jobgen, S.C.; Li, P.; Lee, M.-J.; Smith, S.B.; Spencer, T.E.; Fried, S.K.; Wu, G. Dietary l-Arginine Supplementation Reduces White Fat Gain and Enhances Skeletal Muscle and Brown Fat Masses in Diet-Induced Obese Rats. *J. Nutr.* **2008**, *139*, 230–237.

339. Mirand, P.P.; Arnal-Bagnard, M.-A.; Mosoni, L.; Faulconnier, Y.; Chardigny, J.-M.; Chilliard, Y. Cis-9, Trans-11 and Trans-10, Cis-12 Conjugated Linoleic Acid Isomers Do Not Modify Body Composition in Adult Sedentary or Exercised Rats. *J. Nutr.* **2004**, *134*, 2263–2269.

340. Poirier, H.; Shapiro, J.S.; Kim, R.J.; Lazar, M.A. Nutritional supplementation with trans-10, cis-12-conjugated linoleic acid induces inflammation of white adipose tissue. *Diabetes* **2006**, *55*, 1634–1641.

341. Park, Y.; Storkson, J.M.; Albright, K.J.; Liu, W.; Pariza, M.W. Evidence that the trans-10,cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. *Lipids* **1999**, *34*, 235–241.

342. Wang, Y.; Jones, P.J.H. Dietary conjugated linoleic acid and body composition. *Am. J. Clin. Nutr.* **2004**, *79*, 1153S-1158S.

343. Wang, Y.W.; Jones, P.J.H. Conjugated linoleic acid and obesity control: efficacy and mechanisms. *Int. J. Obes. Relat. Metab. Disord.* **2004**, *28*, 941–955.

344. Medina, E.A.; Horn, W.F.; Keim, N.L.; Havel, P.J.; Benito, P.; Kelley, D.S.; Nelson, G.J.; Erickson, K.L. Conjugated linoleic acid supplementation in humans: Effects on circulating leptin concentrations and appetite. *Lipids* **2000**, *35*, 783–788.

345. Tricon, S.; Burdge, G.C.; Williams, C.M.; Calder, P.C.; Yaqoob, P. The effects of conjugated linoleic acid on human health-related outcomes. *Proc. Nutr. Soc.* **2005**, *64*, 171–182.

346. Zambell, K.L.; Keim, N.L.; Van Loan, M.D.; Gale, B.; Benito, P.; Kelley, D.S.; Nelson, G.J. Conjugated linoleic acid supplementation in humans: Effects on body composition and energy expenditure. *Lipids* **2000**, *35*, 777–782.

347. Whigham, L.D.; Watras, A.C.; Schoeller, D.A. Efficacy of conjugated linoleic acid for reducing fat mass: a meta-analysis in humans. *Am. J. Clin. Nutr.* **2007**, *85*, 1203-1211 9p.

348. DeLany, J.P.; West, D.B. Changes in body composition with conjugated linoleic acid. *J. Am. Coll. Nutr.* **2000**, *19*, 487S-493S.

349. West, D.B.; Blohm, F.Y.; Truett, A.A.; DeLany, J.P. Conjugated Linoleic Acid Persistently Increases Total Energy Expenditure in AKR/J Mice without Increasing Uncoupling Protein Gene Expression. *J. Nutr.* **2000**, *130*, 2471–2477.

350. Terpstra, A.H.M.; Javadi, M.; Beynen, A.C.; Kocsis, S.; Lankhorst, A.E.; Lemmens, A.G.; Mohede, I.C.M.

Dietary Conjugated Linoleic Acids as Free Fatty Acids and Triacylglycerols Similarly Affect Body Composition and Energy Balance in Mice. *J. Nutr.* **2003**, *133*, 3181–3186.

351. Den Hartigh, L.J.; Wang, S.; Goodspeed, L.; Wietecha, T.; Houston, B.; Omer, M.; Ogimoto, K.; Subramanian, S.; Gowda, G.A.N.; O'Brien, K.D.; et al. Metabolically distinct weight loss by 10,12 CLA and caloric restriction highlight the importance of subcutaneous white adipose tissue for glucose homeostasis in mice. *PLoS One* **2017**, *12*, 1–25.

352. Ma, L.; Lengi, A.J.; McGilliard, M.L.; Bauman, D.E.; Corl, B.A. Short communication: Effect of trans-10,cis-12 conjugated linoleic acid on activation of lipogenic transcription factors in bovine mammary epithelial cells. *J. Dairy Sci.* **2014**, *97*, 5001–5006.

353. Rubin, D.; Herrmann, J.; Much, D.; Pfeuffer, M.; Laue, C.; Winkler, P.; Helwig, U.; Bell, D.; Auinger, A.; Darabaneanu, S.; et al. Influence of different CLA isomers on insulin resistance and adipocytokines in pre-diabetic, middle-aged men with PPARγ2 Pro12Ala polymorphism. *Genes Nutr.* **2012**, *7*, 499–509.

354. Stachowska, E.; Kijowski, J.; Dziedziejko, V.; Siennicka, A.; Chlubek, D. Conjugated Linoleic Acid Regulates Phosphorylation of PPARγ by Modulation of ERK 1/2 and p38 Signaling in Human Macrophages/Fatty Acid-Laden Macrophages. *J. Agric. Food Chem.* **2011**, *59*, 11846–11852.

355. Yamasaki, M.; Ikeda, A.; Oji, M.; Tanaka, Y.; Hirao, A.; Kasai, M.; Iwata, T.; Tachibana, H.; Yamada, K. Modulation of body fat and serum leptin levels by dietary conjugated linoleic acid in Sprague-Dawley rats fed various fat-level diets. *Nutrition* **2003**, *19*, 30–35.

356. Kang, K.; Pariza, M.W. trans-10,cis-12-Conjugated Linoleic Acid Reduces Leptin Secretion from 3T3-L1 Adipocytes. *Biochem. Biophys. Res. Commun.* **2001**, *287*, 377–382.

357. Yeganeh, A.; Taylor, C.G.; Tworek, L.; Poole, J.; Zahradka, P. Trans-10,cis-12 conjugated linoleic acid (CLA) interferes with lipid droplet accumulation during 3T3-L1 preadipocyte differentiation. *Int. J. Biochem. Cell Biol.* **2016**, *76*, 39–50.

358. Tsuboyama-Kasaoka, N.; Takahashi, M.; Tanemura, K.; Kim, H.J.; Tange, T.; Okuyama, H.; Kasai, M.; Ikemoto, S.; Ezaki, O. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. *Diabetes* **2000**, *49*, 1534–42.

359. Riserus, U.; Arner, P.; Brismar, K.; Vessby, B. Treatment with dietary trans10cis12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. *Diabetes Care* **2002**, *25*, 1516–1521.

360. Taylor, C.G.; Zahradka, P. Dietary conjugated linoleic acid and insulin sensitivity and resistance in rodent models. *Am. J. Clin. Nutr.* **2004**, *79*, 1164–1168.

361. Ryder, J.W.; Portocarrero, C.P.; Song, X.M.; Cui, L.; Yu, M.; Combatsiaris, T.; Galuska, D.; Bauman, D.E.; Barbano, D.M.; Charron, M.J.; et al. Isomer-Specific Antidiabetic Properties of Conjugated Linoleic Acid. *Diabetes* **2001**, *50*, 1149 LP – 1157.

362. Kennedy, A.; Martinez, K.; Chung, S.; LaPoint, K.; Hopkins, R.; Schmidt, S.F.; Andersen, K.; Mandrup, S.; McIntosh, M. Inflammation and insulin resistance induced by trans- 10, cis- 12 conjugated linoleic acid depend on intracellular calcium levels in primary cultures of human adipocytes . *J. Lipid Res.* **2010**, *51*, 1906–1917.

363. Vyas, D.; Kadegowda, A.K.G.; Erdman, R.A. Dietary conjugated linoleic acid and hepatic steatosis: Species-specific effects on liver and adipose lipid metabolism and gene expression. *J. Nutr. Metab.* **2012**, 13.

364. Degrace, P.; Moindrot, B.; Mohamed, I.; Gresti, J.; Du, Z.Y.; Chardigny, J.M.; Sébédio, J.L.; Clouet, P. Upregulation of liver VLDL receptor and FAT/CD36 expression in LDLR -/- apoB100/100 mice fed trans-10,cis-12 conjugated linoleic acid. *J. Lipid Res.* **2006**, *47*, 2647–2655.

365. Liu, L.-F.; Purushotham, A.; Wendel, A.A.; Belury, M.A. Combined effects of rosiglitazone and conjugated linoleic acid on adiposity, insulin sensitivity, and hepatic steatosis in high-fat-fed mice. *Am. J. Physiol. Liver Physiol.* **2007**, *292*, G1671–G1682.

366. Lin, Y.; Schuurbiers, E.; Van der Veen, S.; De Deckere, E.A.M. Conjugated linoleic acid isomers have differential effects on triglyceride secretion in Hep G2 cells. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **2001**, *1533*, 38–46.

367. Degrace, P.; Demizieux, L.; Gresti, J.; Chardigny, J.-M.; Sébédio, J.-L.; Clouet, P. Association of liver steatosis with lipid oversecretion and hypotriglyceridaemia in C57BL/6j mice fed trans-10,cis-12-linoleic acid. *FEBS Lett.* **2003**, *546*, 335–339.

368. Macarulla, M.T.; Fernández-Quintela, A.; Zabala, A.; Navarro, V.; Echevarría, E.; Churruca, I.; Rodríguez, V.M.; Portillo, M.P. Effects of conjugated linoleic acid on liver composition and fatty acid oxidation are isomer-dependent in hamster. *Nutrition* **2005**, *21*, 512–519.

369. Park, Y.; Albright, K.J.; Liu, W.; Storkson, J.M.; Cook, M.E.; Pariza, M.W. Effect of conjugated linoleic acid on body composition in mice. *Lipids* **1997**, *32*, 853–858.

370. Rasooly, R.; Kelley, D.S.; Greg, J.; Mackey, B.E. Dietary trans 10, cis 12-conjugated linoleic acid reduces the expression of fatty acid oxidation and drug detoxification enzymes in mouse liver. *Br. J. Nutr.* **2007**, *97*, 58–66.

371. Jourdan, T.; Djaouti, L.; Demizieux, L.; Gresti, J.; Vergès, B.; Degrace, P. Liver Carbohydrate and Lipid Metabolism of Insulin-Deficient Mice Is Altered by trans-10, cis-12 Conjugated Linoleic Acid. *J. Nutr.* **2009**, *139*, 1901–1907.

372. Scientific Opinion on the safety of “conjugated linoleic acid (CLA)-rich oil” (Clarinol®) as a Novel Food ingredient. *EFSA J.* **2010**, *8*, 1601.

373. Houseknecht, K.L.; Heuvel, J.P.V.; Moya-Camarena, S.Y.; Portocarrero, C.P.; Peck, L.W.; Nickel, K.P.; Belury, M.A. Dietary Conjugated Linoleic Acid Normalizes Impaired Glucose Tolerance in the Zucker Diabetic Fattyfa/faRat. *Biochem. Biophys. Res. Commun.* **1998**, *244*, 678–682.

374. DeLany, J.P.; Blohm, F.; Truett, A.A.; Scimeca, J.A.; West, D.B. Conjugated linoleic acid rapidly reduces body fat content in mice without affecting energy intake. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **1999**, *276*.

375. West, D.B.; Delany, J.P.; Camet, P.M.; Blohm, F.; Truett, A.A.; Scimeca, J. Effects of conjugated linoleic acid on body fat and energy metabolism in the mouse. *Am. J. Physiol. Integr. Comp. Physiol.* **1998**, *275*, R667–R672.

376. Choi, J.S.; Jung, M.H.; Park, H.S.; Song, J. Effect of conjugated linoleic acid isomers on insulin resistance and mRNA levels of genes regulating energy metabolism in high-fat–fed rats. *Nutrition* **2004**, *20*, 1008–1017.

377. Hwang, D.-M.; Kundu, J.K.; Shin, J.-W.; Lee, J.-C.; Lee, H.J.; Surh, Y.-J. cis- 9, trans- 11-Conjugated linoleic acid down-regulates phorbol ester-induced NF-κB activation and subsequent COX-2 expression in hairless mouse skin by targeting IκB kinase and PI3K-Akt . *Carcinogenesis* **2007**, *28*, 363–371.

378. Engl, J.; Sturm, W.; Sandhofer, A.; Kaser, S.; Tschoner, A.; Tatarczyk, T.; Weiss, H.; Tilg, H.; Patsch, J.R.; Ebenbichler, C.F. Effect of pronounced weight loss on visceral fat, liver steatosis and adiponectin isoforms. *Eur. J. Clin. Invest.* **2008**, *38*, 238–244.

379. Mollica, M.P.; Trinchese, G.; Cavaliere, G.; De Filippo, C.; Cocca, E.; Gaita, M.; Della-Gatta, A.; Marano, A.; Mazzarella, G.; Bergamo, P. C9, t11-Conjugated linoleic acid ameliorates steatosis by modulating mitochondrial uncoupling and Nrf2 pathway. *J. Lipid Res.* **2014**, *55*, 837–849.

380. Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J.P.E.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signal.* **2013**, *18*, 1818–1892.

381. Crozier, A.; Jaganath, I.B.; Clifford, M.N. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat. Prod. Rep.* **2009**, *26*, 1001–1043.

382. Strack, D. 10 - Phenolic Metabolism. In; Dey, P.M., Harborne, J.B.B.T.-P.B., Eds.; Academic Press: London, 1997; pp. 387–416 ISBN 978-0-12-214674-9.

383. Estruch, R.; Martínez-González, M.Á.; Corella, D.; Salas-Salvadó, J.; Ruiz-Gutiérrez, V.; Covas, M.I.; Fiol, M.; Gómez-Gracia, E.; López-Sabater, M.C.; Vinyoles, E.; et al. Effects of a Mediterranean-Style Diet on Cardiovascular Risk Factors: A Randomized Trial. *Ann. Intern. Med.* **2006**, *145*, 1–11.

384. Wang, S.; Moustaid-Moussa, N.; Chen, L.; Mo, H.; Shastri, A.; Su, R.; Bapat, P.; Kwun, I.; Shen, C.-L. Novel insights of dietary polyphenols and obesity. *J. Nutr. Biochem.* **2014**, *25*, 1–18.

385. Cory, H.; Passarelli, S.; Szeto, J.; Tamez, M.; Mattei, J. The Role of Polyphenols in Human Health and Food Systems: A Mini-Review. *Front. Nutr.* **2018**, *5*, 87.

386. Tomas-Barberán, F.A.; Cienfuegos-Jovellanos, E.; Marín, A.; Muguerza, B.; Gil-Izquierdo, A.; Cerdá, B.; Zafrilla, P.; Morillas, J.; Mulero, J.; Ibarra, A.; et al. A New Process To Develop a Cocoa Powder with Higher Flavonoid Monomer Content and Enhanced Bioavailability in Healthy Humans. *J. Agric. Food Chem.* **2007**, *55*, 3926–3935.

387. Gee, J.M.; DuPont, M.S.; Day, A.J.; Plumb, G.W.; Williamson, G.; Johnson, I.T. Intestinal Transport of Quercetin Glycosides in Rats Involves Both Deglycosylation and Interaction with the Hexose Transport Pathway. *J. Nutr.* **2000**, *130*, 2765–2771.

388. Thilakarathna, S.H.; Rupasinghe, H.P.V. Flavonoid bioavailability and attempts for bioavailability enhancement. *Nutrients* **2013**, *5*, 3367–3387.

389. Bladé, C.; Baselga-Escudero, L.; Salvadó, M.J.; Arola-Arnal, A. miRNAs, polyphenols, and chronic disease. *Mol. Nutr. Food Res.* **2013**, *57*, 58–70.

390. Serra, A.; Bladé, C.; Arola, L.; Macià, A.; Motilva, M.-J. Flavanol metabolites distribute in visceral adipose depots after a long-term intake of grape seed proanthocyanidin extract in rats. *Br. J. Nutr.* **2013**, *110*, 1411–1420.

391. Monagas, M.; Urpi-Sarda, M.; Sánchez-Patán, F.; Llorach, R.; Garrido, I.; Gómez-Cordovés, C.; Andres-Lacueva, C.; Bartolomé, B. Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food Funct.* **2010**, *1*, 233–253.

392. Nijveldt, R.J.; van Nood, E.; van Hoorn, D.E.C.; Boelens, P.G.; van Norren, K.; van Leeuwen, P.A.M. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425.

393. Kumar, S.; Pandey, A.K. Chemistry and Biological Activities of Flavonoids: An Overview. *Sci. World J.* **2013**, *2013*, 162750.

394. Kawabata, K.; Mukai, R.; Ishisaka, A. Quercetin and related polyphenols: new insights and implications for their bioactivity and bioavailability. *Food Funct.* **2015**, *6*, 1399–1417.

395. He, F.; Pan, Q.-H.; Shi, Y.; Duan, C.-Q. Biosynthesis and genetic regulation of proanthocyanidins in plants. *Molecules* **2008**, *13*, 2674–2703.

396. Gu, L.; Kelm, M.A.; Hammerstone, J.F.; Beecher, G.; Holden, J.; Haytowitz, D.; Gebhardt, S.; Prior, R.L. Concentrations of Proanthocyanidins in Common Foods and Estimations of Normal Consumption. *J. Nutr.* **2004**, *134*, 613–617.

397. LEE, Y.A.; CHO, E.J.; TANAKA, T.; YOKOZAWA, T. Inhibitory Activities of Proanthocyanidins from Persimmon against Oxidative Stress and Digestive Enzymes Related to Diabetes. *J. Nutr. Sci. Vitaminol. (Tokyo).* **2007**, *53*, 287–292.

398. Martinez-Micaelo, N.; González-Abuín, N.; Ardèvol, A.; Pinent, M.; Blay, M.T. Procyanidins and inflammation: Molecular targets and health implications. *BioFactors* **2012**, *38*, 257–265.

399. Quesada, H.; del Bas, J.M.; Pajuelo, D.; Díaz, S.; Fernandez-Larrea, J.; Pinent, M.; Arola, L.; Salvadó, M.J.; Bladé, C. Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. *Int. J. Obes.* **2009**, *33*, 1007–1012.

400. de la Garza, A.L.; Milagro, F.I.; Boque, N.; Campion, J.; Martinez, J.A. Natural inhibitors of pancreatic lipase as new players in obesity treatment. *Planta Med.* **2011**, *77*, 773–785.

401. Sugiyama, H.; Akazome, Y.; Shoji, T.; Yamaguchi, A.; Yasue, M.; Kanda, T.; Ohtake, Y. Oligomeric Procyanidins in Apple Polyphenol Are Main Active Components for Inhibition of Pancreatic Lipase and Triglyceride Absorption. *J. Agric. Food Chem.* **2007**, *55*, 4604–4609.

402. Feringa, H.H.H.; Laskey, D.A.; Dickson, J.E.; Coleman, C.I. The Effect of Grape Seed Extract on Cardiovascular Risk Markers: A Meta-Analysis of Randomized Controlled Trials. *J. Am. Diet. Assoc.* **2011**, *111*, 1173–1181.

403. Razavi, S.-M.; Gholamin, S.; Eskandari, A.; Mohsenian, N.; Ghorbanihaghjo, A.; Delazar, A.;

Rashtchizadeh, N.; Keshtkar-Jahromi, M.; Argani, H. Red Grape Seed Extract Improves Lipid Profiles and Decreases Oxidized Low-Density Lipoprotein in Patients with Mild Hyperlipidemia. *J. Med. Food* **2013**, *16*, 255–258.

404. Heiss, C.; Jahn, S.; Taylor, M.; Real, W.M.; Angeli, F.S.; Wong, M.L.; Amabile, N.; Prasad, M.; Rassaf, T.; Ottaviani, J.I.; et al. Improvement of Endothelial Function With Dietary Flavanols Is Associated With Mobilization of Circulating Angiogenic Cells in Patients With Coronary Artery Disease. *J. Am. Coll. Cardiol.* **2010**, *56*, 218–224.

405. Pinent, M.; Cedó, L.; Montagut, G.; Blay, M.; Ardévol, A. Procyanidins Improve some Disrupted Glucose Homoeostatic Situations: An Analysis of Doses and Treatments According to Different Animal Models. *Crit. Rev. Food Sci. Nutr.* **2012**, *52*, 569–584.

406. Bladé, C.; Arola, L.; Salvadó, M.-J. Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. *Mol. Nutr. Food Res.* **2010**, *54*, 37–59.

407. Fernández-Hernando, C.; Moore, K.J. MicroRNA modulation of cholesterol homeostasis. *Arterioscler. Thromb. Vasc. Biol.* **2011**, *31*, 2378–2382.

408. Del Bas, J.M.; Fernández-Larrea, J.; Blay, M.; Ardèvol, A.; Salvadó, M.J.; Arola, L.; Bladé, C. Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats. *FASEB J.* **2005**, *19*, 479–481.

409. Quesada, H.; Díaz, S.; Pajuelo, D.; Fernández-Iglesias, A.; Garcia-Vallvé, S.; Pujadas, G.; Salvadó, M.J.; Arola, L.; Bladé, C. The lipid-lowering effect of dietary proanthocyanidins in rats involves both chylomicron-rich and VLDL-rich fractions. *Br. J. Nutr.* **2012**, *108*, 208–217.

410. Pons, Z.; Guerrero, L.; Margalef, M.; Arola, L.; Arola-Arnal, A.; Muguerza, B. Effect of low molecular grape seed proanthocyanidins on blood pressure and lipid homeostasis in cafeteria diet-fed rats. *J. Physiol. Biochem.* **2014**, *70*, 629–637.

411. Aragonès, G.; Suárez, M.; Ardid-Ruiz, A.; Vinaixa, M.; Rodríguez, M.A.; Correig, X.; Arola, L.; Bladé, C. Dietary proanthocyanidins boost hepatic NAD+ metabolism and SIRT1 expression and activity in a dose-dependent manner in healthy rats. *Sci. Rep.* **2016**, *6*, 24977.

412. Del Bas, J.M.; Ricketts, M.-L.; Vaqué, M.; Sala, E.; Quesada, H.; Ardevol, A.; Salvadó, M.J.; Blay, M.; Arola, L.; Moore, D.D.; et al. Dietary procyanidins enhance transcriptional activity of bile acid-activated FXR in vitro and reduce triglyceridemia in vivo in a FXR-dependent manner. *Mol. Nutr. Food Res.* **2009**, *53*, 805–814.

413. Del Bas, J.M.; Ricketts, M.L.; Baiges, I.; Quesada, H.; Ardevol, A.; Salvadó, M.J.; Pujadas, G.; Blay, M.; Arola, L.; Bladé, C.; et al. Dietary procyanidins lower triglyceride levels signaling through the nuclear receptor small heterodimer partner. *Mol. Nutr. Food Res.* **2008**, *52*, 1172–1181.

414. Pascual-Serrano, A.; Arola-Arnal, A.; Suárez-García, S.; Bravo, F.I.; Suárez, M.; Arola, L.; Bladé, C. Grape seed proanthocyanidin supplementation reduces adipocyte size and increases adipocyte number in obese rats. *Int. J. Obes.* **2017**, *41*, 1246–1255.

415. Caimari, A.; Del Bas, J.M.; Crescenti, A.; Arola, L. Low doses of grape seed procyanidins reduce adiposity and improve the plasma lipid profile in hamsters. *Int. J. Obes.* **2013**, *37*, 576–583.

416. Pinent, M.; Bladé M. Cinta; Salvadó, M.J.; Arola, L.; Ardévol, A. Intracellular Mediators of Procyanidin-Induced Lipolysis in 3T3-L1 Adipocytes. *J. Agric. Food Chem.* **2005**, *53*, 262–266.

417. Pinent, M.; Blay, M.; Bladé, M.C.; Salvadó, M.J.; Arola, L.; Ardévol, A. Grape Seed-Derived Procyanidins Have an Antihyperglycemic Effect in Streptozotocin-Induced Diabetic Rats and Insulinomimetic Activity in Insulin-Sensitive Cell Lines. *Endocrinology* **2004**, *145*, 4985–4990.

418. Ardévol, A.; Motilva, M.J.; Serra, A.; Blay, M.; Pinent, M. Procyanidins target mesenteric adipose tissue in Wistar lean rats and subcutaneous adipose tissue in Zucker obese rat. *Food Chem.* **2013**, *141*, 160–166.

419. Gonzalez-Abuin, N.; Pinent, M.; Casanova-Marti, A.; Arola, L.; Blay, M.; Ardevol, A. Procyanidins and their healthy protective effects against type 2 diabetes. *Curr. Med. Chem.* **2015**, *22*, 39–50.

420. Li, B.-Y.; Cheng, M.; Gao, H.-Q.; Ma, Y.-B.; Xu, L.; Li, X.-H.; Li, X.-L.; You, B.-A. Back-regulation of six oxidative stress proteins with grape seed proanthocyanidin extracts in rat diabetic nephropathy. *J. Cell.*

Biochem. **2008**, *104*, 668–679.

421. Cienfuegos-Jovellanos, E.; Quiñones, M. del M.; Muguerza, B.; Moulay, L.; Miguel, M.; Aleixandre, A. Antihypertensive Effect of a Polyphenol-Rich Cocoa Powder Industrially Processed To Preserve the Original Flavonoids of the Cocoa Beans. *J. Agric. Food Chem.* **2009**, *57*, 6156–6162.

422. Negishi, H.; Xu, J.-W.; Ikeda, K.; Njelekela, M.; Nara, Y.; Yamori, Y. Black and Green Tea Polyphenols Attenuate Blood Pressure Increases in Stroke-Prone Spontaneously Hypertensive Rats. *J. Nutr.* **2004**, *134*, 38–42.

423. Quiñones, M.; Miguel, M.; Muguerza, B.; Aleixandre, A. Effect of a cocoa polyphenol extract in spontaneously hypertensive rats. *Food Funct.* **2011**, *2*, 649–653.

424. Thandapilly, S.J.; LeMaistre, J.L.; Louis, X.L.; Anderson, C.M.; Netticadan, T.; Anderson, H.D. Vascular and Cardiac Effects of Grape Powder in the Spontaneously Hypertensive Rat. *Am. J. Hypertens.* **2012**, *25*, 1070–1076.

425. Quiñones, M.; Sánchez, D.; Muguerza, B.; Moulay, L.; Laghi, S.; Miguel, M.; Aleixandre, A. Long-term intake of CocoanOX attenuates the development of hypertension in spontaneously hypertensive rats. *Food Chem.* **2010**, *122*, 1013–1019.

426. Quiñones, M.; Guerrero, L.; Suarez, M.; Pons, Z.; Aleixandre, A.; Arola, L.; Muguerza, B. Low-molecular procyanidin rich grape seed extract exerts antihypertensive effect in males spontaneously hypertensive rats. *Food Res. Int.* **2013**, *51*, 587–595.

427. Ibars, M.; Ardid-Ruiz, A.; Suárez, M.; Muguerza, B.; Bladé, C.; Aragonès, G. Proanthocyanidins potentiate hypothalamic leptin/STAT3 signalling and Pomc gene expression in rats with diet-induced obesity. *Int. J. Obes.* **2017**, *41*, 129–136.

428. Serrano, J.; Casanova-Martí, À.; Gil-Cardoso, K.; Blay, M.T.; Terra, X.; Pinent, M.; Ardévol, A. Acutely administered grape-seed proanthocyanidin extract acts as a satiating agent. *Food Funct.* **2016**, *7*, 483–490.

429. Wallace, T.C.; Giusti, M.M. Anthocyanins. *Adv. Nutr.* **2015**, *6*, 620–622.

430. Wu, T.; Jiang, Z.; Yin, J.; Long, H.; Zheng, X. Anti-obesity effects of artificial planting blueberry (Vaccinium ashei) anthocyanin in high-fat diet-treated mice. *Int. J. Food Sci. Nutr.* **2016**, *67*, 257–264.

431. Wu, T.; Tang, Q.; Yu, Z.; Gao, Z.; Hu, H.; Chen, W.; Zheng, X.; Yu, T. Inhibitory effects of sweet cherry anthocyanins on the obesity development in C57BL/6 mice. *Int. J. Food Sci. Nutr.* **2014**, *65*, 351–359.

432. Karlsen, A.; Retterstøl, L.; Laake, P.; Paur, I.; Kjølsrud-Bøhn, S.; Sandvik, L.; Blomhoff, R. Anthocyanins Inhibit Nuclear Factor-κB Activation in Monocytes and Reduce Plasma Concentrations of Pro-Inflammatory Mediators in Healthy Adults. *J. Nutr.* **2007**, *137*, 1951–1954.

433. Cimino, F.; Speciale, A.; Anwar, S.; Canali, R.; Ricciardi, E.; Virgili, F.; Trombetta, D.; Saija, A. Anthocyanins protect human endothelial cells from mild hyperoxia damage through modulation of Nrf2 pathway. *Genes Nutr.* **2013**, *8*, 391–399.

434. Luo, X.; Fang, S.; Xiao, Y.; Song, F.; Zou, T.; Wang, M.; Xia, M.; Ling, W. Cyanidin-3-glucoside suppresses TNF-α-induced cell proliferation through the repression of Nox activator 1 in mouse vascular smooth muscle cells: involvement of the STAT3 signaling. *Mol. Cell. Biochem.* **2012**, *362*, 211–218.

435. Zhu, Y.; Ling, W.; Guo, H.; Song, F.; Ye, Q.; Zou, T.; Li, D.; Zhang, Y.; Li, G.; Xiao, Y.; et al. Anti-inflammatory effect of purified dietary anthocyanin in adults with hypercholesterolemia: A randomized controlled trial. *Nutr. Metab. Cardiovasc. Dis.* **2013**, *23*, 843–849.

436. Zhang, P.-W.; Chen, F.-X.; Li, D.; Ling, W.-H.; Guo, H.-H. A CONSORT-compliant, randomized, double-blind, placebo-controlled pilot trial of purified anthocyanin in patients with nonalcoholic fatty liver disease. *Medicine (Baltimore).* **2015**, *94*, e758.

437. Li, D.; Zhang, Y.; Liu, Y.; Sun, R.; Xia, M. Purified Anthocyanin Supplementation Reduces Dyslipidemia, Enhances Antioxidant Capacity, and Prevents Insulin Resistance in Diabetic Patients. *J. Nutr.* **2015**, *145*, 742–748.

438. Qin, Y.; Xia, M.; Ma, J.; Hao, Y.; Liu, J.; Mou, H.; Cao, L.; Ling, W. Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl

ester transfer protein in dyslipidemic subjects. *Am. J. Clin. Nutr.* **2009**, *90*, 485–492.

439. Zhu, Y.; Huang, X.; Zhang, Y.; Wang, Y.; Liu, Y.; Sun, R.; Xia, M. Anthocyanin Supplementation Improves HDL-Associated Paraoxonase 1 Activity and Enhances Cholesterol Efflux Capacity in Subjects With Hypercholesterolemia. *J. Clin. Endocrinol. Metab.* **2014**, *99*, 561–569.

440. Zhu, Y.; Xia, M.; Yang, Y.; Liu, F.; Li, Z.; Hao, Y.; Mi, M.; Jin, T.; Ling, W. Purified Anthocyanin Supplementation Improves Endothelial Function via NO-cGMP Activation in Hypercholesterolemic Individuals. *Clin. Chem.* **2011**, *57*, 1524–1533.

441. Wang, Y.; Zhang, Y.; Wang, X.; Liu, Y.; Xia, M. Supplementation with Cyanidin-3-O-β-Glucoside Protects against Hypercholesterolemia-Mediated Endothelial Dysfunction and Attenuates Atherosclerosis in Apolipoprotein E–Deficient Mice. *J. Nutr.* **2012**, *142*, 1033–1037.

442. Bravo, F.I.; Mas-Capdevila, A.; Margalef, M.; Arola-Arnal, A.; Muguerza, B. Novel Antihypertensive Peptides Derived from Chicken Foot Proteins. *Mol. Nutr. Food Res.* **2019**, *63*, 1801176.

443. Mas-Capdevila, A.; Pons, Z.; Aleixandre, A.; Bravo, F.I.; Muguerza, B. Dose-Related Antihypertensive Properties and the Corresponding Mechanisms of a Chicken Foot Hydrolysate in Hypertensive Rats. *Nutrients* **2018**, *10*, 1295.

444. Daliri, E.B.-M.; Oh, D.H.; Lee, B.H. Bioactive Peptides. *Foods (Basel, Switzerland)* **2017**, *6*, 32.

445. Biesalski, H.-K.; Dragsted, L.O.; Elmadfa, I.; Grossklaus, R.; Müller, M.; Schrenk, D.; Walter, P.; Weber, P. Bioactive compounds: Definition and assessment of activity. *Nutrition* **2009**, *25*, 1202–1205.

446. Korhonen, H.; Pihlanto, A. Bioactive peptides: Production and functionality. *Int. Dairy J.* **2006**, *16*, 945–960.

447. Li-Chan, E.C.Y. Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. *Curr. Opin. Food Sci.* **2015**, *1*, 28–37.

448. Miralles, B.; Amigo, L.; Recio, I. Critical Review and Perspectives on Food-Derived Antihypertensive Peptides. *J. Agric. Food Chem.* **2018**, *66*, 9384–9390.

449. Maruyama, S.; Suzuki, H. A Peptide Inhibitor of Angiotensin I Converting Enzyme in the Tryptic Hydrolysate of Casein. *Agric. Biol. Chem.* **1982**, *46*, 1393–1394.

450. Bhat, Z.F.; Kumar, S.; Bhat, H.F. Bioactive peptides of animal origin: a review. *J. Food Sci. Technol.* **2015**, *52*, 5377–5392.

451. Ryan, J.T.; Ross, R.P.; Bolton, D.; Fitzgerald, G.F.; Stanton, C. Bioactive peptides from muscle sources: meat and fish. *Nutrients* **2011**, *3*, 765–791.

452. Lemes, A.C.; Sala, L.; Ores, J. da C.; Braga, A.R.C.; Egea, M.B.; Fernandes, K.F. A Review of the Latest Advances in Encrypted Bioactive Peptides from Protein-Rich Waste. *Int. J. Mol. Sci.* 2016, *17*.

453. Mas-Capdevila, A.; Iglesias-Carres, L.; Arola-Arnal, A.; Suarez, M.; Muguerza, B.; Bravo, F.I. Long-term administration of protein hydrolysate from chicken feet induces antihypertensive effect and confers vasoprotective pattern in diet-induced hypertensive rats. *J. Funct. Foods* **2019**, *55*, 28–35.

454. Miguel, M.; Muguerza, B.; Sánchez, E.; Delgado, M.A.; Recio, I.; Ramos, M.; Aleixandre, M.A. Changes in arterial blood pressure in hypertensive rats caused by long-term intake of milk fermented by Enterococcus faecalis CECT 5728. *Br. J. Nutr.* **2005**, *94*, 36–43.

455. Quiñones, M.; Muguerza, B.; Miguel, M.; Aleixandre, A. Evidence that nitric oxide mediates the blood pressure lowering effect of a polyphenol-rich cocoa powder in spontaneously hypertensive rats. *Pharmacol. Res.* **2011**, *64*, 478–481.

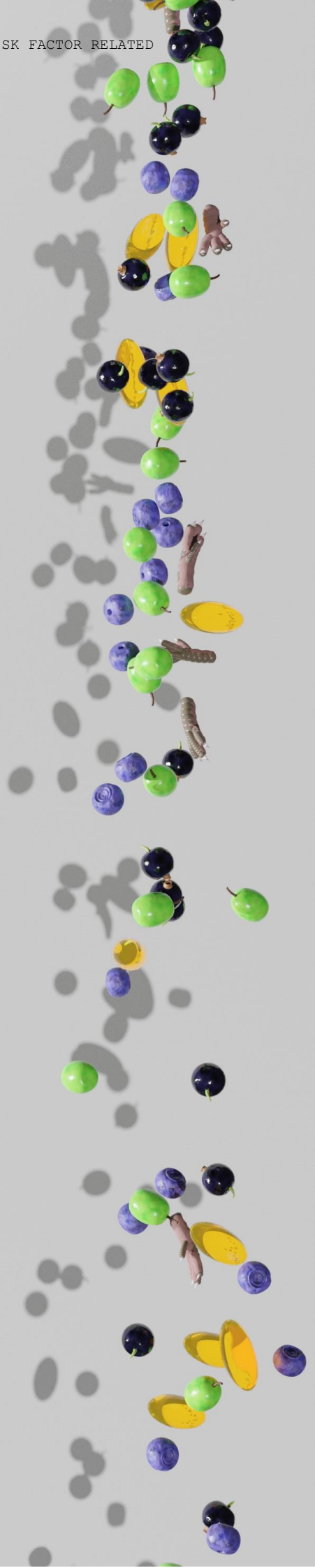
456. Wu, J.-Y.; Kao, H.-J.; Li, S.-C.; Stevens, R.; Hillman, S.; Millington, D.; Chen, Y.-T. ENU mutagenesis identifies mice with mitochondrial branched-chain aminotransferase deficiency resembling human maple syrup urine disease. *J. Clin. Invest.* **2004**, *113*, 434–440.

457. Samuel, V.T.; Shulman, G.I. Mechanisms for insulin resistance: common threads and missing links. *Cell* **2012**, *148*, 852–871.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González



HYPOTHESIS AND OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Hypothesis and objectives

Obesity is defined as an excess of fat accumulation that presents a risk to health. It frequently occurs concurrently with other metabolic risk factors related to lifestyle, such as dyslipidemia, impaired glucose tolerance or non-alcoholic fatty liver disease (NAFLD), resulting in metabolic syndrome (MetS).

Many bioactive compounds have been studied as strategies to prevent obesity and associated pathologies. Conjugated linoleic acid (CLA) is one of the most investigated for weight loss, although controversial metabolic effects after its administration have been reported. However, the results in animal studies might not be comparable with those obtained in human studies, since much higher CLA doses are used in preclinical trials than the doses in clinical trials. Beneficial effects of the flavonoids proanthocyanidins and anthocyanidins in the control of metabolic disturbances related to MetS have also been reported. In this sense, the grape seed extract GSPE, rich in proanthocyanidins and the berry-derived supplement Medox, rich in anthocyanidins, has been shown to improve metabolic risk factors associated with obesity such as blood lipid profile and glucose homeostasis. In addition, the blood pressure lowering effect of some bioactive peptides are well known. In this sense, Hpp11 is a chicken feet hydrolysate whose antihypertensive activity has been demonstrated after its acute and chronic administration.

However, nowadays the use of a single family of bioactive compounds seems not to be sufficient to correct complex, multisystemic and very well regulated situations such as body weight and associated pathologies. Therefore, we hypothesize that the **co-administration of different bioactive compounds, including CLA, the flavonoids proanthocyanidins and anthocyanidins and the antihypertensive hydrolysate Hpp11, could reduce body weight and associated pathologies, improving the metabolic profile of obese animals with MetS.**

Therefore, the aim of this thesis is to elucidate whether the administration of a multifunctional ingredient can be useful to correct the set of metabolic risk factors associated with obesity in rats fed cafeteria diet as an animal model of human MetS .

In order to assess the established assumption, specific objectives were proposed:

1. **To evaluate the effects of low doses of CLA administration on obesity and associated pathologies.**

CLA has been reported to exhibit effects on body weight in different animal models, but some studies have also associated its consumption with liver impairment and insulin resistance. Nevertheless, these studies have mainly been carried out with diets supplemented with high doses of

CLA. Therefore, it was considered the need to prove the efficacy of minor doses of CLA on body weight and rule out any detrimental effects associated with its consumption to these lower doses.

In order to assess this objective the following goal was proposed:

a) To evaluate the effect of low doses of CLA intake on body weight and other main cardiometabolic risk factors associated with obesity in cafeteria-diet fed rats **[Manuscript 1].**

2. To establish the effects on obesity and related pathologies of a multifunctional ingredient based on CLA at low doses, the flavonoids proanthocyanidins and anthocyanidins and the chicken feet hydrolysate Hpp11.

Beneficial properties of individual dietary complements on body weight and other cardiometabolic risk factors have been reported, but the consumption of a single family of bioactive compounds seems not to be sufficient to improve the obesity and associated pathologies. Therefore, the study of the co-administration of different bioactive compounds as a multifunctional ingredient was considered of interest, since these components could both produce a synergetic effect on body weight and act simultaneously on other pathologies associated with obesity.

In order to assess this objective two goals were proposed:

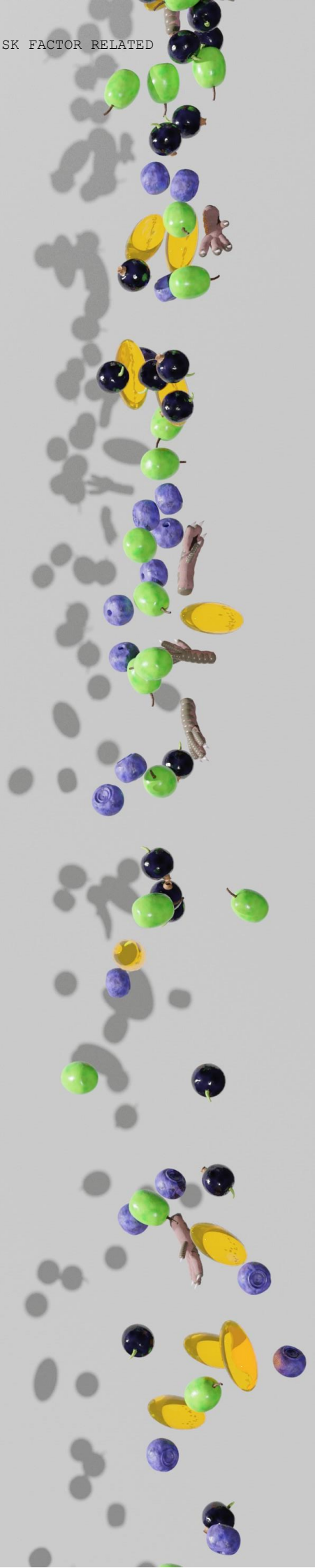
a) To determinate the effect of a multifunctional ingredient on obesity and establish the mechanisms operating in its body weight lowering effects in a model of diet-induced obesity **[Manuscript 2]** and **[Patent]** (see in Annexes).

b) To investigate the effects of the multifunctional ingredient on other cardiometabolic risk factors associated with MetS in cafeteria-diet fed rats **[Manuscript 3]**.

The research work carried out in this Ph.D. has been supported by grants AGL2013-40707-R and AGL2016-77105-R, from the Ministerio de Educación y Ciencia of the Spanish Government. This thesis was performed in the Nutrigenomics Research Group of the Universitat Rovira i Virgili with a predoctoral grant from the Universitat Rovira i Virgili. A European phase was completed in the School of Biological Sciences of the University of Hong Kong, under the supervision of Dr. Hani El-Nezami.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González



RESULTS

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Manuscript 1

Beneficial Effects of a Low-dose of Conjugated Linoleic Acid on Body Weight Gain and other Cardiometabolic Risk Factors in Cafeteria Diet-fed Rats

Miguel Z Martín-González 1,†, Héctor Palacios 1,2,†, Miguel A Rodríguez 2, Lluís Arola 1,3, Gerard Aragonès 1,* and Begoña Muguerza 1

[1] Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, 43007 Tarragona, Spain
[2] Eurecat, Centre Tecnològic de Catalunya, Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT, Unique Scientific and Technical Infrastructures (ICTS), 43204 Reus, Spain.
[3] Eurecat, Centre Tecnològic de Catalunya, Biotechnological Area, 43204 Reus, Spain

***** Correspondence: gerard.aragones@urv.cat; Tel.: +34-977-558-188
† Both authors contributed equally to this manuscript.

Received: 15 January 2020; Accepted: 2 February 2020; Published: 4 February 2020

Published in *Nutrients*

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

nutrients

MDPI

Article

Beneficial Effects of a Low-dose of Conjugated Linoleic Acid on Body Weight Gain and other Cardiometabolic Risk Factors in Cafeteria Diet-fed Rats

Miguel Z Martín-González [1,†], Héctor Palacios [1,2,†], Miguel A Rodríguez [2], Lluís Arola [1,3], Gerard Aragonès [1,*] and Begoña Muguerza [1]

[1] Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, 43007 Tarragona, Spain; miguelmg.1991@gmail.com (M.Z.M.-G.); hector.palacios@eurecat.org (H.P.); lluis.arola@urv.cat (L.A.); begona.muguerza@urv.cat (B.M.)
[2] Eurecat, Centre Tecnològic de Catalunya, Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT, Unique Scientific and Technical Infrastructures (ICTS), 43204 Reus, Spain; miguelangel.rodriguez@eurecat.org
[3] Eurecat, Centre Tecnològic de Catalunya, Biotechnological Area, 43204 Reus, Spain
* Correspondence: gerard.aragones@urv.cat; Tel.: +34-977-558-188
† Both authors contributed equally to this manuscript.

Received: 15 January 2020; Accepted: 2 February 2020; Published: 4 February 2020

Abstract: Conjugated linoleic acid (CLA) is a dietary supplement that has been shown to improve obesity. However, some authors have associated high doses of CLA supplementation with liver impairment and insulin resistance. The aim of this study was to assess whether the consumption of low doses of CLA maintained the beneficial effects on the main metabolic disturbances associated with metabolic syndrome (MetS) but prevented the occurrence of non-desirable outcomes associated with its consumption. Male Wistar rats, fed standard or cafeteria (CAF) diet for 12 weeks, were supplemented with three different low doses of CLA in the last three weeks. Both biochemical and H^1 NMR-based metabolomics profiles were analysed in serum and liver. The consumption of 100 mg/kg CLA, but not doses of 200 and 300 mg/kg, ameliorated the increase in body weight gain as well as the serum concentrations of glucose, insulin, cholesterol, triglyceride, diglyceride, and total phospholipid induced by a CAF diet. In turn, CLA reverted the increase in lactate, alanine, and glucose concentrations in the liver of these animals, but enhanced hepatic cholesterol accumulation without any detrimental effect on liver function. In conclusion, a low dose of CLA corrected the adverse effects associated with MetS without compromising other metabolic parameters.

Keywords: CLA; insulin resistance; leptin; metabolomics; NAFLD; obesity

1. Introduction

The obesity epidemic is a major health risk factor that frequently occurs concurrently with many other cardiovascular risk factors related to lifestyle, such as dyslipidaemia, impaired glucose tolerance or non-alcoholic fatty liver disease (NAFLD), resulting in metabolic syndrome (MetS) [1]. This disease is considered as such when at least three of the following five symptoms are present: high waist circumference, hypertriglyceridaemia, hyperglycaemia, hypertension, and reduced high-density lipoprotein (HDL) cholesterol concentrations. These symptoms provide an easy-to-assess method of diagnosing MetS, but do not provide a full picture of the underlying problem. Metabolomics studies have found many links between MetS and particular metabolites that could be used to better characterise this disease [2]. In this sense, Cheng et al. studied the plasma

concentrations of 45 metabolites in the Framingham Heart Study (n = 1015) and the Malmö Diet and Cancer Study (n = 746). Metabolic risk factors such as obesity, insulin resistance, hypertension and dyslipidaemia were associated with multiple metabolites including branched-chain amino acids (BCAAs), other hydrophobic amino acids, tryptophan breakdown products, and nucleotide metabolites [3]. Other studies have also reported a strong association between plasma metabolites and cardiometabolic risk factors such as increased levels of BCAAs and aromatic amino acids with increased proinflammatory mediators and metabolic disease [4], high alanine levels with insulin resistance [2], and low levels of histidine with inflammation, oxidative stress, and mortality in patients which chronic kidney disease [5]. In addition, reduced plasma levels of lysine and methionine have been pointed to as important contributors and early biomarkers of incipient MetS [4]. Other metabolites such as the phospholipids phosphatidylcholine and phosphatidylethanolamine have also emerged as biomarkers that correlated with features of MetS, as well as adipose tissue dysfunction and inflammation [6].

Wistar rats fed a cafeteria (CAF) diet, which consists of free access to highly palatable, energy dense, unhealthy human food, rich in carbohydrate and fat dietary components, are considered a robust model of human MetS. CAF diet-fed rats present hyperphagia, increased body weight (bw) and develop hyperinsulinaemia, hyperglycaemia and NAFLD [7,8]. In addition, the development of hypertension in animals fed a CAF diet for 10 weeks has also been reported [7]. Therefore, this diet experimental model can be especially suitable to evaluate the effectiveness of different compounds on obesity and other complications related to MetS.

Conjugated linoleic acid (CLA) is a dietary supplement that has been reported, with its effects on body weight loss being one of the most investigated [9,10]. CLA is a group of positional and geometric isomers of the omega-6 essential fatty acid linoleic acid, which present conjugated double bonds naturally produced by ruminal biohydrogenation. These double bonds can take place in different positions, generating a family of isomeric fatty acids, of which *cis*-9 (c9), *trans*-11 (t11), and *trans*-10 (t10), *cis*-12 (c12) CLA are the most naturally abundant and widely studied [11,12]. The administration of CLA to different animal species at doses from 0.5 to 1.5% of total dietary fat has been shown to reduce body fat [13–16], and a meta-analysis of human trials concluded that at an average dose of 3.2 g per day of CLA reduces body fat mass [10]. However, the results in animal studies might not be comparable with those obtained in human studies since much higher CLA doses are used in preclinical trials (27.1–81.7 g/day) than the doses in clinical trials (0.7–6.8 g/day) [10,17]. In addition, depending on the duration of CLA intake, animal species and dose of CLA, several authors have reported controversial metabolic effects of CLA consumption including insulin resistance and hepatic lipid accumulation with a consequent development of type II diabetes [18–21].

Therefore, the aim of this study was to evaluate the effects of a three-week supplementation of CLA on obesity and other cardiometabolic conditions associated with MetS using three low doses of CLA of similar value to that administrated to humans [22] in rats fed a CAF diet. The doses assessed in this study were 100, 200, and 300 mg CLA/kg of bw, corresponding to approximately 0.036%, 0.072%, and 0.11% in the diet, respectively. In addition, this experimental model mimics the classical model of human MetS characterised by exaggerated obesity accompanied by glucose intolerance and inflammation. Furthermore, a potential adverse effects in the liver of these animals was also evaluated by metabolomic, histological and biochemical approaches.

2. Materials and Methods

2.1. The Conjugated Linoleic Acid

CLA (Tonalin® TG 80) was purchased by BASF The Chemical Company (Düsseldorf, Germany) and is a mix of glycerides of which 80% are conjugated linoleic acids. According to the manufacturer, the product was composed of equal amounts of two CLA isomers, c9, t11 and t10, c12.

2.2. Experimental Procedure

Thirty male Wistar rats of 5 weeks old where purchased from Charles River Laboratories (Barcelona, Spain). The animals were maintained at 22 °C, receiving standard (STD) chow Panlab A04 (Barcelona, Spain) and tap water *ad libitum* for 10 days. Subsequently, rats were divided into two groups, STD diet-fed rats (n = 6), fed STD chow Panlab A04 and tap water, or CAF diet-fed rats (n = 24), fed fresh CAF diet in addition to STD diet and tap water. CAF diet consisted of biscuits with pate and cheese, bacon, semi-cured cheese, carrots, *ensaïmada* (traditional sweetened pastry) and milk with 20% sucrose (w/v) daily. As previously reported [8], the composition of the STD diet was 4% fat, 76% carbohydrates and 20% protein, whereas the CAF diet was approximately 35% fat, 51% carbohydrates and 14% protein. The different diets were administered for 12 weeks. At week 9, the STD diet-fed animals were orally administered sweetened skim condensed milk, containing 20% sucrose, as vehicle (VH) (n = 6 per group; STD). CAF diet-fed animals was divided into 4 groups (n = 6 in each group) and the animals were orally administered VH (CAF) and VH containing 100, 200 or 300 mg/kg of CLA (CLA100, CLA200 or CLA300, respectively). All doses were administered daily in a volume of 1 mL between 8:00 a.m. and 9:00 a.m. for 3 weeks. To calculate the daily consumption of CLA with respect to total food intake, both animal body weight and total food intake were determined in each animal at the beginning of CLA supplementation (week 9). The daily amount of CLA was approximately 45, 90, and 135 mg in rats weighing 450 g to obtain doses of 100, 200, and 300 mg/kg, respectively. In addition, considering that the food intake of our animals was approximately 100 g per day and that in Tonalin® TG 80 only an 80% is CLA, the percentage of CLA in diet in our study was approximately 0.036, 0.072 and 0.11%. Body weight was recorded weekly during the experiment. In addition, fat and lean mass contents were recorded by nuclear magnetic resonance (NMR) using an EchoMRI-700 (Echo Medical Systems, LLC., Texas, USA). The results are expressed as a percentage of fat or lean mass with respect to the total body weight. At the end of the experiment, rats were fasted for 3 h after the oral administration and then were sacrificed by decapitation. Serum was obtained after blood clotting and centrifugation (2000× *g*, 15 min, 4 °C) and stored at −80 °C. Fasting conditions of the animals were confirmed by evaluating the serum glucose levels with an enzymatic colorimetric assay (QCA, Barcelona, Spain) (data not shown). Livers were dissected, weighted, frozen immediately in liquid nitrogen and stored at −80 °C. The complete experimental design is schematised in Figure 1. The Animal Ethics Committee of University Rovira i Virgili approved all procedures (reference number 7959 by Generalitat de Catalunya). All of the above-mentioned experiments were carried out as authorised (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food, Madrid, Spain).

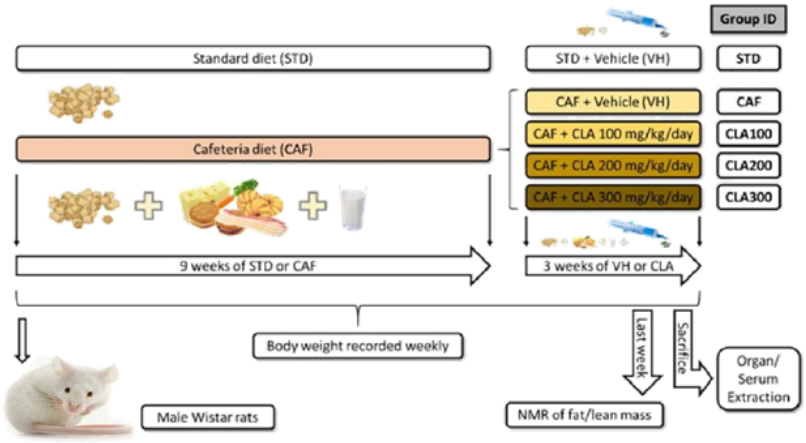


Figure 1. A scheme of the distribution of animals in the study. During the first nine weeks, one group was fed the standard chow diet (STD group), whereas the other group was fed the cafeteria diet (CAF group). After nine weeks, the animals were orally administered either vehicle (VH) or conjugated linoleic acid (CLA) at three doses (100, 200 and 300 mg/kg). On week twelve, the animals were sacrificed. CAF: cafeteria diet; CLA: conjugated linoleic acid; STD: standard chow diet; VH: vehicle.

2.3. Biochemical and Histopathological Analyses

Serum leptin (Ref. #EZRL-83K) and insulin (Ref. #EZRMI-13K) were measured using ELISA kits (Millipore, Madrid, Spain) according to the manufacturer's instructions. The sensitivity of the assays for leptin and insulin were 0.04 and 0.2 ng/mL, respectively. The intra-assay variations were 2.5% for leptin and 1.9% for insulin and the inter-assay variations were 3.2% for leptin and 9.2% for insulin. All samples were diluted 1:2 with assay buffer (0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide and 1% BSA) and tested in duplicates. The enzyme-substrate reaction was developed using 3,3′,5,5′-tetramethylbenzidine and the optical densities were measured at 450 nm in the microtiter plate reader EON Microplate (BioTek, VT, USA). The concentrations were calculated from a standard curve obtained from eight dilutions of lyophilized native rat leptin (range 0.2–30 ng/mL) or insulin (range 0.2–10 ng/mL). GOT (glutamic oxaloacetic transaminase) and GPT (glutamate pyruvate transaminase) enzymatic activity were measured using QCA kits (Comercial Bellés, Tarragona, Spain). A piece of liver was sent for histopathology (Eldine® facilities, Tarragona, Spain). Briefly, liver pieces were thawed and fixed in 4% formaldehyde for 24 h to later undergo several dehydration steps (with ethanol at 70%, 96% and 100%, in addition to xylol/dimethyl benzene) and paraffin infiltration at 52 °C (Citaldel 2000, Thermo Scientific, Madrid, Spain). Sections of 2 μm thickness were cut (Microm HM 355S, Thermo Scientific, Madrid, Spain) and stained with haematoxylin-eosin (Varistain Gemini, Shandon, Madrid, Spain). Stained slides were analysed by a pathologist blinded to experimental groups to measure the steatosis degree, percentage of micro- and macro-steatosis, microgranulomes, lipogranulomes, portal chronic inflammation, sinusoidal dilatation and fibrosis.

2.4. Western Blot Analysis

Liver (60 mg) was mixed with 1 mL of RIPA (radio-immunoprecipitation assay) lysis buffer (100 mM Tri-HCl, 300 mM NaCl, 7% Tween. 10% Na-Deox, at pH 7.4) and proteases inhibitors as recommended by the TissueLyser LT protocol of Qiagen (S.G Servicios Hospitalarios, Barcelona, Spain). Samples were homogenised with the TissueLyser in 3 pulses of 15 s and 50 oscillations/s. After that, they were centrifuged at 12,000× *g* for 20 min at 4 °C and the supernatant collected and stored at −20 °C for later analysis. Quantification of the protein concentration was measured following the recommended protocol of the Pierce BCA (bicinchoninic acid) protein assay kit (Thermo Scientific, Cedex, France). Quantified samples were prepared for western blot analysis by mixing them with Bio-Rad's Laemmli sample buffer and heated at 99 °C for 5 min. SDS-polyacrylamide gel electrophoresis (PAGE) was prepared using the TGFX Fas Cast Acrylamide Kit (Bio-Rad, Barcelona, Spain), and 25 μg of protein was loaded and run in electrophoresis buffer (25 mM Tris-Base, 1% SDS and 192 mM glycine). Proteins were transferred into PVDF (polyvinylidene difluoride) membranes using the recommended protocol by the Trans-Blot Turbo Mini PVDF Transfer packs by Bio-Rad. Blocking was performed with 5% non-fat dried milk. After that, membranes were cut around the expected location of the interested proteins and incubated with anti-rabbit p-STAT3 primary antibody (41135, Cell signalling, Barcelona, Spain) and β-Actin (A2066-100 μL, Sigma, Madrid, Spain), both diluted at 1:1000. Next, membranes were incubated with 1:10,000 diluted anti-rabbit horseradish peroxidase secondary antibody (NA9344, GE Healthcare, Barcelona, Spain) and labelled using the chemiluminescent detection reagent ECL Select (GE Healthcare, Barcelona, Spain) and GeneSys software (B:Box series, Syngene, Barcelona, Spain). Quantification by densitometry was performed with ImageJ (W.S Rasband, Bethesda, MD, USA), and the data were normalised by β-Actin.

2.5. Metabolite Extraction Procedure for ^{1}H NMR Spectrometry

Serum extraction was performed following Bligh-Dyer procedure with slight modifications [23]. Briefly, serum (100 μL) was added to 400 μL of methanol and 100 μL of ultrapure water obtained from a Milli-Q Advantage A10 system (Madrid, Spain). After homogenisation, 200 μL of chloroform (Prolabo®, VWR, Llinars del Vallès, Spain) was added and homogenised to yield a monophasic

solution. Next, an additional 600 µL of chloroform (Prolabo®, VWR, Llinars del Vallès, Spain) and 200 µL of ultrapure water were added and the samples were centrifuged at 8500× *g* for 15 min, at 4 °C, to obtain two phases separated by protein debris. The upper aqueous phase (hydrophilic metabolites) was freeze-dried and stored at −80 °C while the remaining lipidic phase (lipophilic metabolites) was dried under nitrogen stream and stored at –80 °C until further NMR measurement. Meanwhile, liver extraction was performed following the procedure described in [24] with slight modifications. Briefly, 50 mg of liver was manually homogenised using a micropestle in 1 mL of water/acetonitrile (1/1). The homogenate was centrifuged at 15,000× *g* for 30 min at 4 °C. The aqueous upper phase was separated and lyophilised overnight and, once dried, stored at −80 °C until further analysis. The lipophilic pellet was subsequently mixed with 1 mL of a solution of chloroform/methanol (2:1) at 0 °C, allowed to rest at room temperature for 10 min and then vortexed and centrifuged for 15 min at 6000× *g* at room temperature. The lipophilic supernatant was isolated from debris, dried with nitrogen flux and stored at −80 °C.

2.6. ^{1}H NMR Spectrometry

For NMR measurements, the aqueous extracts were reconstituted in 600 µl of deuterium oxide (D_2O) phosphate buffer (PBS (phosphate-buffered saline) 0.05 mM, pH 7.4, 99.5% D_2O) containing 0.73 mM trisilylpropionic acid (Cortecnet®, Voisins-Le-Bretonneux, France). The dried lipophilic extracts were reconstituted with a solution of deuterated chloroform/deuterated methanol (2:1) containing 1.18 mM tetramethylsilane (TMS) and then vortexed. Both extracts were transferred into 5-mm O.D. NMR glass tubes for NMR measurement. ^{1}H NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz using a 5-mm PABBO (proton enhanced–Smartprobe® (Bruker®) broadband gradient probe). For aqueous extracts, one-dimensional ^{1}H pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY) pre-saturation sequence (RD–90°–t1–90°–tm–90° ACQ) to suppress the residual water peak, and the mixing time was set at 100 ms. Solvent pre-saturation with an irradiation power of 75 Hz was applied during the recycling delay (RD = 5 s) and mixing time. The 90° pulse length was calibrated for each sample and varied from 9.95 to 10.06 µs. The spectral width was 12 kHz (20 ppm), and a total of 256 transients were collected into 64 K data points for each ^{1}H spectrum. In the case of lipophilic extracts, a 90° pulse with pre-saturation sequence (zgpr) was used to supress the small residual water signal absorbed from ambient moisture by methanol. An RD of 5.0 s with acquisition time of 2.94 s were used. The 90° pulse length was calibrated for each sample and varied from 9.92 to 10.04 µs. After 4 dummy scans, a total of 128 scans were collected into 64 K data points with a spectral with of 18.6 ppm. The exponential line broadening applied before Fourier transformation was of 0.3 Hz. The frequency domain spectra were phased, baseline-corrected and referenced to the TSP or TMS signal (δ = 0 ppm) using TopSpin software (version 2.1, Bruker, Mannheim, Germany). The acquired 1H NMR were compared to references of pure compounds from the metabolic profiling AMIX spectra database (Bruker), HMDB, Chenomx NMR suite 8.4 software (Chenomx Inc., Edmonton, Canada) and databases for metabolite identification. In addition, we assigned metabolites by 1H–1H homonuclear correlation (COSY (correlation spectroscopy) and TOCSY (total correlation spectroscopy) and 1H–13C heteronuclear (HSQC) 2D NMR experiments and by correlation with pure compounds run in-house. After pre-processing, specific ^{1}H NMR regions identified in the spectra were integrated and quantified using the AMIX 3.9 software package using TSP signal of buffer as internal reference [24]. A general view and metabolite assignment of a representative serum extract NMR aqueous spectra is presented in Supplementary Figure S1.

2.7. Gene Expression Analysis

Liver was homogenised and RNA extraction was performed using TRIzol LS Reagent (Thermo Fisher, Madrid, Spain). Total extracted RNA was quantified and its purity measured in a Nanodrop 100 Spectrophotometer (Thermo Scientific, Madrid, Spain). RNA quality was assessed on denaturing agarose gels. Reverse transcription was performed to obtain cDNA using the High-Capacity

Complementary DNA Reverse Transcription Kit (Thermo Fisher, Madrid, Spain). Quantitative PCR amplification and detection were performed in the ABI prism 7900HT real-time PCR system (Applied Biosystems) using 96-well plates and SYBR PCR Premix Reagent Ex Taq™ (Takara, Barcelona, Spain) following the commercial protocol. Relative mRNA levels of *Tnf-α* (tumour necrosis factor α), *Ccl2* (C-C motif chemokine ligand 2), *Hmgcr* (3-hydroxy-3-methyl-glutaryl-CoA reductase), *Ldlr* (low-density lipoprotein receptor), *Cyp7a1* (cytochrome P450 family 7 subfamily a member 1), *Asbt* (apical sodium-dependent bile acid transporter) and *Ppia* (peptidylprolyl isomerase A) were analysed by real-time PCR using *Ppia* as the housekeeping gene. Primer specificity was verified by melting curve analysis. The forward (FW) and reverse (RV) primers used in this study were obtained from Biomers.net (Ulm, Germany) and can be found in Supplementary Table S1. The efficiency of qPCR was calculated by evaluating a 2-fold dilution series of cDNA and calculated by $E = 10^{(1/slope)}$. The results are expressed as the logarithm of the cDNA concentration vs the obtained Ct value. The relative expression was calculated by dividing the E^{Ct} of the studied gene by the E^{Ct} of *Ppia* and then dividing by the value of the control, normalising to the STD group. Each sample was performed in triplicate.

2.8. Statistical Analysis

The data are expressed as the means ± standard errors of the means (SEM). Groups were compared by Student's t-test or one-way ANOVA and Duncan's post hoc test. Outliers were determined by Grubbs' test. Statistical analyses were performed using XLSTAT 2017: Data Analysis and Statistical Solution for Microsoft Excel (Addinsoft, Paris, France (2017)). Graphics were prepared using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Body Weight Gain, Body Composition and Serum Leptin Levels

The body weight of all animals immediately before the study was 164 ± 5 g. The STD diet-fed rats gained weight progressively during the course of the experiment. However, as expected, the weight gain in the group fed the CAF diet was significantly higher than that of the STD animals (554 ± 39 g vs 432 ± 20 g, respectively). Notably, in the last week of treatment (week 12), there was a statistically significant reduction in the body weight gain in animals supplemented with 100 mg/kg of CLA compared with that in the CAF group (Figure 2A). The effect of CLA on body weight gain was only found in the animals treated with the lowest dose of CLA and not doses of 200 and 300 mg/kg. Specific tissue weights (Supplementary Table S2) and body composition analysis indicated no statistical differences among groups, although there was a trend to decrease fat mass and increase lean mass in the group treated with CLA at a dose of 100 mg/kg (Figures 2B and 2C).

A very similar pattern was also observed for the leptin levels, which exhibited a slight decrease in animals treated with the lowest dose of CLA, but the difference with respect to that in the CAF group was not statistically significant (Figure 2D). In addition, the CAF diet caused a decrease in hepatic leptin signalling compared with the STD diet measured by the quotient between p-STAT3 and serum leptin concentrations. However, no changes in this quotient were observed in the liver of CAF rats supplemented with CLA with respect to the control group (Supplementary Figure S2).

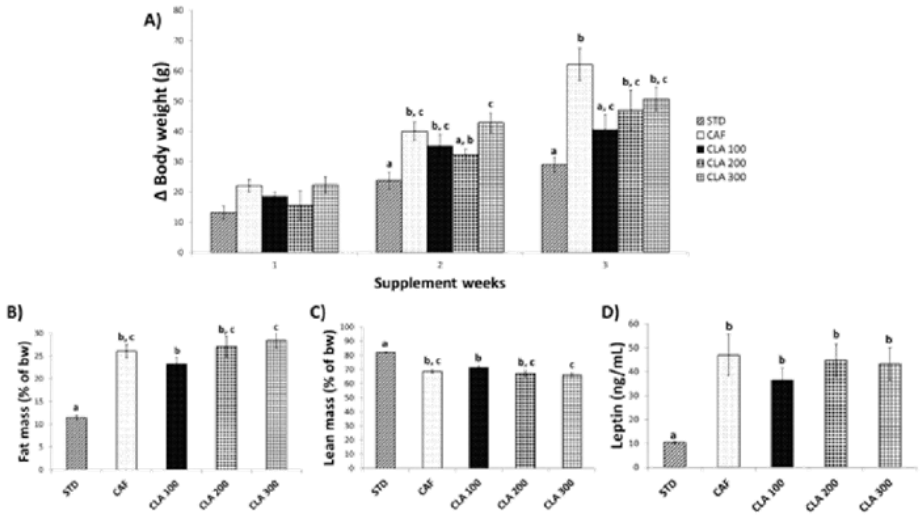


Figure 2. Metabolic parameters. The rats were fed the STD or CAF diet for 9 weeks and then were treated orally with CLA (100, 200 or 300 mg per kg bw) for 3 weeks. (**A**) Body weight gain (g) from the first, second and third week of the supplementation until the last day. (**B**) and (**C**) Body composition (%) assessed by NMR, including fat and lean content, respectively. (**D**) Serum levels of leptin. Data are expressed as the mean ± SEM. ^{a,b,c} denotes $p < 0.05$ assessed by one-way ANOVA and Duncan's post hoc test. CAF: cafeteria diet; NMR: nuclear magnetic resonance; CLA: conjugated linolenic acid; STD: standard chow diet; bw: body weight.

3.2. Glucose and Insulin Metabolism

From here onwards, we continued our study only on the dose of 100 mg/kg as the consumption of this dose, but not doses of 200 and 300 mg/kg, exerted a significant reduction in body weight gain induced by CAF diet. In this context, the CAF diet caused an increase in both glucose and insulin values compared with levels with the STD diet. Notably, the administration of 100 mg/kg of CLA normalised them to basal levels observed in the STD group (Figures 3A and 3B). In addition, a very similar pattern was also observed in some individual serum metabolites identified in aqueous extract including citrate, glycerol and threonine (Table 1). At the hepatic level, animals treated with 100 mg/kg of CLA also displayed lower concentrations of glucose, alanine, lactate and diglycerides than levels in the CAF animals (Figures 3C,D,E ,F), suggesting an improvement in the overall carbohydrate metabolism in animals treated with this dose of CLA. Hepatic individual concentrations of metabolites identified in the aqueous extract are shown in Table 2.

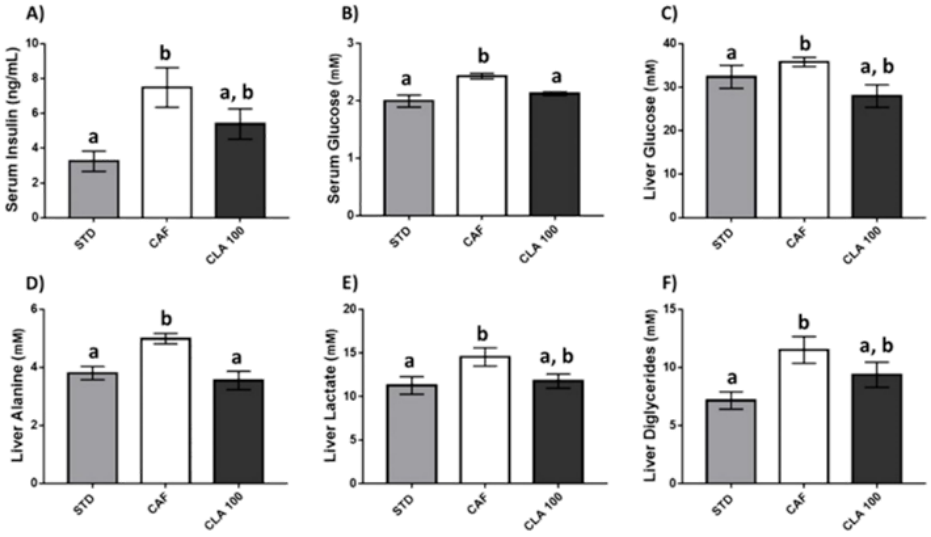


Figure 3. Glucose metabolism. The rats were fed the STD or CAF diet for 9 weeks and then were treated orally with CLA at 100 mg per kg of bw for 3 weeks. (**A**) Serum insulin and (**B**) serum glucose levels. The panels from (**C**) to (**F**) show liver metabolite levels of glucose, alanine, lactate and diglycerides, respectively. Data are expressed as the mean ± SEM. ^{a,b} denotes $p < 0.05$ assessed by one-way ANOVA and Duncan's post hoc test. CAF: cafeteria diet; CLA: conjugated linolenic acid; STD: standard chow diet.

Table 1. Serum individual concentrations (mM) of metabolites identified in aqueous extracts.

	STD	CAF	CLA 100
O-Acetylcarnitine	0.113 ± 0.01	0.119 ± 0.01	0.11 ± 0.01
Formate	0.42 ± 0.03	0.42 ± 0.01	0.46 ± 0.02
Glycerol	1.32 ± 0.1 ^{a}	1.71 ± 0.1 ^{b}	1.52 ± 0.2 ^{a,b}
Acetate	3.52 ± 0.1	3.65 ± 0.1	3.77 ± 0.1
3-Hidroxybutyrate	1.25 ± 0.2	0.87 ± 0.1	1.21 ± 0.3
Glucose	2.00 ± 0.11 ^{a}	2.43 ± 0.05 ^{b}	2.13 ± 0.03 ^{a}
Piruvate	0.44 ± 0.1	0.64 ± 0.1	0.6 ± 0.1
Succinate	0.19 ± 0.01	0.17 ± 0.01	0.2 ± 0.02
Lactate	20.13 ± 2.7	24.56 ± 3.1	24.14 ± 3.2
Citrate	0.17 ± 0.01 ^{a}	0.22 ± 0.01 ^{b}	0.18 ± 0.01 ^{a}
Asparagine	0.46 ± 0.02	0.51 ± 0.04	0.53 ± 0.02
Leucine	0.97 ± 0.02	0.94 ± 0.08	0.96 ± 0.04
Threonine	1.35 ± 0.1 ^{a}	1.67 ± 0.1 ^{b}	1.57 ± 0.1 ^{a,b}
Tryptophan	0.74 ± 0.04	0.87 ± 0.06	0.8 ± 0.05
Tyrosine	0.61 ± 0.04	0.76 ± 0.07	0.65 ± 0.03
Proline	0.89 ± 0.1	1.14 ± 0.1	1.04 ± 0.1
Isoleucine	0.68 ± 0.02	0.62 ± 0.05	0.6 ± 0.03
Glycine	1.9 ± 0.1	1.57 ± 0.1	1.64 ± 0.13
Glutamate	1.01 ± 0.05	1.11 ± 0.07	1.11 ± 0.04
Glutamine	5.52 ± 0.1	5.51 ± 0.4	5.39 ± 0.1
Methionine	0.61 ± 0.01	0.69 ± 0.04	0.61 ± 0.01
Lysine	2.3 ± 0.1	2.45 ± 0.2	2.57 ± 0.2
Valine	1.11 ± 0.1	1.17 ± 0.1	1.11 ± 0.1
Serine	2.2 ± 0.1 ^{a}	2.6 ± 0.2 ^{a,b}	2.75 ± 0.1 ^{b}

Alanine	3.06 ± 0.1	3.99 ± 0.5	3.54 ± 0.2
Phenylalanine	0.56 ± 0.01	0.61 ± 0.03	0.55 ± 0.01
Taurine	0.29 ± 0.02	0.29 ± 0.01	0.31 ± 0.01
Carnosine	0.26 ± 0.01	0.23 ± 0.03	0.22 ± 0.01
Choline	0.16 ± 0.005 [a]	0.14 ± 0.008 [b]	0.14 ± 0.004 [b]
Betaine	0.82 ± 0.07 [a]	0.54 ± 0.04 [b]	0.58 ± 0.03 [b]
Creatinine	0.09 ± 0.003	0.08 ± 0.007	0.09 ± 0.003
Glutathione	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Allantoin	0.24 ± 0.01 [a]	0.19 ± 0.001 [b]	0.2 ± 0.01 [b]
Creatine	1.95 ± 0.2	2.57 ± 0.2	2.35 ± 0.2
Creatine phosphate	0.3 ± 0.01	0.33 ± 0.03	0.27 ± 0.01
Pantothenate	0.06 ± 0.002	0.07 ± 0.01	0.05 ± 0.007

Values are presented as the mean ± SEM. [a,b] denotes $p < 0.05$ assessed by one-way ANOVA and Duncan's post hoc test. CAF: cafeteria diet; CLA: conjugated linolenic acid; STD: standard chow diet.

Table 2. Hepatic individual concentrations (mM) of metabolites identified in aqueous extracts.

	STD	CAF	CLA 100
Valine	0.48 ± 0.02	0.57 ± 0.04	0.49 ± 0.04
Isoleucine	0.3 ± 0.01 [a]	0.27 ± 0.02 [a,b]	0.22 ± 0.01 [b]
Leucine	1.17 ± 0.1	1.18 ± 0.1	1.04 ± 0.1
Glycine	0.1 ± 0.016	0.1 ± 0.007	0.09 ± 0.015
Alanine	3.8 ± 0.2 [a]	4.99 ± 0.2 [b]	3.54 ± 0.3 [a]
Glutamine	5.51 ± 0.4	4.47 ± 0.4	5.13 ± 0.3
Tyrosine	0.27 ± 0.009 [a]	0.26 ± 0.015 [a]	0.2 ± 0.014 [b]
Histidine	0.61 ± 0.03	0.57 ± 0.03	0.53 ± 0.02
Methionine	0.33 ± 0.01 [a]	0.25 ± 0.01 [b]	0.23 ± 0.01 [b]
Glutamate	2.26 ± 0.1	2.89 ± 0.2	2.41 ± 0.3
Phenylalanine	0.8 ± 0.05	0.84 ± 0.07	0.7 ± 0.04
Glucose	32.38 ± 2.6 [a]	35.79 ± 1.0 [b]	27.95 ± 2.6 [a,b]
Succinate	1.5 ± 0.1	1.23 ± 0.5	1.54 ± 0.3
Acetate	0.37 ± 0.02	0.47 ± 0.05	0.5 ± 0.13
Lactate	11.26 ± 1.0 [a]	14.54 ± 1.0 [b]	11.76 ± 0.8 [a,b]
Fumarate	0.09 ± 0.009	0.07 ± 0.008	0.1 ± 0.01
NAD^+	0.64 ± 0.04	0.54 ± 0.1	0.64 ± 0.03
$NADP^+$	0.37 ± 0.03	0.32 ± 0.03	0.34 ± 0.02
3-Hydroxybutyrate	0.47 ± 0.05	0.35 ± 0.02	0.41 ± 0.05
Uridine	0.55 ± 0.02	0.56 ± 0.09	0.43 ± 0.02
Choline	0.12 ± 0.005 [a]	0.09 ± 0.005 [a,b]	0.08 ± 0.016 [b]
Phosphocholine	1.48 ± 0.1	1.13 ± 0.2	1.05 ± 0.3
Beatine	2.32 ± 0.2 [a]	1.08 ± 0.1[b]	1.04 ± 0.2 [b]
Glutathione	4.07 ± 0.8	2.69 ± 0.4	3.15 ± 0.6
Niacinamide	0.3 ± 0.02 [a]	0.31 ± 0.01 [a]	0.22 ± 0.01 [b]
Ascorbate	1.46 ± 0.1	1.52 ± 0.22	1.6 ± 0.1
Dimethylamine	0.04 ± 0.003 [a]	0.03 ± 0.001 [a,b]	0.02 ± 0.002 [b]
Inosine	1.94 ± 0.1	1.98 ± 0.2	1.59 ± 0.1
Creatinine	0.86 ± 0.2	0.59 ± 0.1	0.44 ± 0.1
Creatine phosphate	1.86 ± 0.1	2.00 ± 0.3	1.59 ± 0.1
Creatine	0.09 ± 0.005	0.09 ± 0.01	0.08 ± 0.01

Values are presented as the mean ± SEM. [a,b] denotes $p < 0.05$ assessed by one-way ANOVA and Duncan's post hoc test. CAF: cafeteria diet; CLA: conjugated linolenic acid; STD: standard chow diet; NAD^+: nicotinamide adenine dinucleotide; $NADP^+$: nicotinamide adenine dinucleotide phosphate.

3.3. Serum Lipid Profile

As expected, the CAF diet caused an increase in total, esterified and free cholesterol serum values compared with those in the STD diet group. However, the administration of 100 mg/kg of CLA normalised cholesterol levels in these animals to that in the STD group (Figures 4A, 4B and 4C). In addition, the administration of a CAF diet increased serum triglyceride, diglyceride and phospholipid concentrations compared to those of the STD group, and the administration of 100 mg/kg of CLA also produced a significant decrease in all of these values compared with those of the CAF animals (Figures 4D,E,F). In a similar way, the concentration of other important lipid forms including linoleic acid, oleic acid, polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs) and docosahexaenoic acid (DHA) were reduced in the serum of CLA-treated animals with respect to the CAF group but did not reach to basal levels observed in the STD group (Table 3).

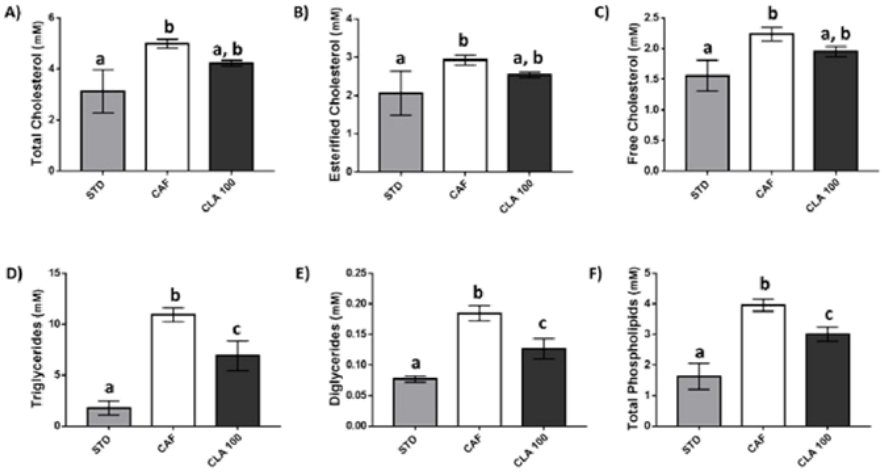


Figure 4. Serum lipid profile. The rats were fed the STD or CAF diet for 9 weeks and then were treated orally with CLA at 100 mg per kg of bw for 3 weeks. The panels from (**A**) to (**F**) show metabolite levels of different forms of cholesterol, triglycerides, diglycerides and total phospholipids, respectively. Data are expressed as the mean ± SEM. [a,b,c] denotes $p < 0.05$ assessed by one-way ANOVA and Duncan's post hoc test. CAF: cafeteria diet; CLA: conjugated linolenic acid; STD: standard chow diet.

3.4. Hepatic Fat Accumulation and Liver Function

The CAF diet caused an increase in serum GOT and GPT enzymatic activity compared with levels in the STD group, and the administration of 100 mg/kg of CLA normalised these enzymatic activities (Figures 5A,B). In addition, CAF animals presented hepatic steatosis with respect to STD rats as indicated by the histological score of steatosis (Figure 5C). However, the administration of 100 mg/kg of CLA did not normalise the steatosis degree to basal levels and did not generate significant changes in the presence of microgranulomes and sinusoidal dilatation (Table 4). In addition, any group of animals developed portal chronic inflammation, fibrosis or lipogranulomes. In contrast, the hepatic gene expression of the inflammatory marker *Ccl2* significantly decreased after the administration of 100 mg/kg of CLA (Figure 5D).

Table 3. Serum individual concentrations (mM) of metabolites identified in lipophilic extracts.

	STD	CAF	CLA 100
Total Cholesterol	3.12 ± 0.8 [a]	4.98 ± 0.1 [b]	4.12 ± 0.1 [a,b]
Free Cholesterol	1.55 ± 0.2 [a]	2.23 ± 0.1 [b]	1.94 ± 0.1 [a,b]

Esterified Cholesterol	2.05 ± 0.5 [a]	2.92 ± 0.1 [b]	2.53 ± 0.1 [a,b]
Triglycerides	1.76 ± 0.6 [a]	10.91 ± 0.6 [b]	6.89 ± 1.4 [c]
Diglycerides	0.07 ± 0.01 [a]	0.18 ± 0.01 [b]	0.12 ± 0.02 [c]
Total Phospholipids	1.62 ± 0.4 [a]	3.95 ± 0.1 [b]	3.01 ± 0.2 [c]
Linoleic acid	1.25 ± 0.4 [a]	3.42 ± 0.1 [b]	2.09 ± 0.1 [c]
Oleic acid	1.71 ± 0.6 [a]	14.33 ± 0.7 [b]	8.88 ± 2 [c]
Sphingomyelin	0.51 ± 0.03	0.48 ± 0.01	0.49 ± 0.02
ARA + EPA	1.97 ± 0.5	2.22 ± 0.2	1.79 ± 0.1
DHA	0.13 ± 0.03 [a]	0.26 ± 0.02 [b]	0.2 ± 0.01 [a,b]
Omega-3	0.6 ± 0.14 [a]	0.98 ± 0.05 [b]	0.75 ± 0.02 [b]
Phosphocholine	2.84 ± 0.7 [a]	5.83 ± 0.2 [b]	4.63 ± 0.2 [b]
PUFA	6.84 ± 1.8	9.56 ± 0.8	7.6 ± 0.2
MUFA	4.94 ± 1.5 [a]	21.24 ± 1 [b]	13.12 ± 2.4 [c]

Values are presented as the mean ± SEM. [a,b,c] denotes $p < 0.05$ assessed by one-way ANOVA and Duncan's post hoc test. CAF: cafeteria diet; CLA: conjugated linolenic acid; STD: standard chow diet; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; PUFA: polyunsaturated fatty acid; MUFA: monounsaturated fatty acid.

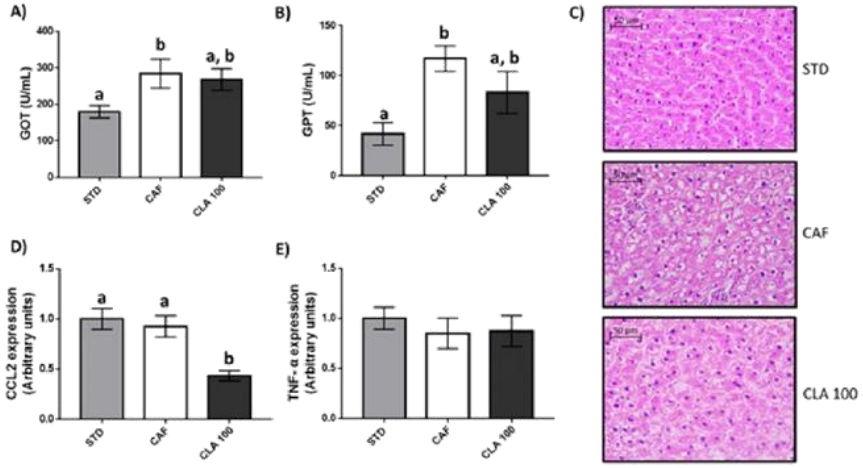


Figure 5. Liver function. The rats were fed the STD or CAF diet for 9 weeks and then were treated orally with CLA at 100 mg per kg of bw for 3 weeks. (**A**) GOT and (**B**) GPT serum enzymatic activities. (**C**) Representative histological sections of liver from from STD, CAF and CLA groups. Hepatic (**D**) *Ccl2* and (**E**) *Tnf-α* relative gene expression. Data are expressed as the mean ± SEM. [a,b] denotes $p < 0.05$ assessed by one-way ANOVA and Duncan's post hoc test. CAF: cafeteria diet; CLA: conjugated linolenic acid; STD: standard chow diet; GOT: Glutamic oxaloacetic transaminase; GPT: glutamate pyruvate transaminase.

Table 4. Summary of liver histological analysis.

	STD	CAF	CLA 100
Steatosis degree (0 to 3 in severity)	0.67 ± 0.3 [a]	1.50 ± 0.2 [b]	1.33 ± 0.2 [a,b]
Sinusoidal dilatation (0 to 2 in severity)	0.17 ± 0.2	0.33 ± 0.2	0.67 ± 0.2
Microgranulomes (number of samples)	2/6	1/6	2/6
Fibrosis degree (0 to 4 in severity)	0	0	0
Portal inflammation (0 to 2 in severity)	0	0	0
Lipogranulomes (number of samples)	0/6	0/6	0/6

Values are presented as the mean ± SEM. [a,b] denotes $p < 0.05$ assessed by one-way ANOVA and Duncan's post hoc test.

At the metabolomics level, the administration of 100 mg/kg of CLA did not significantly change the lipid profile in the liver of these animals (Table 5), although the concentrations of total and esterified cholesterol were significantly increased with respect to the STD animals (Figures 6A and 6B). No changes in free cholesterol values were observed among the three groups of animals (Figure 6C). In order to understand these cholesterol alterations, the gene expression of key enzymes involved in cholesterol metabolism was studied in the liver. Accordingly, *Asbt*, *Ldlr*, and *Cyp7a1* relative gene expression was not influenced by CLA supplementation (Figures 6D,E,F). However, animals supplemented with CLA showed a significant downregulation in liver expression of *Hmgcr* with respect to the STD group (Figure 6G).

Table 5. Summary of metabolites from the liver lipidic extraction.

	STD	CAF	CLA 100
Total Cholesterol	9.42 ± 0.7 ^{a}	16.91 ± 2.6 ^{a,b}	24.53 ± 4.6 ^{b}
Free Cholesterol	1.26 ± 0.08	1.38 ± 0.16	1.41 ± 0.13
Esterified Cholesterol	1.05 ± 0.1 ^{a}	7.83 ± 1.9 ^{a,b}	15.17 ± 4.5 ^{b}
Triglycerides	42.02 ± 3.5 ^{a}	81.03 ± 11.3 ^{b}	87.01 ± 11.1 ^{b}
Diglycerides	7.14 ± 0.7 ^{a}	11.51 ± 1.1 ^{b}	9.37 ± 1 ^{a,b}
Sphingomyelin	2.88 ± 0.1	2.76 ± 0.4	2.84 ± 0.3
ARA + EPA	28.66 ± 2.3	25.02 ± 3.5	25.61 ± 2
Plasmalogen	1.36 ± 0.1 ^{a}	1.62 ± 0.2 ^{a}	2.58 ± 0.3 ^{b}
Total Phospholipids	48.1 ± 5.5	45.42 ± 7.4	45.34 ± 5.5
Phosphoethanolamine	17.37 ± 3.1	20.32 ± 2.6	20.68 ± 1.7
Linoleic acid	32.29 ± 2.8	36.05 ± 6.1	44.94 ± 5.7
Oleic acid	16.96 ± 2.4 ^{a}	86.78 ± 14.6 ^{b}	87 ± 16.3 ^{b}
Omega-3	0.05 ± 0.002 ^{a}	0.03 ± 0.004 ^{b}	0.03 ± 0.002 ^{b}
DHA	4.19 ± 0.6	4.22 ± 0.8	4.64 ± 0.2
PUFA	120.63 ± 10.5	112.11 ± 15.6	118.57 ± 7.1
MUFA	38.89 ± 4.5 ^{a}	109.45 ± 19.6 ^{b}	120.16 ± 23.7 ^{b}
Total fatty acids	150.44 ± 9.8 ^{a}	277.54 ± 35.5 ^{b}	290.55 ± 9.8 ^{b}

Values are presented as the mean ± SEM. ^{a,b} denotes $p < 0.05$ assessed by one-way ANOVA and Duncan's post hoc test. CAF: cafeteria diet; CLA: conjugated linolenic acid; STD: standard chow diet; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; PUFA: polyunsaturated fatty acid; MUFA: monounsaturated fatty acid.

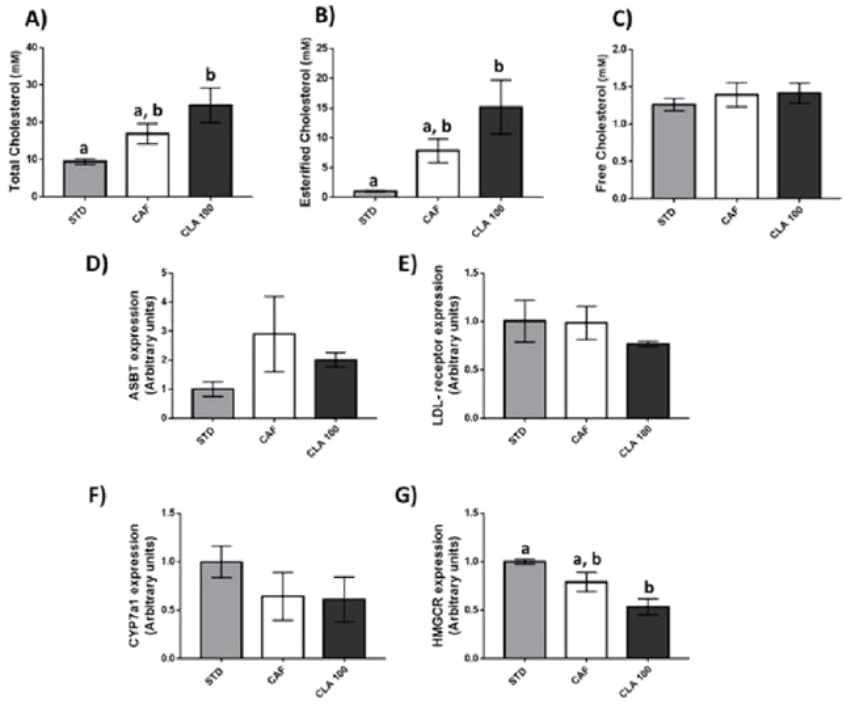


Figure 6. Hepatic cholesterol metabolism. The rats were fed the STD or CAF diet for 9 weeks and then were treated orally with CLA at 100 mg per kg of bw for 3 weeks. The panels from (**A**) to (**C**) show metabolite levels of different forms of cholesterol including total, esterified and free cholesterol, respectively. The panels from (**D**) to (**G**) illustrate the hepatic relative gene expression levels of *Asbt*, *Ldlr*, *Cyp7a1* and *Hmgcr*, respectively. Data are expressed as the mean ± SEM. ^{a,b} denotes $p < 0.05$ assessed by one-way ANOVA and Duncan's post hoc test. CAF: cafeteria diet; CLA: conjugated linolenic acid; STD: standard chow diet.

5. Discussion

MetS is a cluster of conditions including hypertension, high blood glucose, excess body fat around the waist, and abnormal blood lipid levels, which increases the risk of cardiovascular disease and type 2 diabetes. Different bioactive compounds have been widely studied as strategies for preventing the onset of MetS and its comorbidities, with CLA supplementation being one of the most investigated for weight loss [25]. However, animal studies have mainly been carried out with diets supplemented with 0.5–1.5%, which implies a daily CLA supplementation approximately 50 times times higher than those successfully used in clinical trials [10,17]. Therefore, since a greater level of inflammation, insulin resistance, and steatosis are related to the use of higher doses [26], low doses of CLA were tested in this study. Nevertheless, as the beneficial effect of body fat loss is also related to the dose of CLA used [27], the aim of this study was to test whether low doses of CLA were able to promote an ameliorating effect on obesity without inducing metabolic adverse effects. In addition, as differential effects of both CLA isomers have been reported, an equal ratio of c9,t11 and t10,c12 was used. In fact, although the isomer t10,c12 is the isomer related to the weight loss [11], the effects of this isomer on other cardiometabolic risk factors such as insulin sensitivity, markers of cardiovascular risk and liver function have raised safety concerns [28]. However, the isomer c9,t11 has not been reported to develop detrimental effects and it is generally considered safer to supplement [21], but does not have a meaningful impact on obesity [29]. This isomer has also been shown to reduce the appearance of steatosis and the concentration of inflammatory cytokines [30]. It is also known to improve mitochondrial activity in liver, modulating the release of ROS. Concretely, the c9,t11 isomer reduces ROS yield production, H_2O_2 concentration and promotes fatty acid

oxidation rate [31]. This process, however, was reported not to be enough to compensate for the increase in fatty acid accumulation, ultimately leading to hepatic steatosis. Finally, as CAF diet-fed rats present not only obesity, but also other cardiometabolic risk factors, the impact of low doses of an equal ratio of c9,t11 and t10,c12 isomers was also investigated in other abnormalities associated with MetS.

Our results showed that supplementation with low doses of CLA caused a decrease in the body weight gain in the animals fed the CAF diet. The doses assessed in this study (100, 200 and 300 mg CLA/kg), correspond to approximately 0.036, 0.072 and 0.11% in the diet, respectively, and are similar to the human recommended doses [22]. Paradoxically, the most effective dose to reduce the body weight gain at the end of the study was the lowest dose. Supporting these results, mice supplemented with moderate doses of CLA (150 and 500 mg/kg) also showed a decrease in body weight gain, which was not greater for the higher dose [32]. Interestingly, the changes in body weight gain observed after the lowest dose of CLA supplementation in our study were not accompanied by a reduction in body fat mass, nor by a reduction in lean mass, which is the major undesirable effect obtained when a caloric restriction diet is used to reduce body weight [33]. In addition, as expected, the CAF group showed increased serum leptin levels reinforcing the well-established association between leptin resistance and obesity. However, CLA supplementation failed to reduce the concentration of this hormone as well as to restore the hepatic leptin sensitivity in these animals, indicating that the anti-obesity effect of CLA is independent of the leptin system.

In this study, CAF diet-fed rats showed elevated serum glucose and insulin levels. The presence of peripheral insulin resistance and increased pancreatic insulin secretion have been reported in this animal model [34]. Nevertheless, despite insulin resistance being one of the most commonly reported detrimental effects of CLA supplementation, the results of this study not only demonstrated no adverse effects on insulin signalling but that the use of a low dose of CLA improved the overall carbohydrate metabolism in these animals. In this sense, serum and liver glucose and circulating insulin were normalised to STD levels after CLA supplementation, counteracting the effect of the CAF diet. In addition, a reduction in both alanine and lactate hepatic levels was found in animals supplemented with CLA, reaching values similar to those of the STD animals. Reduced levels of these hepatic metabolites, namely, alanine [35] and lactate [36], might indicate a lower gluconeogenesis that would result in better insulin signalling because the liver would not need to produce as much glucose. However, a direct confirmation of this hypothesis should be further tested in future studies. In addition, isoleucine and other BCAAs have also been directly linked with the development of type II diabetes [37,38], and our results showed a reduction in hepatic isoleucine in animals supplemented with a low dose of CLA. Increased hepatic diglyceride levels have also been associated with insulin resistance because diglycerides modulate the affinity of the insulin receptor, through protein kinase Ce (PKCe) [39–41], and our results showed that a low dose of CLA significantly reduced their concentrations. The effect of low-dose CLA supplementation on lipid metabolism was also examined in this study. Our results showed an extensive reduction in several lipid metabolite concentrations, suggesting an improvement in the management of fat in CLA-supplemented animals. Supporting these results, an improvement in the lipid profile caused by CLA supplementation has been previously reported in animal [42] and human studies [43]. In fact, diverse lines of research indicate that the CLA effects on lipid metabolism are mediated by the modulation of eicosanoid formation [44], and these effect seems to be more favourably influenced by the c9,t11 isomers than others in both animals [45] and humans [46].

NAFLD is considered as one of the main harmful concomitant outcomes from CLA supplementation [21]. Factors leading to hepatic lipid accumulation are multifactorial and involve increased fatty acid influx and synthesis, as well as altered fatty acid oxidation and triglyceride secretion [47–54]. In this sense, a decrease in the PUFA/MUFA ratio has been considered as a marker of liver steatosis in different metabolomics studies [24]. However, our results indicated that CLA consumption did not reduce this ratio with respect to CAF animals. In addition, the hepatic histopathological analyses performed in our animals showed no signs of liver damage aggravation in rats supplemented with CLA with respect to CAF animals, and even a lower steatosis degree was

observed in animals supplemented with CLA. Supporting these results, serum GOT and GPT enzymatic activities were also normalised in the CLA group with respect to the STD group, suggesting a slight recovery of the hepatic damage associated with the CAF diet. In addition, many CLA supplements are currently formulated to contain an equal ratio of c9,t11 and t10,c12, aiming to obtain the reduction of fat mass and obesity, but compensating with the health benefits of the c9,t11 isomer. This slight improvement of hepatic steatosis by the c9,t11 isomer, besides the low dose of t10,c12, could explain why we did not find signs of worsening liver status, as c9,t11 might be able to compensate the damage by the t10,c12. However, it is difficult to assess and compare among experiments as doses and diet play a major role in the effect of CLA.

Conversely, a significant increase in the hepatic esterified cholesterol content after CLA supplementation was observed in these animals. To uncover the potential mechanism of the hepatic cholesterol-increasing effect of CLA, we investigated the relative gene expression of *Hmgcr*, *Asbt*, *Ldlr*, and *Cyp7a1* that are the key regulators associated with cholesterol metabolism and have been reported to be regulated by dietary intervention [55]. However, our data demonstrated that this increase was not related to changes in hepatic cholesterol synthesis, cholesterol conversion into bile acids, cholesterol caption or hepatic bile acid reabsorption. Thus, further research is worthy to be conducted assess the effects of dietary supplemental CLA on hepatic cholesterol deposition and elucidating its possible mechanism.

Finally, the role of CLA as pro- or anti-inflammatory agent has no clear consensus as of yet among the scientific community. While several studies in rodents and cell culture showed CLA is an anti-inflammatory molecule, some authors do not consider that its anti-inflammatory effects have been sufficiently demonstrated in humans [56]. In addition, other studies have clearly marked CLA as a proinflammatory inductor [57,58], indicating that this effect could be greatly heterogeneous depending on physiological conditions, animal species and the dose of CLA. In addition, initial studies in cells showed that CLA, specifically t10,c12, presented ameliorating effects over the production of reactive oxygen species (ROS), correlating to lower gene expression of inflammatory markers [59]. Some studies in humans revealed consistent improvements in inflammation with a reduction in TNF-α (tumour necrosis factor α) and IL-1β (interleukin 1-β) levels [60], while others found increases in TNF-α values [57]. Thus, although there is no clear consensus of the effect of CLA on inflammation, our results indicated that low doses of CLA have the ability to decrease the gene expression of *Ccl2* in the liver as well as the levels of diglycerides, which have been reported as key markers of hepatic inflammation [61,62]. In addition, the levels of niacinamide were also significantly reduced in CLA-supplemented animals, indicating the ability of these animals to control oxidative stress and, consequently, to reduce ROS-induced damage in this tissue [63]. However, non-significant changes in *Tnf-α* mRNA levels were observed and, therefore, further studies are warranted in order to determine the potential anti-inflammatory effect of low-doses of CLA.

Finally, in this study, the most effective dose of CLA for reducing body fat was 100 mg/kg/day. This dose, using a translation of animal to human doses [64] and estimating the daily intake for an 80 kg human, correspond to an intake of 2 g/day. Although experimental results obtained in our study cannot be directly translatable to humans, the fact that only 100 mg/kg of CLA ameliorate the metabolic alterations induced by a CAF diet suggests that the utilization of CLA in the diets of obese humans could be a good strategy for improving their health outcomes.

6. Conclusions

In summary, long-term supplementation with CLA at a dose of 100 mg/kg caused a decrease in the body weight gain in animals fed a CAF diet. In addition, other important cardiometabolic conditions associated with MetS were also improved after CLA supplementation. In particular, an improvement in glucose and lipid metabolism was observed. Notably, although no major detrimental effect at this dose of CLA was found in our study, a note of caution should be sounded concerning the increased cholesterol values observed in the liver and, thus, further studies are needed to elucidate the molecular mechanisms involved in this phenomenon. In addition, these results corroborated with other studies carried out in humans in which 3 g of CLA (a slightly higher dose

than the one we have studied) was given to normal weight, overweight and obese subjects for up to six months and no adverse effects on insulin sensitivity, blood glucose and liver function were reported [22]. The use of a CLA mixture with equal ratios of both isomers used in this study could also be decisive in this study. However, as the preconditions of our experiment are different from others reported, as we use the CAF diet as an inducer of MetS, we cannot rule out that this lack of detrimental qualities of CLA might not be due to a different metabolic and physiological status. Studies with rats have been performed using high-fat diets, inducing different metabolic alterations in which CLA might have a different impact. A synergic effect coming from the low dose of CLA, with a better experimental model that mimicked human MetS, might be the reason why we are not finding signs of inflammation, insulin resistance and NAFLD but significant beneficial effects on MetS. Thus, our results suggest that long-term supplementation with low-dose CLA might have a deeper, healthy impact on obesity.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: A summary of the rat-specific primer sequences used for qPCR analysis, Table S2: Relative tissue weights, Figure S1: General view and metabolite assignment of representative serum extract NMR aqueous spectra, Figure S2: Hepatic leptin sensitivity.

Author Contributions: Conceptualisation, L.A., G.A. and B.M.; methodology, M.Z.M.-G. and H.P.; formal analysis, M.Z.M.-G. H.P. and M.A.R.; writing-original draft preparation, M.Z.M.-G.; writing-review and editing, G.A. and B.M..; funding acquisition, L.A. and B.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministerio de Economía, Industria y Competitividad (AGL2013-40707-R and AGL2016-77105-R) and by the Universitat Rovira i Virgili (2017R2B-05). G.A. is a Serra-Hunter Fellow.

Acknowledgments: We gratefully acknowledge the aid of Niurka Dariela Llópiz and Rosa Pastor, the laboratory technicians.

Conflicts of Interest: The authors declare no conflicts of interest

References

1. Bremer, A.A.; Mietus-Snyder, M.; Lustig, R.H. Toward a Unifying Hypothesis of Metabolic Syndrome. *Pediatrics* **2012**, *129*, 557–570.
2. Lent-Schochet, D.; McLaughlin, M.; Ramakrishnan, N.; Jialal, I. Exploratory metabolomics of metabolic syndrome: A status report. World J. *Diabetes* **2019**, *10*, 23–36.
3. Cheng, S.; Rhee, E.P.; Larson, M.G.; Lewis, G.D.; McCabe, E.L.; Shen, D.; Palma, M.J.; Roberts, L.D.; Dejam, A.; Souza, A.L.; et al. Metabolite profiling identifies pathways associated with metabolic risk in humans. *Circulation* **2012**, *125*, 2222–2231.
4. Newgard, C.B. Interplay between lipids and branched-chain amino acids in development of insulin resistance. *Cell Metab*. **2012**, *15*, 606–614.
5. Watanabe, M.; Suliman, M.E.; Qureshi, A.R.; Garcia-Lopez, E.; Bárány, P.; Heimbürger, O.; Stenvinkel, P.; Lindholm, B. Consequences of low plasma histidine in chronic kidney disease patients: Associations with inflammation, oxidative stress, and mortality. *Am. J. Clin. Nutr*. **2008**, *87*, 1860–1866.
6. van der Veen, J.N.; Kennelly, J.P.; Wan, S.; Vance, J.E.; Vance, D.E.; Jacobs, R.L. The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. *Biochim. Biophys. Acta - Biomembr*. **2017**, *1859*, 1558–1572.
7. Sampey, B.P.; Vanhoose, A.M.; Winfield, H.M.; Freemerman, A.J.; Muehlbauer, M.J.; Fueger, P.T.; Newgard, C.B.; Makowski, L. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: Comparison to high-fat diet. *Obesity* **2011**, *19*, 1109–1117.
8. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Acute administration of single oral dose of grape seed polyphenols restores blood pressure in a rat model of metabolic syndrome: role of nitric oxide and prostacyclin. *Eur. J. Nutr*. **2016**, *55*, 749–758.
9. Evans, M.E.; Brown, J.M.; McIntosh, M.K. Isomer-specific effects of conjugated linoleic acid (CLA) on adiposity and lipid metabolism. *J. Nutr. Biochem*. **2002**, *13*, 508–516.

10. Whigham, L.D.; Watras, A.C.; Schoeller, D.A. Efficacy of conjugated linoleic acid for reducing fat mass: a meta-analysis in humans. *Am. J. Clin. Nutr.* **2007**, *85*, 1203-1211.
11. Pariza, M.W.; Park, Y.; Cook, M.E. The biologically active isomers of conjugated linoleic acid. *Prog. Lipid Res.* **2001**, *40*, 283–298.
12. Shen, W.; Baldwin, J.; Collins, B.; Hixson, L.; Lee, K.T.; Herberg, T.; Starnes, J.; Cooney, P.; Chuang, C.C.; Hopkins, R.; et al. Low level of trans-10, cis-12 conjugated linoleic acid decreases adiposity and increases browning independent of inflammatory signaling in overweight Sv129 mice. *J. Nutr. Biochem.* **2015**, *26*, 616–625.
13. Kim, M.R.; Park, Y.; Albright, K.J.; Pariza, M.W. Differential responses of hamsters and rats fed high-fat or low-fat diets supplemented with conjugated linoleic acid. *Nutr. Res.* **2002**, *22*, 715–722.
14. Koba, K.; Akahoshi, A.; Yamasaki, M.; Tanaka, K.; Yamada, K.; Iwata, T.; Kamegai, T.; Tsutsumi, K.; Sugano, M. Dietary conjugated linolenic acid in relation to CLA differently modifies body fat mass and serum and liver lipid levels in rats. *Lipids* **2002**, *37*, 343–350.
15. Ostrowska, E.; Muralitharan, M.; Cross, R.F.; Bauman, D.E.; Dunshea, F.R. Dietary Conjugated Linoleic Acids Increase Lean Tissue and Decrease Fat Deposition in Growing Pigs. *J. Nutr.* **2018**, *129*, 2037–2042.
16. Szymczyk, B.; Pisulewski, P.M.; Szczurek, W.; Hanczakowski, P. Effects of conjugated linoleic acid on growth performance, feed conversion efficiency, and subsequent carcass quality in broiler chickens. *Br. J. Nutr.* **2001**, *85*, 465–473.
17. Benjamin, S.; Prakasan, P.; Sreedharan, S.; Wright, A.D.; Spener, F. Pros and cons of CLA consumption: An insight from clinical evidences. *Nutr Metab (Lond).* **2015**, *12*, 4.
18. Lamarche, B.; Desroches, S. Metabolic syndrome and effects of conjugated linoleic acid in obesity and lipoprotein disorders: the Québec experience. *Am. J. Clin. Nutr.* **2004**, *79*, 1149S-1152S.
19. Tsuboyama-Kasaoka, N.; Takahashi, M.; Tanemura, K.; Kim, H.J.; Tange, T.; Okuyama, H.; Kasai, M.; Ikemoto, S.; Ezaki, O. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. *Diabetes* **2000**, *49*, 1534–1542.
20. Poirier, H.; Shapiro, J.S.; Kim, R.J.; Lazar, M.A. Nutritional supplementation with trans-10, cis-12-conjugated linoleic acid induces inflammation of white adipose tissue. *Diabetes* **2006**, *55*, 1634–1641.
21. Vyas, D.; Kadegowda, A.K.G.; Erdman, R.A. Dietary conjugated linoleic acid and hepatic steatosis: Species-specific effects on liver and adipose lipid metabolism and gene expression. *J. Nutr. Metab.* **2012**, *2012*.
22. Scientific Opinion on the safety of "conjugated linoleic acid (CLA)-rich oil" (Clarinol®) as a Novel Food ingredient. *EFSA J.* **2010**, *8*, 1601.
23. Ulmer, C.Z.; Jones, C.M.; Yost R.A., Garrett, T.J.; Bowden, J.A. Optimization of Folch, Bligh-Dyer, and Matyash Sample-to-Extraction Solvent Ratios for Human Plasma-Based Lipidomics Studies. *Anal Chim Acta* **2018**, *1037*, 351–357.
24. Vinaixa M, Rodríguez MA, Rull A, Beltrán R, Bladé C, Brezmes J, Cañellas N, Joven J, Correig X. Metabolomic assessment of the effect of dietary cholesterol in the progressive development of fatty liver disease. *J Proteome Res.* **2010**, *9*, 2527–2538.
25. Egras, A.M.; Hamilton, W.R.; Lenz, T.L.; Monaghan, M.S. An evidence-based review of fat modifying supplemental weight loss products. *J. Obes.* **2011**, *2011*, 297515. doi: 10.1155/2011/297315
26. Bezan, P.N.; Holland, H.; De Castro, G.S.; Cardoso, J.F.R.; Ovidio, P.P.; Calder, P.C.; Jordao, A.A. High dose of a conjugated linoleic acid mixture increases insulin resistance in rats fed either a low fat or a high fat diet. *Exp. Clin. Endocrinol. Diabetes* **2018**, *126*, 379–386.
27. DeLany, J.P.; Blohm, F.; Truett, A.A.; Scimeca, J.A.; West, D.B. Conjugated linoleic acid rapidly reduces body fat content in mice without affecting energy intake. *Am. J. Physiol.—Regul. Integr. Comp. Physiol.* **1999**, *276*, R1172–1179.
28. Taylor, C.G.; Zahradka, P. Dietary conjugated linoleic acid and insulin sensitivity and resistance in rodent models. *Am. J. Clin. Nutr.* **2004**, *79*, 1164–1168.
29. Park, Y.; Storkson, J.M.; Albright, K.J.; Liu, W.; Pariza, M.W. Evidence that the trans-10,cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. *Lipids.* **1999**, *34*, 235–41.
30. Engl, J.; Sturm, W.; Sandhofer, A.; Kaser, S.; Tschoner, A.; Tatarczyk, T.; Weiss, H.; Tilg, H.; Patsch, J.R.; Ebenbichler, C.F. Effect of pronounced weight loss on visceral fat, liver steatosis and adiponectin isoforms. *Eur J Clin Invest.* **2008**, *38*, 238–44.

31. Mollica, M.P.; Trinchese, G.; Cavaliere, G.; De Filippo, C.; Cocca, E.; Gaita, M.; Della-Gatta, A.; Marano, A.; Mazzarella, G.; Bergamo, P. c9;t11-Conjugated linoleic acid ameliorates steatosis by modulating mitochondrial uncoupling and Nrf2 pathway. *J Lipid Res*. **2014**, *55*, 837–49.
32. Parra, P.; Serra, F.; Palou, A. Moderate doses of conjugated linoleic acid isomers mix contribute to lowering body fat content maintaining insulin sensitivity and a noninflammatory pattern in adipose tissue in mice. *J. Nutr. Biochem.* **2010**, *21*, 107–115.
33. Willoughby D, Hewlings S, Kalman D. Body composition changes in weight loss: strategies and supplementation for maintaining lean body mass: A brief review. *Nutrients*. **2018**, *10*, 1876.
34. Pinto Júnior, D.A.C.; Seraphim, P.M. Cafeteria diet intake for fourteen weeks can cause obesity and insulin resistance in wistar rats. *Rev. Nutr*. **2012**, *25*, 313–319.
35. Petersen, M.C.; Vatner, D.F.; Shulman, G.I. Regulation of hepatic glucose metabolism in health and disease. *Nat Rev Endocrinol*. **2017**, *13*, 572–587.
36. Rui, L. Energy metabolism in the liver. *Compr. Physiol*. **2014**, *4*, 177–197.
37. Chen, X.; Yang, W. Branched-chain amino acids and the association with type 2 diabetes. *J. Diabetes Investig*. **2015**, *6*, 369–370.
38. Connelly, M.A.; Wolak-Dinsmore, J.; Dullaart, R.P.F. Branched chain amino acids are associated with insulin resistance independent of leptin and adiponectin in subjects with varying degrees of glucose tolerance. *Metab. Syndr. Relat. Disord*. **2017**, *15*, 183–186.
39. Yu, C.; Chen, Y.; Cline, G.W.; Zhang, D.; Zong, H.; Wang, Y.; Bergeron, R.; Kim, J.K.; Cushman, S.W.; Cooney, G.J.; et al. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J. Biol. Chem*. **2002**, *277*, 50230–50236.
40. Arnold, R.S.; Newton, A.C. Diacylglycerol directly stimulates the insulin receptor tyrosine kinase. *FEBS Lett*. **1996**, *380*, 58–62.
41. Erion, D.M.; Shulman, G.I. Diacylglycerol-mediated insulin resistance. *Nat. Med*. **2010**, *16*, 400–402.
42. Kloss, R.; Linscheid, J.; Johnson, A.; Lawson, B.; Edwards, K.; Linder, T.; Stocker, K.; Petitte, J.; Kern, M. Effects of conjugated linoleic acid supplementation on blood lipids and adiposity of rats fed diets rich in saturated versus unsaturated fat. *Pharmacol. Res*. **2005**, *51*, 503–507.
43. Blankson, H.; Stakkestad, J.A.; Fagertun, H.; Thom, E.; Wadstein, J.; Gudmundsen, O. Conjugated Linoleic Acid Reduces Body Fat Mass in Overweight and Obese Humans. *J. Nutr*. **2018**, *130*, 2943–2948.
44. Belury, M.A. Dietary conjugated linoleic acid in health: Physiological Effects and Mechanisms of Action. *Annu. Rev. Nutr*. **2002**, *22*, 505–531.
45. Valeille, K.; Gripois, D.; Blouquit, M.-F.; Souidi, M.; Riottot, M.; Bouthegourd, J.-C.; Sérougne, C.; Martin, J.-C. Lipid atherogenic risk markers can be more favourably influenced by the cis -9, trans -11-octadecadienoate isomer than a conjugated linoleic acid mixture or fish oil in hamsters. *Br. J. Nutr*. **2004**, *91*, 191–199.
46. Tricon, S.; Burdge, G.C.; Kew, S.; Banerjee, T.; Russell, J.J.; Jones, E.L.; Grimble, R.F.; Williams, C.M.; Yaqoob, P.; Calder, P.C. Opposing effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid on blood lipids in healthy humans. *Am. J. Clin. Nutr*. **2004**, *80*, 614–620.
47. Clément, L.; Poirier, H.; Niot, I.; Bocher, V.; Guerre-Millo, M.; Krief, S.; Staels, B.; Besnard, P. Dietary trans-10,cis-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. *J. Lipid Res*. **2002**, *43*, 1400–1409.
48. Ide, T. Interaction of fish oil and conjugated linoleic acid in affecting hepatic activity of lipogenic enzymes and gene expression in liver and adipose tissue. *Diabetes* **2005**, *54*, 412–423.
49. Takahashi, Y.; Kushiro, M.; Shinohara, K.; Ide, T. Activity and mRNA levels of enzymes involved in hepatic fatty acid synthesis and oxidation in mice fed conjugated linoleic acid. *Biochim. Biophys. Acta—Mol. Cell Biol. Lipids* **2003**, *1631*, 265–273.
50. Javadi, M.; Beynen, A.C.; Hovenier, R.; Lankhorst, Æ.; Lemmens, A.G.; Terpstra, A.H.M.; Geelen, M.J.H. Prolonged feeding of mice with conjugated linoleic acid increases hepatic fatty acid synthesis relative to oxidation. *J. Nutr. Biochem*. **2004**, *15*, 680–687.
51. Park, Y.; Pariza, M.W. Mechanisms of body fat modulation by conjugated linoleic acid (CLA). *Food Res. Int*. **2007**, *40*, 311–323.
52. Yeonhwa, P.; Jayne, M.S.; Karen, J.A.; Wei, L.; Michael, W.P. Evidence that the trans-10,cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. *Lipids* **1999**, *34*, 235–241.

53. Belury, M.A.; Kempa-Steczko, A. Conjugated linoleic acid modulates hepatic lipid composition in mice. *Lipids* **1997**, *32*, 199–204.
54. Kelley, D.S.; Bartolini, G.L.; Warren, J.M.; Simon, V.A.; Mackey, B.E.; Erickson, K.L. Contrasting effects of t 10, c 12- and c 9, t 11-conjugated linoleic acid isomers on the fatty acid profiles of mouse liver lipids. *Lipids* **2004**, *39*, 135–141.
55. Chen, Z.-Y.; Ma, K.Y.; Liang, Y.; Peng, C.; Zuo, Y. Role and classification of cholesterol-lowering functional foods. *J. Funct. Foods*. **2011**, *3*, 61–69.
56. Reynolds, C.M.; Roche, H.M. Conjugated linoleic acid and inflammatory cell signalling. Prostaglandins Leukot. Essent. *Fat. Acids* **2010**, *82*, 199–204.
57. Haghighatdoost, F.; Nobakht M.; Gh, B.F. Effect of conjugated linoleic acid on blood inflammatory markers: a systematic review and meta-analysis on randomized controlled trials. *Eur. J. Clin. Nutr*. **2018**, *72*, 1071–1082.
58. Derakhshandeh-Rishehri, S.-M.; Rahbar, A.R.; Ostovar, A. Effects of Conjugated Linoleic Acid Intake in the Form of Dietary Supplement or Enriched Food on C-Reactive Protein and Lipoprotein (a) Levels in Humans: A Literature Review and Meta-Analysis. *Iran. J. Med. Sci*. **2019**, *44*, 359–373.
59. Dipasquale, D.; Basiricò, L.; Morera, P.; Primi, R.; Tröscher, A.; Bernabucci, U. Anti-inflammatory effects of conjugated linoleic acid isomers and essential fatty acids in bovine mammary epithelial cells. *Animal* **2018**, *12*, 2108–2114.
60. Song, H.J.; Grant, I.; Rotondo, D.; Mohede, I.; Sattar, N.; Heys, S.D.; Wahle, K.W.J. Effect of CLA supplementation on immune function in young healthy volunteers. *Eur. J. Clin. Nutr*. **2005**, *59*, 508–517.
61. Pingitore, P.; Sasidharan, K.; Ekstrand, M.; Prill, S.; Lindén, D.; Romeo, S. Human Multilineage 3D Spheroids as a Model of Liver Steatosis and Fibrosis. *Int. J. Mol. Sci*. **2019**, *20*, 1629.
62. Ipsen, D.H.; Lykkesfeldt, J.; Tveden-Nyborg, P. Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. *Cell. Mol. Life Sci*. **2018**, *75*, 3313–3327.
63. Tilak, J.C.; Adhikari, S.; Devasagayam, T.P.A. Antioxidant properties of Plumbago zeylanica, an Indian medicinal plant and its active ingredient, plumbagin. *Redox Rep*. **2004**, *9*, 219–227.
64. Reagan-Shaw, S.; Nihal, M.; Ahmad N. Dose translation from animal to human studies revisited. *FASEB J* **2008**, *22*, 659–661.

Supplemental data for:

Beneficial Effects of a Low-dose of Conjugated Linoleic Acid on Body Weight Gain and Other Cardiometabolic Risk Factors in Cafeteria Diet-fed Rats

Miguel Martín-González [1,#], Héctor Palacios [1,2,#], Miguel A Rodríguez [2], Lluís Arola [1,3], Gerard Aragonès [1,*] and Begoña Muguerza [1]

[1] Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, Tarragona, Spain.

[2] Eurecat, Centre Tecnològic de Catalunya, Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT, Unique Scientific and Technical Infrastructures (ICTS), Reus, Spain.

[3] Eurecat, Centre Tecnològic de Catalunya, Biotechnological Area, Reus, Spain.

# Both authors contributed equally to this manuscript

* Correspondence: gerard.aragones@urv.cat; Tel.: +34-977-558-188

Table S1. Primer sequences used in qPCR amplification.

Gene	Sequence	Product size (bp)	GenBank accession nº
Ccl2	Forward 5′-GCTGCTACTCATTCACTGGC Reverse 5′-GGTGCTGAAGTCCTTAGGGT	241	NM_031530.1
Tnf-α	Forward 5′- ACCACGCTCTTCTGTCTACTG Reverse 5′ - CTTGGTGGTTTGCTACGAC	169	NM_012675.3
Ppia	Forward 5′ - CTTCGAGCTGTTTGCAGACAA Reverse 5′- AAGTCACCACCCTGGCACAT	138	NM_017101.1
Hmgcr	Forward 5′ - CCTGGTTCTTGTTCACGCTC Reverse 5′ - GCTCGATGTCCATGCTGATC	210	NM_013134.2
Ldlr	Forward 5′ - AGTGCGATGGCCCTAACAAA Reverse 5′ - CTCGTTGGTCTTGCACTCCT	128	NM_175762.2
Cyp7a1	Forward 5′ - GTTGATTCCGTACCTGGGCT Reverse 5′ - TGCTTCTGTGTCCAAATGCC	230	NM_012942.2
Asbt	Forward 5′ - TGGGCTTCCTCTGTCAGTTT Reverse 5′ - GCAAAGGGGCATCATTCCAA	224	NM_017222.2

Ccl2, C-C motif chemokine ligand 2; *Tnf-α*, tumour necrosis factor α; *Ppia*, peptidylprolyl isomerase A; *Hmgcr* reductase, 3-hidroxi-3-metil-glutaril-CoA reductase; *Ldlr*, Low-Density Lipoprotein receptor; *Cyp7a1*, Cytochrome P450 Family 7 Subfamily A Member 1; *Asbt*, Apical Sodium Dependent Bile Acid Transporter.

Table S2. Relative tissue weights.

	STD	CAF	CLA 100	CLA 200	CLA 300
Liver (%)	2.92 ± 0.1	2.98 ± 0.1	2.94 ± 0.1	3.00 ± 0.1	2.80 ± 0.1
Muscle (%)	0.59 ± 0.01^{a}	0.45 ± 0.03^{b}	0.49 ± 0.02^{b}	0.48 ± 0.02^{b}	0.44 ± 0.01^{b}
eWAT (%)	2.03 ± 0.2^{a}	3.61 ± 0.3^{b}	3.73 ± 0.4$^{b, c}$	3.68 ± 0.3$^{b, c}$	4.57 ± 0.1^{c}
iWAT (%)	0.23 ± 0.03^{a}	1.03 ± 0.2^{b}	0.68 ± 0.2$^{a, b}$	1.22 ± 0.4^{b}	1.34 ± 0.3^{b}
rWAT (%)	0.83 ± 0.04^{a}	1.72 ± 0.2^{b}	1.77 ± 0.2^{b}	1.75 ± 0.2^{b}	2.02 ± 0.2^{b}
BAT (%)	0.12 ± 0.01^{a}	0.22 ± 0.02^{c}	0.20 ± 0.01$^{b, c}$	0.18 ± 0.01^{b}	0.22 ± 0.01^{c}

BAT, brown adipose tissue; Bw, body weight; eWAT, iWAT and rWAT, epididymal, inguinal and retroperitoneal white adipose tissue, respectively. Values are presented as the mean ± SEM of six animals per group. Different letters represent significant differences between groups (one-way ANOVA and Duncan's post hoc test; P<0·05).

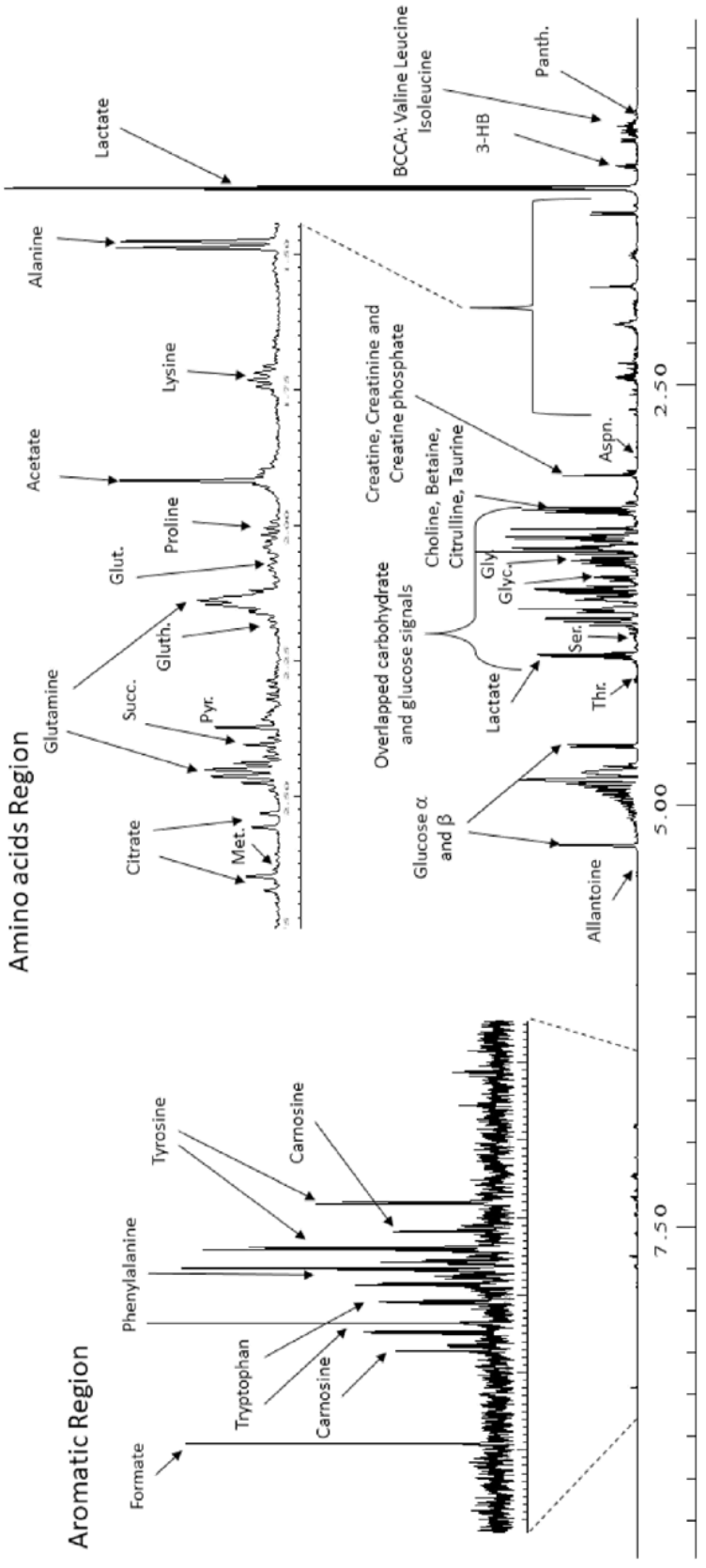


Figure S1. General view and metabolite assignment of representative serum extract NMR aqueous spectra. BCCA: Branched Chain Aminoacids; 3-HB: 3-Hydroxybutirate; Panth: Panthotenate; Glut: Glutamate; Gluth: Glutathione; Aspn: Asparagine; Pyr: Pyruvate; Succ: Succinate; Met: Methionine; Gly: Glycine; Glyc: Glycerol; Ser: Serine; Thr: Threonine;.

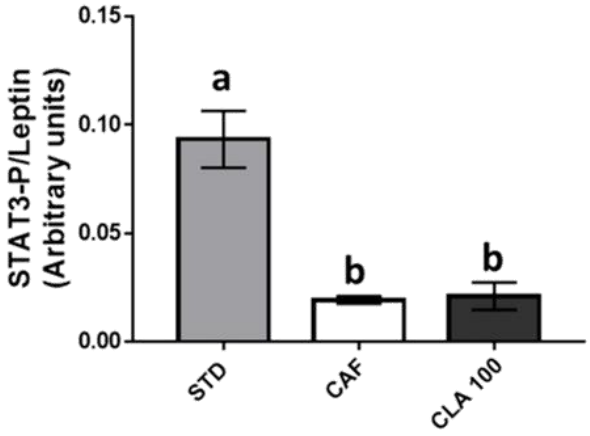


Figure S2. Hepatic leptin sensitivity. Leptin sensitivity values was obtained by the ratio between p-STAT3 (assessed by western blotting) and serum leptin levels as previously described [1]. The rats were fed the STD or CAF diet for 9 weeks and then were treated orally with CLA at 100 mg per kg of bw for 3 weeks. Data are expressed as the mean ± SEM. ^{a,b} denotes $p < 0.05$ assessed by one-way ANOVA and Duncan's post hoc test. CAF: cafeteria diet; CLA: conjugated linolenic acid; STD: standard chow diet.

[1] Ardid-Ruiz A, Ibars M, Mena P, Del Rio D, Muguerza B, Bladé C, Arola L, Aragonès G, Suárez M. Potential Involvement of Peripheral Leptin/STAT3 Signaling in the Effects of Resveratrol and Its Metabolites on Reducing Body Fat Accumulation. *Nutrients*. **2018,** *10*, E1757

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Manuscript 2

A Novel Dietary Multifunctional Ingredient Reduces Body Weight and Improves Hypothalamic Leptin Sensitivity in Cafeteria Diet-fed Rats

Miguel Z Martín-González [1], Héctor Palacios [1,2], Maria Ibars [1], Andrea Ardid-Ruiz[1], Albert Gibert-Ramos [1], Miguel A Rodríguez [2], Manuel Suárez[1], Begoña Muguerza [1,*] and Gerard Aragonès [1]

[1] Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, 43007 Tarragona, Spain
[2] Eurecat, Centre Tecnològic de Catalunya, Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT, Unique Scientific and Technical Infrastructures (ICTS), 43204 Reus, Spain.

* Correspondence: begona.muguerza@urv.cat; Tel.: +34-977-558-216

Submitted to *Nutrients*

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

nutrients

MDPI

Article

A Novel Dietary Multifunctional Ingredient Reduces Body Weight and Improves Hypothalamic Leptin Sensitivity in Cafeteria Diet-fed Rats

Miguel Z Martín-González [1], Héctor Palacios [1,2], Maria Ibars [1], Andrea Ardid-Ruiz [1], Albert Gibert-Ramos [1], Miguel A Rodríguez [2], Manuel Suárez[1], Begoña Muguerza [1,*] and Gerard Aragonès [1]

[1] Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, 43007 Tarragona, Spain
[2] Eurecat, Centre Tecnològic de Catalunya, Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT, Unique Scientific and Technical Infrastructures (ICTS), 43204 Reus, Spain.

* Correspondence: begona.muguerza@urv.cat; Tel.: +34-977-558-216

Received: date; Accepted: date; Published: date

Abstract: In recent years, thousands of dietary bioactive compounds have been further identified for their ability to prevent obesity. However, the use of a single family of bioactive compounds has not been sufficient to manage highly regulated mechanisms such as body weight and energy metabolism. Therefore, we aimed to evaluate the effect of a multifunctional ingredient (MIX) based on the co-administration of a low dose of conjugated linoleic acid (100 mg/kg) with the protein hydrolysate from chicken feet, Hpp11 (55 mg/kg), and a mixture of grape-seed proanthocyanidins (25 mg/kg) and berry anthocyanins (100 mg/kg) on body weight management. The daily oral administration of the MIX for three weeks to obese rats fed a cafeteria diet significantly reversed obesity and hyperleptinemia without modifying faecal lipid excretion and liver functionality. Notably, animals administered the MIX exhibited greater hypothalamic activation of STAT3 in POMC-expressing neurons than non-supplemented animals. In addition, this leptin signalling activation was directly associated with a significant increase in energy expenditure and fat oxidation but not with changes in energy intake. Our results strongly suggest that the co-administration of different bioactive compounds could be a promising strategy to complement the existing therapies against obesity and leptin resistance.

Keywords: anthocyanidins; bioactive peptides; CLA; multi-ingredient; obesity; proanthocyanidins

1. Introduction

Dietary obesity results from an altered energy regulatory mechanism in the arcuate nucleus of the hypothalamus, which gives rise to a chronic energy imbalance as a result of over nutrition, lack of physical activity or both [1]. This hypothalamic area receives signals from peripheral organs that provide information about energy status in the body [2]. One of these chemical signals is leptin, the key hormone in energy regulation, which is mainly produced in adipocytes, and its plasma levels are proportional to body fat stores [3]. Leptin activates a signal transduction pathway through the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in different neurons to suppress food intake and allow energy expenditure [4]. In particular, leptin activates anorexigenic proopiomelanocortin (POMC) neurons that produce the α-MSH peptide responsible for binding melanocortin receptor 4 (MC4R), subsequently producing satiety signals and promoting energy expenditure [5]. However, in diet-induced obesity, leptin is not able to maintain energy balance despite the observation of highly increased plasmatic levels of this hormone. This loss in leptin

sensitivity is known as leptin resistance, and the main molecular mechanisms involved in this phenomenon have been described in recent years [6,7].

As current pharmacological treatments to control body weight and leptin sensitivity in diet-induced obesity have limited efficacy, the scientific community supports that dietary bioactive compounds could be a promising strategy to complement existing therapies. In this sense, in recent years, thousands of these compounds, such as flavonoids, fatty acids and bioactive peptides, have been investigated for their ability to prevent obesity [8]. However, the use of a single family of bioactive compounds has not been sufficient to correct highly regulated mechanisms such as body weight and energy balance. Therefore, it is plausible to hypothesize that the co-administration of different bioactive compounds could promote body weight management in a much more effective way than the administration of individual bioactive compounds. In this context, we have developed a multifunctional ingredient (MIX) consisting of different natural dietary bioactive compounds that have individually been shown to exert certain beneficial effects against different targets associated with obesity. In particular, our MIX was based on the co-administration of a low dose of conjugated linoleic acid (CLA) with the protein hydrolysate from chicken feet, Hpp11, a mixture of proanthocyanidins extracted from grape seeds and anthocyanins from berries.

CLA is one of the most investigated dietary bioactive compounds in weight management, although controversial metabolic effects after its administration have been widely reported [9-11]. However, we have recently demonstrated that the daily administration of a low dose of CLA at 100 mg/kg (0.036% of the diet) caused a significant decrease in body weight gain in animals fed a cafeteria diet without any deleterious effect on insulin sensitivity or liver function [12]. Additionally, it has also been reported that several polyphenolic-rich extracts obtained from natural sources are able to reduce adiposity and improve leptin signalling [13]. In particular, we demonstrated that the consumption of 25 mg/kg grape seed proanthocyanidins for three weeks was able to reduce plasmatic leptin concentrations in obese rats and improve both hypothalamic and peripheral leptin signalling [14]. This capacity has also been observed in several pure phenolic compounds, most notably resveratrol [15]. Finally, chicken feet proteins have recently been demonstrated to be a great source of hydrolysates with antihypertensive properties [16]. Although there are no reported effects of these hydrolysates on body weight management, previous studies have suggested that some angiotensin-converting enzyme inhibitors could also play a role in body fat and obesity [17].

Therefore, the aim of this study was to evaluate the capacity of a novel dietary multifunctional ingredient to regulate body weight in animals fed a cafeteria diet. In addition, both plasma leptin concentrations and hypothalamic leptin signalling were evaluated to investigate the primary potential mechanism by which this dietary multi-ingredient-based supplement alleviated obesity in these animals.

2. Materials and Methods

2.1. Multifunctional ingredient composition

CLA (Tonalin® TG 80) was purchased from BASF Chemical Company (Düsseldorf, Germany) and consisted of a mix of glycerides, of which 80% were conjugated linoleic acids. According to the manufacturer, the product was composed of equal amounts of two CLA isomers, *c9,t11* and *t10,c12*. Grape-seed proanthocyanidin extract (GSPE) was kindly provided by *Les Dérives Résiniques et Terpéniques* (Dax, France), and its composition was previously characterized [18]. Anthocyanin extract (MEDOX®) was provided by MedPalett AS (Sandnes, Norway) and contained purified anthocyanins isolated from bilberries (*Vaccinium myrtillus*) and blackcurrant (*Ribes nigrum*) with a mixture of 3-*O*-rutinosides of cyanidin and delphinidin and 3-*O*-β-galactosides, 3-*O*-β-glucosides, and 3-*O*-β-arabinosides of cyanidin, peonidin, delphinidin, petunidin and malvidin. The 3-*O*-β-glucosides of cyanidin and delphinidin constituted at least 40–50% of the total anthocyanins [19]. The composition and manufacturing method of the protein hydrolysate from chicken feet, Hpp11, was previously described [16,20].

2.2. Experimental Procedure

Male Wistar rats (n=32) were purchased from Charles River Laboratories (Barcelona, Spain). The animals were housed under a 12 h light-dark cycle at 22°C and fed a standard chow diet (Panlab A04, Barcelona, Spain) *ad libitum* during the adaptation week. Then, the animals were fed a cafeteria diet for 11 weeks. The cafeteria diet was composed of 14% protein, 35% fat and 51% carbohydrates and consisted of bacon, carrots, cookies, foie gras, cupcakes, cheese and sugary milk. For the last three weeks, the animals were randomly distributed into two groups of 16 animals and orally treated with the MIX or vehicle (VH). The MIX was composed of CLA (100 mg/kg), GSPE (25 mg/kg), anthocyanins (100 mg/kg) and Hpp11 (55 mg/kg) suspended in 1 mL of glucose solution 50% (w/v). VH was composed of 400 mg/kg maltodextrin (Sigma, Madrid, Spain) diluted in the same sugary solution. Both treatments were administered daily between 8:00 a.m. and 9:00 a.m. for 3 weeks. At the end of the experiment, the rats were fasted for 3 hours after oral supplementation and then sacrificed by decapitation. Serum was obtained after blood clotting and centrifugation (2,000 x *g*, 15 minutes, 4°C) and stored at -80°C. The liver, skeletal muscle and epididymal white adipose tissue (eWAT) were excised, weighed, immediately frozen in liquid nitrogen and stored at -80°C until further analysis, while the brain was frozen whole on dry ice to preserve morphology, embedded in Shandon M-1 embedding matrix (Thermo Fisher Scientific Inc., Bilbao, Spain) and stored at -80°C.

The study was conducted in accordance with the European Directive 86/609/CEE and the Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food (Madrid, Spain) and was approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 7959 by Generalitat de Catalunya).

2.3. Body Weight and Composition Analysis

Body weight and food intake were monitored weekly until the end of the experiment. In addition, the total body composition of live animals was assessed by proton nuclear magnetic resonance (NMR) using an EchoMRI-700 system (Echo Medical Systems, Houston, TX, USA) one day before starting MIX administration and one day before sacrifice. Fat mass was directly measured in triplicate for each animal, and the results were expressed as a percentage of total body weight.

2.4. Indirect calorimetry

Indirect calorimetry analyses were performed using an Oxylet Pro System (Panlab, Barcelona, Spain). Rats were put in metabolic cages for 24 hours, prior to the administration of the treatment, starting at 9:00 a.m., with the change in light, and were housed under a 12:12 hour day:night cycle. Measurements were collected for 16 hours around the change in light, 8 hours during the day and 8 hours at night. The procedure was performed 4 days before sacrifice on eight animals per group. *Metabolism 2.1.02* software (Panlab, Barcelona, Spain) was used to measure O_2 consumption (VO_2) and CO_2 production (VCO_2) in the chambers and automatically calculated respiratory quotient (RQ) as VCO_2/VO_2 and energy expenditure using the formula: VO_2 x 1.44 x (3.815 + (1.232 x RQ) (Kcal/day/kg$^{0.75}$), according to the Weir equation [21]. A nitrogen excretion rate of 135 µg/kg/min was assumed [22].

2.5. Biochemical Analyses

Serum leptin concentrations were measured using an ELISA kit from Millipore (Madrid, Spain) according to the manufacturer's instructions (Ref. #EZRL-83K). The sensitivity of the assay was 0.04 ng/mL. The intra-assay and inter-assay variations were 2.5% and 3.2%, respectively. All samples were diluted 1:2 with assay buffer (0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide and 1% BSA) and tested in duplicate. The enzyme-substrate reaction was developed using 3,3',5,5'-tetramethylbenzidine, and the optical densities were measured at 450 nm in a EON Microplate microtiter plate reader (BioTek, Vermont, USA). The concentrations were calculated from a standard curve obtained from eight dilutions of lyophilized native rat leptin (range 0.2-30 ng/mL). Serum AST (aspartate aminotransferase) and ALT (alanine transaminase) enzymatic activities were measured using QCA kits (Comercial Bellés, Tarragona, Spain).

2.6. Histopathological Analysis

Liver pieces were thawed and fixed in 4% formaldehyde for 24 hours before several subsequent dehydration steps and paraffin infiltration at 52°C. Sections were cut at a thickness of 2 µm (Microm HM 355S, Thermo Scientific, Madrid, Spain), stained with haematoxylin-eosin and analysed by a pathologist blinded to the experimental groups to measure the degree of steatosis and fibrosis, the percentage of microsteatosis and macrosteatosis and the presence of lipogranulomas.

2.7. Faecal Lipid Analysis

Faeces were collected over 24 hours on the last day of the experiment, weighed and frozen at -80°C until lipid extraction. Prior to extraction, the samples were lyophilized for 3 days, weighed, and stored at -80°C after drying until further analysis. Then, 1 g was diluted with 5 ml of 0.9% NaCl (Sigma, Madrid, Spain) and 5 mL of chloroform/methanol (2:1). The homogenate was centrifuged at 1,000 x *g* for 10 min. The lipidic lower phase was separated and dried with nitrogen flux. Total lipids were measured by gravimetry and subsequently dissolved isopropanol. Triglyceride (Ref. #992320) and cholesterol (Ref. #995280) levels were analysed with colorimetric kits (QCA, Barcelona, Spain).

2.8. Hepatic ^{1}H-NMR Spectrometry

Liver extraction was performed according to previous reports [12] with slight modifications. Briefly, 50 mg of liver was manually homogenized using a micropestle in 1 mL of water/acetonitrile (1/1). The homogenate was centrifuged at 15,000 × *g* for 30 minutes at 4°C. The lipophilic pellet was subsequently mixed with 1 mL of a solution of chloroform/methanol (2:1) at 0°C, allowed to rest at room temperature for 10 minutes and then vortexed and centrifuged for 15 minutes at 6,000 × *g* at room temperature. The lipophilic supernatant was isolated from the debris, dried with nitrogen flux and stored at -80°C until further analysis. ^{1}H-NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz using a 5-mm PABBO (proton-enhanced Smartprobe® (Bruker®) broadband gradient probe) and were compared to pure compound references as previously described [23]. After pre-processing, specific ^{1}H-NMR regions identified in the spectra were integrated and quantified using the AMIX 3.9 software package using the TSP signal of buffer as an internal reference.

2.9. Leptin Signalling Analysis

Brain samples were cut using a Leica cryostat to obtain 10 µm-thick coronal sections. Sections were washed and prefixed in 4% paraformaldehyde solution (w/v) for 10 minutes and then unmasked sequentially for 10 min each in 0.3% glycine (w/v) and 0.3% SDS (w/v). Afterwards, the sections were washed and then blocked for 1 hour. Double staining was performed by simultaneous incubation with the following primary antibodies overnight at 4°C: mouse anti-pSTAT3 (1:100, Cell Signalling Technology Europe, B. V, Leiden, Netherlands) and guinea pig anti-ACTH (1:500, National Hormone & Peptide Program, Los Angeles, CA, USA). The sections were then washed and incubated at room temperature for 1 hour with secondary donkey anti-rabbit Alexa 594 and donkey anti-goat Alexa 488 (1:200, Thermo Fisher Scientific Inc., Bilbao, Spain). Nuclear counterstaining was performed by 4′,6-diamidino-2-phenylindole (DAPI, 1:2,000) incubation together with the secondary antibody. Sections were prepared using Vectashield (Vector Laboratories, Burlingame, CA, USA) mounting media. Images were captured with a Nikon TE2000-E microscope equipped with a Hamamatsu C8484 digital camera. Images were taken using NIS-Elements software (Nikon, Melville, NY, USA) with the same exposure time and avoiding pixel saturation. Only sections processed in the same experiments were compared. ImageJ (NIH, Bethesda, MD, USA) was used to quantify pSTAT3-positive cells. The researcher was blinded to the experimental groups of the images for the cell counting analysis. Sections were matched by anatomical landmarks according to bregma (anterior, Bregma: -2.3 mm; medial, Bregma -2,80 mm; posterior, Bregma -3.30 mm) using Paxino's and Watson's Rat Brain Atlas (Seventh Edition). At least two sections per region per mouse were quantified and then averaged. To quantify the number of pSTAT3-positive cells, only signals overlapping with DAPI were included.

In addition, leptin signaling in the liver, calf skeletal muscle and eWAT was also assessed by calculating the activation of STAT3 using an ELISA kit with a phospho-specific antibody for STAT3 phosphorylation (pSTAT3) at tyrosine 705 (Abcam, Cambridge, UK). As cellular pSTAT3 levels are mainly attributable to leptin action, leptin sensitivity in the peripheral tissues was objectively estimated as the ratio of pSTAT3 levels in each tissue to the leptin concentration in serum as previously reported [15].

2.10. Statistical Analysis

The data are expressed as the means ± standard errors of the means (SEMs). Groups were compared by Student's t-test or paired t-test. Outliers were determined by Grubbs' test. Statistical analyses were performed using XLSTAT 2017: Data Analysis and Statistical Solution for Microsoft Excel 2017 (Addinsoft, Paris, France). Graphics were prepared using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. MIX reversed the obesity induced by the cafeteria diet without compromising liver function.

As shown in Figure 1A, the body weight of all animals at the beginning of the study was 173 ± 3 g. During the course of the experiment, the animals fed the cafeteria diet continuously and consistently gained weight (4.92 ± 0.2 g/day) until they reached a final body weight of 552 ± 14 g in the last week of the experiment. Notably, there was a strongly significant reduction of 8.1% in the final body weight of animals supplemented with the MIX compared with the animals supplemented with the VH (Figure 1B). In addition, body weight gain was 11.8% lower in animals supplemented with the MIX than in those in the VH group (Figure 1C), and this reduction was directly associated with a significant decrease in total body fat mass (Figure 1D) and was not accompanied by a decrease in lean mass (Figure 1E).

Furthermore, as rapid and severe body fat mass loss could result in fat accumulation within the liver, hepatic lipid content was quantified with ^{1}H-NMR spectroscopy in all animals. As shown in Table 1, supplementation with the MIX did not significantly change the lipid profile in the liver of these animals, although the concentrations of total and esterified cholesterol were significantly increased with respect to the animals supplemented with the VH. However, no significant changes in free cholesterol and triglyceride concentrations were observed in MIX-supplemented animals with respect to VH animals. In addition, serum transaminase enzymatic activities, mainly AST and ALT, and haematoxylin-eosin staining were used to evaluate hepatic damage in these animals. Importantly, our data confirmed that supplementation with the MIX did not result in significant changes in serum transaminases and did not exacerbate the degree and type of hepatic steatosis induced by the cafeteria diet (Table 2). In addition, no significant changes in the hepatic transcripts of inflammatory genes were observed in the MIX-supplemented group compared to the VH group (Figure S1 in the Supplemental Data).

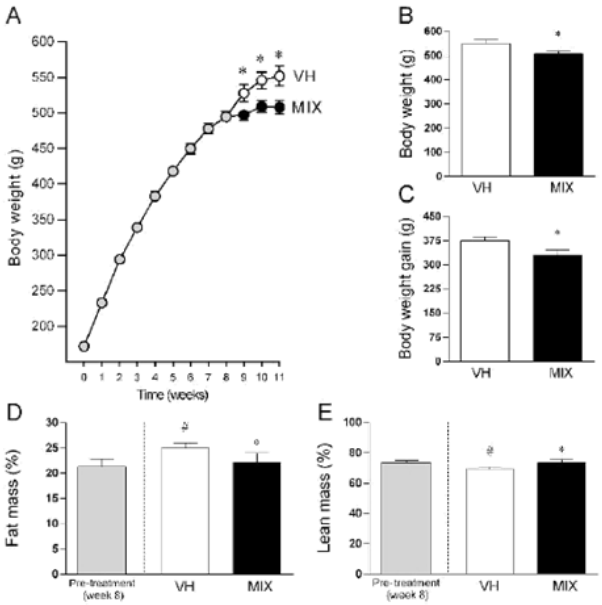


Figure 1. Body weight management. The rats were fed a cafeteria diet for 11 weeks and were orally treated with VH or MIX for the last three weeks (weeks 8 to 11). (**A**) Changes in body weight throughout the experiment. (**B**) Final body weight at the end of the experiment. (**C**) Body weight gain at the end of the experiment. (**D**) and (**E**) Body composition as assessed by ^{1}H-NMR, including fat and lean content, pre- and post-treatment with VH or MIX. Data are expressed as the mean ± SEM. * indicates $p < 0.05$ as assessed by Student's t-test comparing the VH group to the MIX group. # indicates $p < 0.05$ as assessed by paired t-test comparing pre- and post-treatment fat and lean mass. ^{1}H-NMR: proton nuclear magnetic resonance; MIX: multifunctional ingredient; VH: vehicle.

Table 1. Individual concentrations (mM) of lipid metabolites identified in liver

	VH	MIX	p value*
Total cholesterol	15.66 ± 1.5	29.05 ± 3.6	<0.01
Free cholesterol	3.8 ± 0.2	4.16 ± 0.2	*ns*
Esterified cholesterol	12.06 ± 1.5	24.79 ± 3.4	<0.01
Triglycerides	17.76 ± 2.5	21.56 ± 2.3	*ns*
Diglycerides	5.2 ± 0.3	6.08 ± 0.3	0.05
Sphingomyelin	0.93 ± 0.03	1.02 ± 0.03	0.04
ARA + EPA	11.88 ± 0.4	12.83 ± 0.5	*ns*
Plasmalogen	0.3 ± 0.01	0.4 ± 0.01	*ns*
Total phospholipids	24.73 ± 0.9	26.05 ± 0.7	*ns*
Linoleic acid	9.21 ± 0.9	11.18 ± 1.1	*ns*
Oleic acid	44.91 ± 5	55.07 ± 4.5	*ns*
Omega-3	4.39 ± 0.2	5.81 ± 0.5	0.01
PUFA	51.49 ± 1.9	53.07 ± 1.5	*ns*
MUFA	62.32 ± 6.7	77.6 ± 6.4	*ns*
Total fatty acids	140.65 ± 10.4	166.95 ± 10.7	*ns*

Values are presented as the mean ± SEM. * assessed by Student's t-test; *ns* indicates not significant.
ARA: arachidonic acid; EPA: eicosapentaenoic acid; MIX: multifunctional ingredient; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; VH: vehicle.

Table 2. Summary of biomarkers of hepatic damage

SERUM BIOMARKERS	VH	MIX	p value*
Aspartate aminotransferase (U/mL)	190.9 ± 16.1	177.8 ± 15.3	*ns*
Alanine transferase (U/mL)	70.3 ± 9.1	58.8 ± 8.1	*ns*
LIVER HISTOLOGICAL ANALYSIS	**VH**	**MIX**	**p value***
Steatosis degree (0 to 3 in severity)	1.8 ± 0.2	2.1 ± 0.2	*ns*
Microsteatosis (% of total steatosis)	82.2 ± 6	80.6 ± 6	*ns*
Microsteatosis (% of total steatosis)	11.6 ± 2	13.1 ± 2	*ns*
Fibrosis degree (0 to 4 in severity)	0	0	*ns*
Portal inflammation (0 to 2 in severity)	0	0	*ns*
Lipogranulomas (number of samples)	0/16	0/16	*ns*

Values are presented as the mean ± SEM. * assessed by Student's t-test; *ns* indicates not significant.
MIX: multifunctional ingredient; VH: vehicle.

3.2. MIX improved hyperleptinemia and energy expenditure without modifying faecal lipid excretion and food intake.

Decreased body fat mass could be a consequence of reduced intestinal lipid absorption. Thus, to analyse the mechanism underlying the reversed obesity observed in animals supplemented with the MIX, we directly measured faecal lipid excretion in these animals. As shown in Table 3, faecal cholesterol and triglyceride concentrations and total lipid content were not affected by MIX consumption, indicating that intestinal lipid absorption was not altered by the dietary incorporation of the MIX in animals fed a cafeteria diet. However, supplementation with the MIX for three weeks significantly reduced the hyperleptinemia induced by the cafeteria diet (28.6% lower), indicating that supplementation with the MIX during this period was able to normalize the leptin levels of cafeteria diet-fed rats (Figure 2). Then, as leptin is reported to maintain energy balance in mammals, we reasoned that MIX consumption could decrease food intake, enhance energy expenditure or lead to the utilization of fat as the main energy source. Thus, we directly measured the daily food intake, energy expenditure and respiratory quotient (RQ) of both groups of rats. Notably, although food intake was not affected by MIX consumption, animals supplemented with the MIX had significantly increased VO_2 values and, subsequently, energy expenditure (Table 4 and Figure S2 in the Supplemental Data). In addition, a significant increase in fat oxidation was also observed in the MIX group compared with the VH group, although no significant changes were observed in RQ values. Together, these results indicate that compared with VH supplementation, MIX consumption increased energy expenditure and favoured lipid use as an energetic substrate in cafeteria diet-fed rats, resulting in reduced energy balance and body fat mass percentage.

Table 3. Faecal lipid and water content

	VH	MIX	p value*
Total Lipids (mg/g collected faeces)	12.7 ± 2.3	10.15 ± 1.7	*ns*
Triglycerides (mg/g collected faeces)	0.37 ± 0.1	0.52 ± 0.1	*ns*
Total Cholesterol (mg/g collected faeces)	1.26 ± 0.2	1.11 ± 0.3	*ns*
Water Content (%)	32.51 ± 4.1	32.35 ± 4	*ns*

Values are presented as the mean ± SEM. * assessed by Student's T-test; *ns* indicates not significant.
MIX: multifunctional ingredient; VH: vehicle.

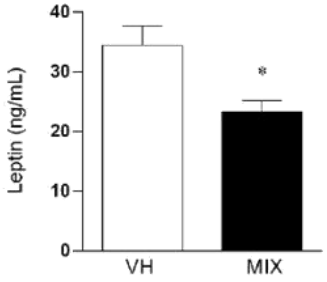


Figure 2. Serum leptin concentrations. The rats were fed a cafeteria diet for 11 weeks and were orally treated with VH or MIX for the last three weeks (weeks 8 to 11). Serum leptin values were measured using an ELISA kit from Millipore (Madrid, Spain). Data are expressed as the mean ± SEM. * denotes $p < 0.05$ assessed by Student's t-test. MIX: multifunctional ingredient; VH: vehicle.

Table 4. Summary of food intake, substrate oxidation and energy expenditure

	VH	MIX	p value*
Food intake (g/day/animal)	26.36 ± 1.76	21.11 ± 1.03	*ns*
Respiratory quotient	0.79 ± 0.02	0.76 ± 0.01	*ns*
Energy expenditure (Kcal/day/kg$^{0.75}$)	101.30 ± 3.8	111.81 ± 3.3	0.04
Carbohydrate oxidation (kJ/min/kg$^{0.75}$)	321.42 ± 20.3	276.99 ± 21.9	*ns*
Fat oxidation (kJ/min/kg$^{0.75}$)	185.91 ± 23.5	230.44 ± 17.7	0.05

Values are presented as the mean ± SEM. * assessed by Student's T-test; *ns* indicates not significant. MIX: multifunctional ingredient; VH: vehicle.

3.3. MIX enhanced hypothalamic leptin signalling in POMC-expressing cells

To address whether the decrease observed in serum leptin concentrations could indicate that supplementation with MIX affects the functionality of POMC-expressing neurons, we assessed the leptin signalling pathway through the detection of STAT3 activation in the hypothalamus using a phospho-specific antibody recognizing Tyr705-phosphorylated STAT3 (p-STAT3). Indeed, a statistically significant increase in the levels of p-STAT3 in POMC-expressing neurons was detected in the animals supplemented with the MIX (Figure 3A and Figure S3 in the Supplemental Data), indicating that the consumption of this multifunctional ingredient for three weeks was sufficient to rescue hypothalamic leptin signalling in these animals. Alternatively, to assess the contribution of the leptin-related metabolic signals derived from peripheral tissues to the regulation of energy homeostasis, we also investigated the leptin signal transduction in the liver, skeletal muscle and eWAT. Indeed, an increase in leptin sensitivity was also observed in the liver and eWAT of the animals supplemented with the MIX (Figure 3B and 3C) compared with the VH group, although no significant differences were observed in the skeletal muscle (Figure 3D).

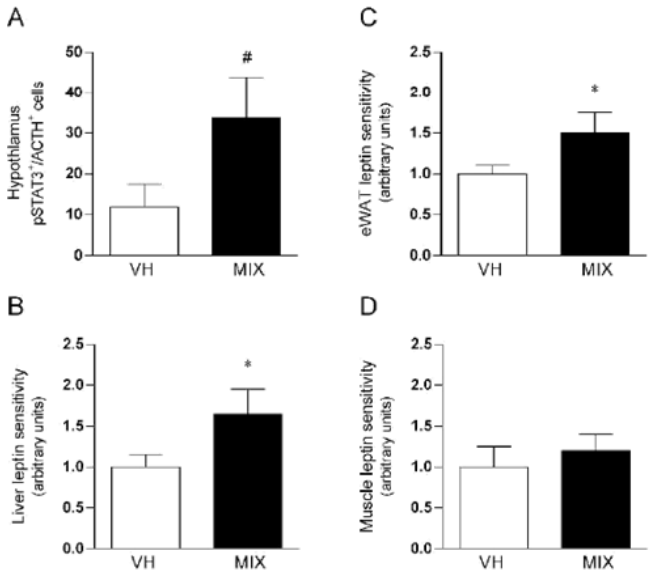


Figure 3. Leptin signalling. Rats were fed a cafeteria diet for 11 weeks and were orally treated with VH or MIX for the last three weeks (weeks 8 to 11). Leptin sensitivity in hypothalamic POMC neurons (**A**), liver (**B**), eWAT (**C**) and skeletal muscle (**D**). Data are expressed as the mean ± SEM (n=6 rats/group). * denotes $p < 0.05$ and # denotes $p<0.01$ as assessed by Student's t-test comparing the VH group to the MIX group. eWAT: epididymal white adipose tissue; MIX: multifunctional ingredient; VH: vehicle.

4. Discussion

Previous results from our group and other authors indicated that the individual consumption of different families of bioactive compounds is able to exert certain beneficial effects against different metabolic alterations induced by a cafeteria diet. However, several studies over the past few years have reported that the use of a single family of bioactive compounds at physiological doses is usually not sufficient to effectively correct highly regulated processes such as body weight and energy metabolism [24-26]. Thus, the objective of this study was to determine whether the co-administration of a novel dietary multifunctional ingredient was able to properly manage body weight in animals fed a cafeteria diet.

Our results demonstrated that the co-administration of different bioactive compounds in a single multifunctional ingredient promoted much more effective body weight management than the administration of individual bioactive compounds. In particular, the co-administration of a low dose of CLA (100 mg/kg) with the protein hydrolysate from chicken feet, Hpp11 (55 mg/kg), and a mixture of grape-seed proanthocyanidins (25 mg/kg) and berry anthocyanidins (100 mg/kg) resulted in a marked decrease in body weight and fat mass in cafeteria diet-fed rats. In particular, our results showed that the administration of MIX to cafeteria diet-fed animals for three weeks resulted in a marked reduction in both body weight and fat mass without any impact on lean body mass. This protection against lean body mass loss is of value since several studies reported that the loss of lean body mass could have multiple negative health implications [27]. In this sense, in an effort to offset this lean body mass, several studies have incorporated exercise or dietary supplement products in conjunction with a weight loss program.

The effect of MIX administration on hepatic lipid metabolism was also examined in this study since marked body weight loss could promote hepatic fat accumulation. Donnelly et al. reported a few years ago that more than 30% of hepatic triglycerides in non-alcoholic fatty liver disease are derived from the circulating free fatty acids released by lipolysis in white adipose tissue [28]. Our results showed that the administration of the MIX did not cause liver injury or hepatic triglyceride

accumulation. Supporting these results, an improvement in the hepatic lipid profile caused by the same doses of CLA and GSPE supplementation used in this study has been previously reported in cafeteria diet-fed rats [12,29]. In addition, the histopathological analyses performed in this study showed no signs of liver damage aggravation in rats supplemented with the MIX, and even lower serum AST and ALT enzymatic activities were observed in MIX-supplemented animals than in VH-supplemented animals, suggesting a slight improvement in the hepatic damage associated with the cafeteria diet. Nevertheless, a significant increase in the esterified cholesterol content after MIX supplementation was observed in these animals. Increased cholesterol concentrations in the liver have been related to oxidative stress [30]. Thus, further research is needed to assess the effects of MIX administration on esterified cholesterol deposition in the liver.

Our results also showed an increase in energy expenditure and fat utilization as energetic substrates in animals supplemented with the MIX, resulting in a reduced energy balance compared to the control animals. Remarkably, food intake and intestinal lipid absorption were not affected by the administration of the MIX. Supporting these results, different studies with CLA, anthocyanins and GSPE demonstrated the ability of these bioactive compounds to induce fat oxidation in obese animals [31-33]. However, only CLA supplementation increased energy expenditure in obese animals [33,34]. Thus, it is plausible to mainly attribute the observed differences in energy expenditure to the CLA, although the effects on fat oxidation could be caused by a synergic effect of the co-administration of CLA with the other functional ingredients.

Because of the increased energy expenditure and lipid oxidation observed in the animals supplemented with MIX, we also studied hypothalamic leptin signalling, which is reported to maintain energy balance in mammals. Our results showed that MIX administration restored serum leptin values and increased the leptin sensitivity of first-order neurons in the hypothalamus. This improvement in central leptin sensitivity could mediate the decreased body weight and fat mass and the increased energy expenditure observed in animals supplemented with the MIX.

The induction of central leptin resistance in diet-induced obesity has been mainly attributed to hypothalamic inflammation as a result of the induction of pro-inflammatory signalling molecules such as JNK and NF-κB [35,36]. Remarkably, previous results demonstrated that GSPE supplementation reduced hypothalamic inflammation [14], suggesting that this local anti-inflammatory activity of proanthocyanidins in this tissue could be one of the mechanisms by which MIX administration restored hypothalamic leptin signalling. In addition, sirtuin-1 activity has also been highlighted as a mediator of hypothalamic leptin action [37,38]; thus, the hypothalamic overexpression of sirtuin-1 induced by GSPE supplementation could be another mechanism by which MIX administration reduced hypothalamic leptin resistance. However, further studies are warranted to elucidate the molecular mechanism by which MIX administration re-establishes central leptin sensitivity in cafeteria diet-fed animals.

5. Conclusions

Our results demonstrated that the co-administration of different bioactive compounds in a single multifunctional ingredient promoted much more effective body weight management than the expected effect resulting from their individual administration in cafeteria diet-fed animals. The effect of the MIX on body weight, which could be mediated by improved hypothalamic leptin sensitivity, was directly associated with a significant increase in energy expenditure and fat oxidation but not with changes in energy intake or intestinal lipid absorption. In addition, MIX administration was not accompanied by any adverse hepatic effects associated with weight loss, although a note of caution concerning the increased hepatic values of esterified cholesterol observed in the liver is warranted. In summary, the MIX could be a good candidate nutraceutical or included in functional foods to complement existing therapies for the management of obesity.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Hepatic expression of the inflammatory genes *Ccl2*, *Tnf-α* and *iNOS*. Figure S2: Indirect calorimetry analysis, Figure S3: Representative image of hypothalamic immunostaining.

Author Contributions: Conceptualization, M.S., B.M. and G.A.; methodology, M. M-G, M. I, A.G-R. and H.P.; formal analysis, M. M-G, M.I. and M.A.R.; writing-original draft preparation, M.M-G.; writing-review and editing, B.M. and G.A.; funding acquisition, B.M. and G.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministerio de Economía, Industria y Competitividad (AGL2013-40707-R and AGL2016-77105-R) and by the Universitat Rovira i Virgili (2017R2B-05). G.A. is a Serra-Hunter Fellow.

Acknowledgements: We gratefully acknowledge the aid of Niurka Dariela Llópiz and Rosa Pastor, the laboratory technicians.

Conflicts of Interest: The authors declare no conflicts of interest

References

1. Di Angelantonio, E. et al. Body-mass index and all-cause mortality: individual-participant-data meta-analysis of 239 prospective studies in four continents. *Lancet* **2016**, *388*, 776–786.
2. Horvath, T. L. The hardship of obesity: a soft-wired hypothalamus. *Nat Neurosci.* **2005**, *8*, 561–565.
3. Friedman, J. M. Leptin and the endocrine control of energy balance. *Nature Metabolism* **2019**, *1*, 754–764.
4. Myers, M. G.; Cowley, M.; Münzberg, H. Mechanisms of leptin action and leptin resistance. *Annu. Rev. Physiol.* **2008**, *70*, 537–556.
5. Barateiro, A.; Mahú, I.; Domingos, A. I. Leptin Resistance and the Neuro-Adipose Connection. *Front. Endocrinol. (Lausanne).* **2017**, *8*, 45.
6. Myers, M. G. Leptin Keeps Working, Even in Obesity. *Cell Metab.* **2015**, *21*, 791–792.
7. Pan, H.; Guo, J.; Su, Z. Advances in understanding the interrelations between leptin resistance and obesity. *Physiol. Behav.* **2014**, *130C*, 157–169.
8. Konstantinidi, M.; Koutelidakis, A. E. Functional Foods and Bioactive Compounds: A Review of Its Possible Role on Weight Management and Obesity's Metabolic Consequences. *Medicines (Basel).* **2019**, *6*, E94.
9. Lamarche, B.; Desroches, S. Metabolic syndrome and effects of conjugated linoleic acid in obesity and lipoprotein disorders: the Québec experience. *Am. J. Clin. Nutr.* **2004**, *79*, 1149S-1152S.
10. Tsuboyama-Kasaoka, N.; Takahashi, M.; Tanemura, K.; Kim, H.J.; Tange, T.; Okuyama, H.; Kasai, M.; Ikemoto, S.; Ezaki, O. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. *Diabetes* **2000**, *49*, 1534–1542.
11. Poirier, H.; Shapiro, J.S.; Kim, R.J.; Lazar, M.A. Nutritional supplementation with trans-10, cis-12-conjugated linoleic acid induces inflammation of white adipose tissue. *Diabetes* **2006**, *55*, 1634–1641.
12. Martín-González, M. Z.; Palacios, H.; Rodríguez, M. A.; Arola, L.; Aragonès, G.; Muguerza, B. Beneficial Effects of a Low-dose of Conjugated Linoleic Acid on Body Weight Gain and Other Cardiometabolic Risk Factors in Cafeteria Diet-fed Rats. *Nutrients* **2020**, *In press*.
13. Aragonès, G; Ardid-Ruiz, A; Ibars, M; Suárez, M; Bladé, C. Modulation of leptin resistance by food compounds. *Mol. Nutr. Food. Res.* **2016**, *60*, 1789–1803.
14. Ibars, M., Ardid-Ruiz, A., Suárez, M., Muguerza, B., Bladé, C., Aragonès, G. Proanthocyanidins potentiate hypothalamic leptin/STAT3 signalling and Pomc gene expression in rats with diet-induced obesity. *Int J Obes (Lond).* **2017**, *41*, 129-136.
15. Ardid-Ruiz, A; Ibars, M; Mena, P; Del Rio, D; Muguerza, B; Bladé, C; Arola, L; Aragonès, G; Suárez, M. Potential Involvement of Peripheral Leptin/STAT3 Signaling in the Effects of Resveratrol and Its Metabolites on Reducing Body Fat Accumulation. *Nutrients*. **2018**, *10*, E1757.
16. Bravo, F. I.; Mas-Capdevila, A.; Margalef, M.; Arola-Arnal, A.; Muguerza, B. Novel Antihypertensive Peptides Derived from Chicken Foot Proteins. *Mol Nutr Food Res*. **2019,** *63*, e1801176.
17. Weisinger, R. S.; Stanley, T. K.; Begg, D. P.; Weisinger, H. S.; Spark, K.J.; Jois, M. Angiotensin converting enzyme inhibition lowers body weight and improves glucose tolerance in C57BL/6J mice maintained on a high fat diet. *Physiol Behav*. **2009**, *98*, 192-7.

18. Margalef, M.; Pons, Z.; Iglesias-Carres, L.; Bravo, F. I.; Muguerza, B.; Arola-Arnal, A. Lack of Tissue Accumulation of Grape Seed Flavanols after Daily Long-Term Administration in Healthy and Cafeteria-Diet Obese Rats. *J Agric Food Chem* **2015**, *63*, 9996–10003.
19. Qin, Y.; Xia, M.; Ma, J.; Hao, Y.; Liu, J.; Mou, H.; Cao, L.; Ling, W. Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects. *Am J Clin Nutr* **2009**, *90*, 485–492.
20. Mas-Capdevila, A.; Pons, Z.; Aleixandre, A.; Bravo, F. I.; Muguerza, B. Dose-Related Antihypertensive Properties and the Corresponding Mechanisms of a Chicken Foot Hydrolysate in Hypertensive Rats. *Nutrients* **2018**, *10*, E1295.
21. Weir, J. New methods for calculating metabolic rate with special reference to protein metabolism. *J Physiol.* **1949**, *109*, 1–9.
22. Carraro, F.; Stuart, C. A.; Hartl, W. H.; Rosenblatt, J.; Wolfe, R. R. Effect of exercise and recovery on muscle protein synthesis in human subjects. *Am J Physiol-Endocrinol Metab*. **1990**, *259*, E470–E476.
23. Vinaixa M, Rodríguez MA, Rull A, Beltrán R, Bladé C, Brezmes J, Cañellas N, Joven J, Correig X. Metabolomic assessment of the effect of dietary cholesterol in the progressive development of fatty liver disease. *J Proteome Res.* **2010**, *9*, 2527–2538.
24. Rondanelli, M.; Opizzi, A.; Perna, S.; Faliva, M.; Solerte, S.B.; Fioravanti, M.; Klersy, C.; Edda, C.; Maddalena, P.; Luciano, S.; et al. Improvement in insulin resistance and favourable changes in plasma inflammatory adipokines after weight loss associated with two months' consumption of a combination of bioactive food ingredients in overweight subjects. *Endocrine* **2013**, *44*, 391–401.
25. Liu, R.H. Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action. **J. Nutr**. *2004*, *134*, 3479S-3485S.
26. Wang, F.; Zhao, S.; Li, F.; Zhang, B.; Qu, Y.; Sun, T.; Luo, T.; Li, D. Investigation of antioxidant interactions between radix Astragali and Cimicifuga foetida and identification of synergistic antioxidant compounds. *PLoS One* **2014**, *9*.
27. Willoughby, D.; Hewlings, S.; Kalman, D. Body Composition Changes in Weight Loss: Strategies and Supplementation for Maintaining Lean Body Mass, a Brief Review. *Nutrients*. **2018**, *10*, E1876.
28. Donnelly, K.L.; Smith, C.I.; Schwarzenberg, S.J.; Jessurun, J.; Boldt, M.D.; Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest.* **2005**, *115*, 1343-51.
29. Bladé, C.; Aragonès, G.; Arola-Arnal, A.; Muguerza, B.; Bravo, FI.; Salvadó, MJ.; Arola, L.; Suárez, M. Proanthocyanidins in health and disease. *Biofactors*. **2016**, *42*, 5-12.
30. Enríquez-Cortina, C.; Bello-Monroy, O.; Rosales-Cruz, P.; Souza, V.; Miranda, R.U.; Toledo-Pérez, R.; Luna-López, A.; Simoni-Nieves, A.; Hernández-Pando, R.; Gutiérrez-Ruiz, M.C.; Calvisi, D.F.;, Marquardt J.U.; Bucio, L.; Gomez-Quiroz, L.E. Cholesterol overload in the liver aggravates oxidative stress-mediated DNA damage and accelerates hepatocarcinogenesis. *Oncotarget*. **2017**, *8*, 104136-104148.
31. Guo H, Ling W. The update of anthocyanins on obesity and type 2 diabetes: Experimental evidence and clinical perspectives. *Rev Endocr Metab Disord* **2015**, *16*, 1–13.
32. Casanova E, Baselga-Escudero L, Ribas-Latre A, Cedó L, Arola-Arnal A, Pinent M et al. Chronic intake of proanthocyanidins and docosahexaenoic acid improves skeletal muscle oxidative capacity in diet-obese rats. J Nutr Biochem **2014**, *25*, 1003–1010.
33. Terpstra AHM, Beynen AC, Everts H, Kocsis S, Katan MB, Zock PL. The decrease in body fat in mice fed conjugated linoleic acid is due to increases in energy expenditure and energy loss in the excreta. *J Nutr* **2002**, *132*, 940–945.
34. Den Hartigh LJ, Wang S, Goodspeed L, Wietecha T, Houston B, Omer M et al. Metabolically distinct weight loss by 10,12 CLA and caloric restriction highlight the importance of subcutaneous white adipose tissue for glucose homeostasis in mice. *PLoS One* **2017**, *12*, 1–25.
35. Benzler J, Ganjam GK, Pretz D, Oelkrug R, Koch CE, Legler K et al. Central inhibition of IKKβ/NF-κB signaling attenuates high-fat diet-induced obesity and glucose intolerance. *Diabetes* **2015**, 64, 2015–2027.
36. de Git KCG, Adan RAH. Leptin resistance in diet-induced obesity: the role of hypothalamic inflammation. *Obes Rev* **2015**, *16*, 207–224.
37. Sasaki T, Kikuchi O, Shimpuku M, Susanti VY, Yokota-Hashimoto H, Taguchi R et al. Hypothalamic SIRT1 prevents age-associated weight gain by improving leptin sensitivity in mice. *Diabetologia* **2014**, *57*, 819–831.

38. Sasaki T. Age-associated weight gain, leptin, and SIRT1: a possible role for hypothalamic SIRT1 in the prevention of weight gain and aging through modulation of leptin sensitivity. *Front Endocrinol* **2015**, 6, 109.

Supplemental data for:

A Novel Dietary Multifunctional Ingredient Reduces Body Weight and Improves Hypothalamic Leptin Sensitivity in Cafeteria Diet-fed Rats

Miguel Z Martín-González [1], Héctor Palacios [1,2], Maria Ibars [1], Andrea Ardid-Ruiz [1], Albert Gibert-Ramos [1], Miguel A Rodríguez [2], Manuel Suárez [1], Begoña Muguerza [1,*] and Gerard Aragonès [1]

[1] Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, Tarragona, Spain.

[2] Eurecat, Centre Tecnològic de Catalunya, Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT, Unique Scientific and Technical Infrastructures (ICTS), Reus, Spain.

[3] Eurecat, Centre Tecnològic de Catalunya, Biotechnological Area, Reus, Spain.

* Correspondence: begona.muguerza@urv.cat; Tel.: +34-977-558-216

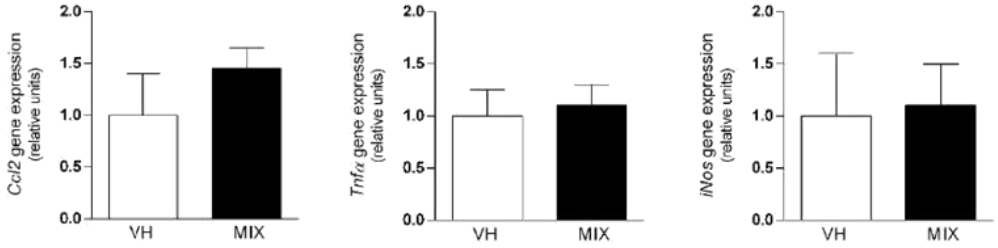


Figure S1. Hepatic expression of the inflammatory genes *Ccl2*, *Tnf-α* and iNos. Total RNA was extracted from the liver using TRIzol LS Reagent (Thermo Fisher, Madrid, Spain) and an RNeasy Mini Kit (Qiagen, Madrid, Spain) according to the manufacturers' protocols. The quantity and purity of RNA were measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Madrid, Spain). Only samples with an adequate RNA concentration (A260/A280 ≥ 1.8) and purity (A230/A260 ≥ 2.0) were selected for reverse transcription. Complementary DNA (cDNA) was generated using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher), and 10 ng was subjected to quantitative PCR (qPCR) with iTaq Universal SYBR Green Supermix (Bio-Rad, Barcelona, Spain) using a 7900HT Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The thermal profile settings were 50°C for 2 min, 95°C for 2 min, and then 40 cycles of 95°C for 15 s and 60 °C for 2 min. A cycle threshold (Ct) value was generated by setting the threshold during the geometric phase of the cDNA sample amplification. The relative expression of each gene was calculated by referring to cyclophilin peptidylprolyl isomerase A (Ppia) mRNA levels and was normalized to the STD group. The ΔΔCt method was used and corrected for primer efficiency. MIX: multifunctional ingredient; VH: vehicle.

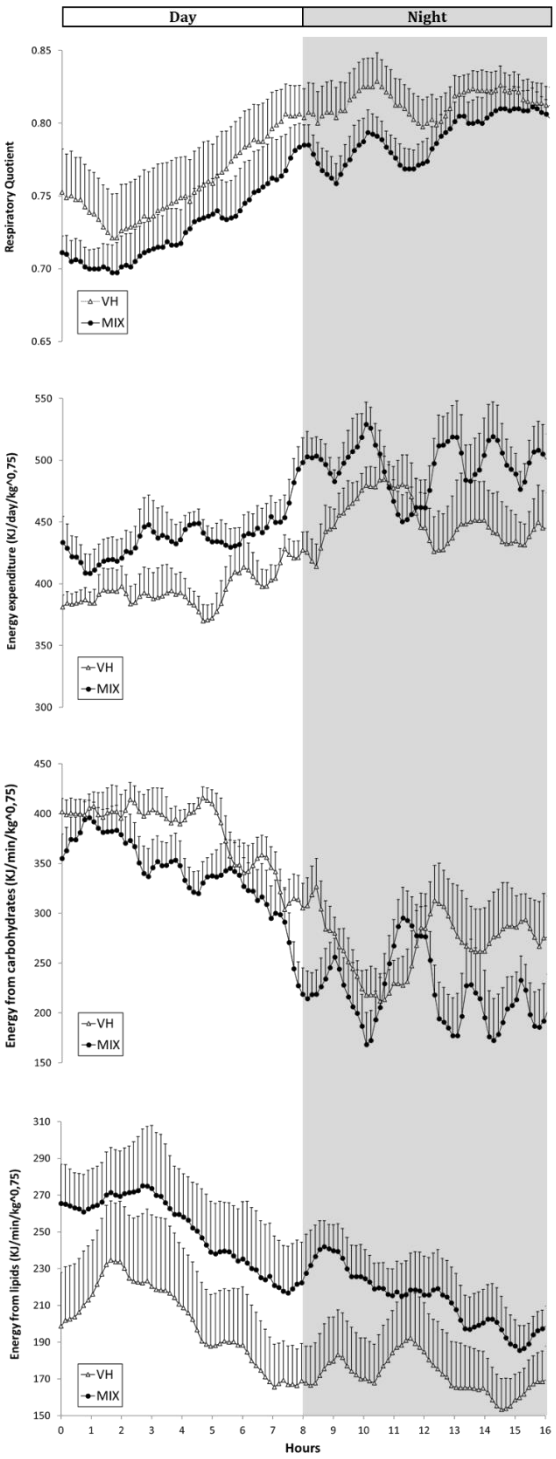


Figure S2. Indirect calorimetry analysis. Rats were put in metabolic cages for 24 hours, prior to the administration of the appropriate treatment, starting at 9:00 a.m., with the change in light, and were housed under a 12:12 hours day:night cycle. Measurements were collected for 16 hours around the change in light, for 8 hours during the light and for 8 hours at night. The procedure was performed 4 days before the last experimental day on eight animals per group. Metabolism 2.1.02 software (Panlab, Barcelona, Spain) was used to measure O2 consumption (VO2) and CO2 production (VCO2) in the chambers and automatically calculated respiratory quotient (RQ) as VCO2/VO2 and energy expenditure using the formula: VO2 x 1.44 x (3.815 + (1.232 x RQ) (Kcal/day/kg0.75). A nitrogen excretion rate of 135 μg/kg/min was assumed. Data are expressed as the mean ± SEM. MIX: multifunctional ingredient; VH: vehicle.

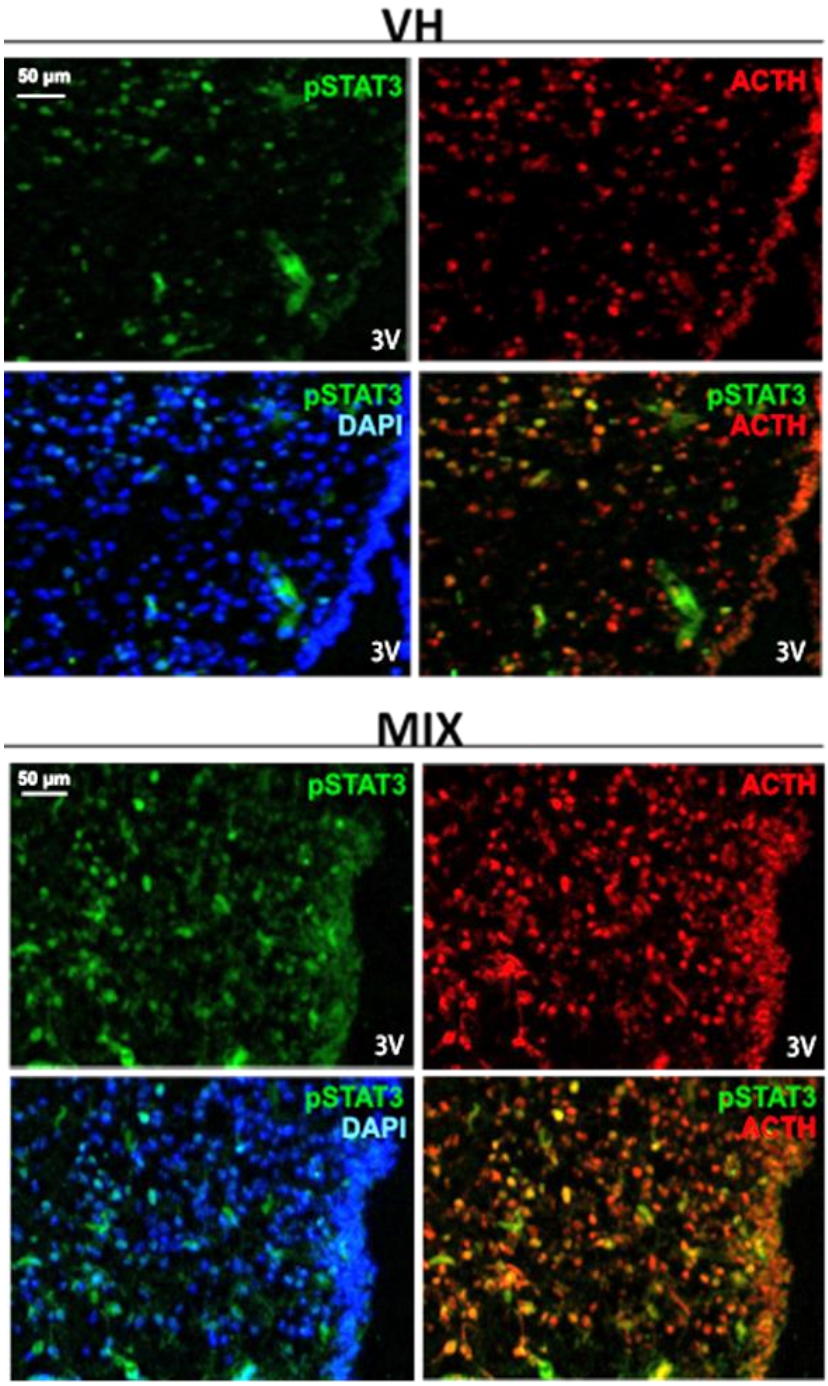


Figure S3. Representative image of hypothalamic immunostaining. (A-B) Immunostaining for pSTAT3 (green), ACTH (red) and DAPI (blue) in the arcuate (ARC) and dorsomedial hypothalamic (DMH) nucleus of cafeteria diet-fed rats supplemented with either VH or MIX for three weeks. Double staining was performed by simultaneous incubation with mouse anti-pSTAT3 and guinea pig anti-ACTH primary antibodies. Sections were then washed and incubated with secondary donkey anti-rabbit Alexa 594 and donkey anti-goat Alexa 488. Nuclear counterstaining was performed by 4',6-diamidino-2-phenylindole (DAPI) incubation. Images were taken using NIS-Elements software (Nikon, Melville, NY, USA) with the same exposure time and avoiding pixel saturation. Only sections processed in the same experiments were compared. ImageJ (NIH, Bethesda, MD, USA) was used for quantification. Experimenters were blinded to the experimental groups of the images for the cell counting analysis. Sections were matched by anatomical landmarks according to bregma (anterior, bregma: -2.3 mm; medial, bregma -2,80 mm; posterior. MIX: multifunctional ingredient; VH: vehicle.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Manuscript 3

A multifunctional ingredient for the management of metabolic syndrome in cafeteria diet-fed rats

Miguel Z Martín-González [1], Héctor Palacios [1,2], Anna Mas-Capdevila[1,2], Miguel A Rodríguez [2], Francisca I. Bravo[1], Begoña Muguerza [1]*, and Gerard Aragonès [1]

[1] Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, 43007 Tarragona, Spain
[2] Eurecat, Centre Tecnològic de Catalunya, Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT, Unique Scientific and Technical Infrastructures (ICTS), 43204 Reus, Spain.

* Correspondence: begona.muguerza@urv.cat; Tel.: +34-977-558-216

In preparation

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

nutrients

MDPI

Article

A multifunctional ingredient for the management of metabolic syndrome in cafeteria diet-fed rats

Miguel Z Martín-González [1], Héctor Palacios [1,2], Anna Mas-Capdevila [1,2], Miguel A Rodríguez [2], Francisca I Bravo [1], Begoña Muguerza [1,*] and Gerard Aragonès [1]

[1] Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Nutrigenomics Research Group, 43007 Tarragona, Spain
[2] Eurecat, Centre Tecnològic de Catalunya, Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT, Unique Scientific and Technical Infrastructures (ICTS), 43204 Reus, Spain.

* Correspondence: begona.muguerza@urv.cat; Tel.: +34-977-558-216

Received: date; Accepted: date; Published: date

Abstract: The body weight lowering properties of a multifunctional ingredient (MIX) based on CLA at low doses, the flavonoids proanthocyanidins and anthocyanidins and the chicken feet hydrolysate Hpp11 has been previously reported. The aim of this study was to evaluate the effect of long-term administration of the MIX on other cardiometabolic risk factor associated with metabolic syndrome (MetS) in rats fed a cafeteria diet (CAF). Male Wistar rats were fed CAF for 11 weeks and during the last 3 weeks, animals were orally administered MIX or vehicle. Lipid tolerance tests were performed previous and after MIX administration. At the end of the experimental period, serum and inguinal white adipose tissue (iWAT) metabolite profiles were analysed by metabolomics and biochemical approaches. The spectrum of serum and iWAT metabolites changed after 3 weeks of treatment, suggesting an improvement of lipid and glucose homeostasis in the MIX administered animals. In addition, MIX also exhibited antihypertensive properties. These results suggest that MIX could be a good candidate to ameliorate the cardiometabolic risk factors related to MetS.

Keywords: ^{1}H-NMR; blood pressure; conjugated linoleic acid; flavonoids; glucose and lipid homeostasis; chicken feet hydrolysate.

1. Introduction

Obesity is a major health risk factor that frequently occurs concurrently with many other risk cardiovascular factors related to lifestyle, such as dyslipidemia, impaired glucose tolerance, hypertension (HTN) and non-alcoholic fatty liver disease (NAFLD), resulting in metabolic syndrome (MetS) [1]. The components related to MetS are high waist circumference, hypertriglyceridemia, hyperglycemia, HTN and reduced high-density lipoprotein cholesterol (HDL-C). MetS is considered when at least three of them are present [2]. These symptoms provide an easy-to-assess method of diagnosis, but do not provide a full picture of the undergoing problem. Multiple approaches have been used to study and analyse the molecular metabolic alterations associated with the MetS. In this sense, metabolomics studies have found many links between MetS and particular metabolites that could be used to better characterize this disease [3].

Wistar rats fed a cafeteria (CAF) diet, which have free access to unhealthy human food, highly palatable, energy dense and rich in carbohydrates and fats, are considered a robust experimental model of human MetS [4]. These animals show hyperphagia and obesity and develop hyperinsulinemia, hyperglycaemia, HTN and NAFLD [4,5]. Therefore, this experimental approach is especially suitable to evaluate the effectiveness of different bioactive compounds both on obesity and other cardiovascular risk factors related to MetS. In this context, many dietary supplements such as polypehnols, fatty acids and bioactive peptides have been investigated as strategies to prevent the development and onset of obesity and MetS. These compounds have gained interest in recent years

because, given their natural food origins, they are usually considered as harmless and safer in their supplementation than classical pharmaceutical compounds [6–8]. This have prompted scientific interest as alternative medicinal approach to prevent or ameliorate some of the cardiometabolic risk factors related to MetS [9,10].

In this context, conjugated linoleic acid (CLA) is one of the most investigated dietary bioactive compounds for their body weight lowering properties [11]. CLA is a fatty acid produced by a fermentative bacteria, *Butyrivibrio fibrisolvens*, that isomerizes linoleic acid into conjugated linoleic acid, creating a double carbon bond [12]. These double bonds can take place in different positions generating a family of isomeric fatty acids, of which cis-9, trans-11 and trans-10, cis-12 are the most naturally abundant and deeply studied [13,14]. Nevertheless, some detrimental metabolic effects of its consumption have also been reported in some animal studies [15,16]. These controversial findings could be attributed to the used doses, since much higher CLA doses are employed in preclinical studies than the used in clinical trials [11,17]. In this sense, the use of an equal mixture of the isomers c9,t11 and t10,c12 at low doses, similar to those considered as safe to be used in humans [18], has been reported to cause a decrease in the body weight gain without compromising other metabolic parameters [19,20]. Alternatively, different health benefits have also been reported for bioactive peptides [21-24]. These peptides are protein fragments released from their specific precursor proteins, which present biological activities being one of the most studied antihypertensive activity [25]. This blood pressure (BP) lowering effect of some bioactive peptides is mainly mediated by their capacity to inhibit angiotensin-converting enzyme (ACE), key enzyme in the control of arterial BP. Hpp11 is a hydrolysate obtained from chicken feet protein [26], which exhibit BP lowering properties after their short [27] and long-term administration [28]. AVFQHNCQE is one of the antihypertensive peptides identify in the hydrolysate [29], which enhanced nitric oxide (NO) bioavailability and improved endothelium functionality when was administered to hypertensive animals [30].

Finally, different health beneficial effects of proanthocyanidins and anthocyanidins have been also reported [31,32]. Proanthocyanidins are abundant in many fruits and vegetables, most notably in grape seeds [33]. These phenolic compounds present a wide range of biological functions, which include BP lowering effect, protection against body weight gain, improvement lipid metabolism, increase insulin secretion, protection against type 2 diabetes, reduction of inflammation and antioxidant effects, among other effects [31]. Anthocyanidins have also been associated with cardioprotective effects, protection against oxidative damage, anti-inflammatory properties and anti-proliferative effects, among others [34]. In this sense, the grape seed extract (GSPE), rich in proanthocyanidins and the berry-derived supplement Medox, rich in anthocyanidins, has been demonstrated to improve metabolic risk factors related to MetS such as blood lipid profile, glucose homeostasis and vascular function [35–37].

However, nowadays the use of a single family of bioactive compounds seems not to be sufficient to correct complex, multisystemic and very well-regulated situations such as body weight and associated pathologies. In this sense, the co-administration of different bioactive compounds as a multifunctional ingredient (MIX), which includes CLA, the flavonoids proanthocyanidins and anthocyanidins, and the antihypertensive hydrolysate (Hpp11), has demonstrated a marked body weight lowering activity in obese animals fed CAF [38,39]. Therefore, the aim of this study was to investigate if MIX, in addition to reducing body weight, could also improve other cardiometabolic risk factors related to MetS in rats fed a CAF diet as experimental model that mimics the classical model of human MetS.

2. Materials and Methods

2.1. Multifunctional ingredient

The MIX was the combination of 100 mg/kg of conjugated linoleic acid (CLA), 55 mg/kg of the chicken feet hydrolysate Hpp11, 25 mg/kg of GSPE and 100 mg/kg of anthocyanidins obtained by the administration of 500 mg/kg of Medox® (Medox). CLA (Tonalin® TG 80) was purchased from the BASF the Chemical Company (Düsseldorf, Germany) and is a mix of glycerides of which 80% are

CLA. According to the manufacturer, the product was composed of equal amounts of two CLA isomers, cis-9 trans-11 (c9,t11) conjugated linoleic acid and trans-10 cis-12 (t10,c12) conjugated linoleic acid. The chicken-feet hydrolysate Hpp11 was produced by our group following the hydrolysis procedure patented by Bravo et al. [39]. GSPE was obtained from white grape seeds and provided by Les Dérives Résiniques et Terpéniques (Dax, France). Phenolic content of this extract was reported by Margalef et al. [40]. Medox was purchased from the Biolink Group company (Sandness, Norway). Medox contains 20 % of purified anthocyanidins isolated from berries, mainly bilberries and blackcurrant, and obtained by a patented process, designed to optimise the extraction of ortodihydroxy-structured anthocyanidins. The specific anthocyanindin content of Medox is reported by Karlsen et al. [41].

2.2. Experimental procedure in rats

The Animal Ethics Committee of University Rovira i Virgili approved all procedures (reference number 7959 by Generalitat de Catalunya). All of the, above mentioned, experiments were performed as authorized (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food, Madrid, Spain).

Thirty two male normotensive Wistar rats of five-week-old, were purchased from Charles River Laboratories (Barcelona, Spain), with an initial body weight of 172 ± 3 grams. Rats were maintained at 22 ºC under 12 hours light/12h dark conditions. Rats were fed CAF ad libitum for a total of 11 weeks. CAF diet consisted of biscuits with paté, biscuits with cheese, semi cured cheese, bacon, ensaïmada (sweetened pastry), carrots and milk with sucrose 20% (w/v). The composition of the CAF diet was 35% fat, 51% carbohydrates and 14% protein. At the 8th week of the experiment, CAF fed rats were divided into two experimental groups, animals administered daily vehicle (VH) or MIX (VH and MIX groups, respectively). The number of animals was 16 per group. MIX and VH, carried equal amounts of maltodextrin (stabilizing matrix of Medox) (Sigma, Madrid, Spain) and both MIX and VH were orally administered solubilized in 50:50 a sugar:water solution. Both treatments were administered daily in a volume of 1 mL between 08.00 and 09.00 a.m. for 3 weeks. Body weight was recorded weekly during all the experiment. Oral acute lipid tolerance tests were performed on overnight fasted rats 5 days before and 16 days after starting MIX administration (5 days before their sacrifice). In both cases, the animal blood from the tail vein was collected into capillary tubes before (time 0) and 1, 2, and 4 h after the oral administration of lard (2.5 mL/kg). Serum was obtained after blood clotting and centrifugation (2000 x g, 15 min, 4ºC) and stored at -80ºC. BP was measured during the 8th week of experiment and the last week before sacrifice by tail-cuff method [42] with some modifications [43]. Changes in systolic BP (SBP) were expressed as the difference between the mean values of the two mentioned measurements. SBP was recorded always by the same person in a stress-free environment to prevent alterations in the values. Finally, at the end of the experiment, rats were fasted for 3 hours after the last oral administration, and then they were killed by live decapitation. Serum was obtained after blood clotting and centrifugation (2000 x g, 15 min, 4ºC) and stored at -80ºC. Inguinal white adipose tissue (iWAT) was dissected, weighted, frozen immediately in liquid nitrogen and stored at -80ºC. The complete experimental design is schematized in Figure 1.

2.3. Metabolite extraction ^{1}H-NMR (Nuclear magnetic resonance) based metabolomics assays

Serum extraction was carried out following Bligh-Dyer procedure with some modifications [19]. Serum (100 µL) was added to methanol (400 µL) and ultrapure water (100 µL) obtained from a Milli-Q Advantage A10 system (Madrid, Spain). After the homogenisation of the tissue with a micropestle (S.G Servicios Hospitalarios, Barcelona, España), 200 µL of chloroform (Prolabo®, VWR, Llinars del Vallès, Spain) were added and homogenised to yield a monophasic solution. After, an additional 600 µL of chloroform and 200 µL of ultrapure water were added and the samples were centrifuged at 8,500 x g for 15 minutes, at 4°C, to obtain two phases separated by protein debris. The upper aqueous phase (hydrophilic metabolites) was freeze-dried and stored at -80°C while the remaining lipidic phase (lipophilic metabolites) was dried under nitrogen stream and stored at -80°C until future 1H-NMR measurement. Samples of iWAT were extracted following an adapted protocol from Castro et

al. [44]. Samples were pulverized. After that, the tissue (200 mg) was homogenised in methanol (800 µL). Subsequently, chloroform (400 µL) was added and the samples were mixed by vortex, after that an additional 1200 µl of chloroform were added. Subsequently, 800 µl of ultrapure water were added and the samples were homogenised and centrifuged for 10 min, at 4000 x g and 4ºC. The upper aqueous phase was extracted. The lower phase was subjected to several clean-ups steps with chloroform/methanol/water (2/1/1) and followed by centrifugation, for 10 min, at 4000 x g and 4ºC, on which the upper layer was extracted and mixed with the upper phases previously extracted. After two washes the upper phase was frozen at -80ºC for later analysis. The lower lipidic phase was extracted and dried under nitrogen stream, to ensure and frozen at -80ºC.

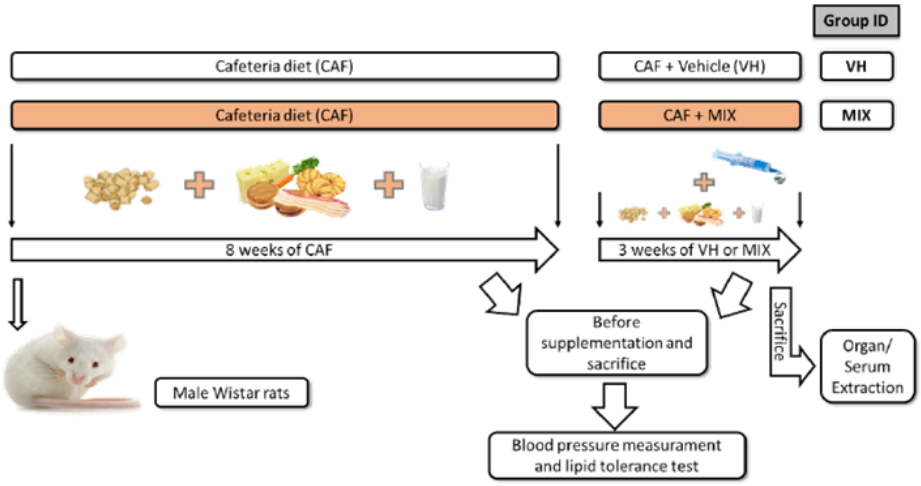


Figure 1. Graphical representation of the experimental design used in this study. VH, cafeteria diet rats administered vehicle; MIX, cafeteria diet rats administered MIX. Rats were fed cafeteria diet for 11 weeks and the last 3 weeks the animals were administered VH or MIX (n=16 per group). The animals were sacrificed after 11 weeks. CAF: cafeteria diet; STD: standard chow diet; MIX: ingredient; VH: vehicle.

2.4 *^{1}H-NMR analyses*

The aqueous extracts, for ^{1}H-NMR measurements were resuspended in 600 µl of deuterium oxide (D2O) phosphate buffer (PBS (phosphate-buffered saline) 0.05 mM, pH 7.4, 99.5% D2O) which contains 0.73 mM trisilylpropionic acid (TSP) (Cortecnet®, Voisins-Le-Bretonneux, France). Regarding the lipophilic extracts, they were reconstituted with a solution of deuterated chloroform/deuterated methanol (2:1), that includes 1.18 mM tetramethylsilane (TMS) and subsequently vortexed. The lipidic and aqueos extracts were transferred into 5-mm O.D. 1H-NMR glass tubes for 1H-NMR measurement. 1H NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Germany) that operates at a proton frequency of 600.20 MHz with a 5-mm PABBO (proton enhanced –Smartprobe® (Bruker®) broadband gradient probe). For the analysis of aqueous extracts, the supressing of the residual water peak was performed with one-dimensional 1H pulse experiments that were carried out using the nuclear Overhauser effect spectroscopy (NOESY) with a pre-saturation sequence (RD–90°–t1–90°–tm–90° ACQ). 100 ms were set as the mixing time. An irradiation power of 75 Hz was applied to pre-saturate solvents, during the recycling delay (RD = 5 s) and mixing time. Each sample was preceded by a calibration of the 90° pulse length, which varied from 9.95 to 10.06 µs. The spectral width was 12 kHz (20 ppm), and 256 transients were obtained into 64 K data points for each 1H spectrum. Regarding the lipophilic extracts, residual water signals in the lipophilic extracts, that could have been absorbed from ambient moisture by methanol, were suppressed with 90° pulses, with pre-saturation sequence (zgpr). An RD of 5.0 s with acquisition time of 2.94 s were used. The 90° pulse length was calibrated for each sample and varied from 9.92 to

10.04 µs. After 4 trial scans, 128 scans were obtained into 64K data points with a spectral with of 18.6 ppm. The exponential line broadening applied before Fourier transformation was of 0.3 Hz. TopSpin software (version 2.1, Bruker) was used to phase the frequency domain spectra, correct its baseline and reference to the TSP or TMS signal (δ = 0 ppm). Comparisons were made between the obtained 1H-NMR with references of pure compounds, obtained from the metabolic profiling AMIX spectra database (Bruker), HMDB, Chenomx NMR suite 8.4 software (Chenomx Inc., Edmonton, Canada) and databases for metabolite identification. Besides, metabolites by 1H–1H homonuclear correlation (COSY (correlation spectroscopy) were assigned and TOCSY (total correlation spectroscopy) and ^{1}H–13C heteronuclear (HSQC) 2D NMR experiments and by correlation with pure compounds run in-house. After pre-processing, specific 1H-NMR regions identified in the spectra were integrated and quantified using the AMIX 3.9 software package using TSP signal of buffer as internal reference.

2.5 *Serum biochemistry and related analysis*

Serum insulin was measured in duplicates, using ELISA kits (Millipore, Madrid, Spain) and the optical densities were measured at 450 nm in the microtiter plate reader EON Microplate (BioTek, Vermont, USA). The concentrations were calculated from a standard curve with provided lyophilized native rat insulin, ranging from 0.2 to 10 ng/mL. At the end of the experimental period, fasting values of glucose were confirmed by evaluating the serum glucose levels with an enzymatic colorimetric assay (QCA, Barcelona, Spain) and these values were used to determine the indices homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β-cell function (HOMA-β) and Quantitative insulin sensitivity check index (QUICKI). These indices were calculated as follows, HOMA-IR = fasting insulin (µU/mL) × fasting glucose (mM)/22.5; HOMA-β = 20 × fasting insulin (µU/mL)/(fasting glucose (mM) - 3.5); QUICKI = 1/log fasting insulin (µU/mL) + log fasting glucose (mg/dL). Serum triglycerides levels from serum obtained at the end of the experiment and oral acute lipid tolerance tests were measured by Triglycerides QCA kit (Comercial Bellés, Tarragona, Spain), following the manufacturer's recommended protocol. Serum HDL-C levels were obtained by precipitation of every other lipoprotein and measuring the remaining cholesterol with a Cholesterol QCA kit (Comercial Bellés, Tarragona, Spain), following standard procedures. Serum low-density lipoprotein-cholesterol (LDL-C) levels were measured in a similar manner, but required an initial quantification of total cholesterol, measured with a Cholesterol QCA kit and results for LDL-C were defined as the substation of total cholesterol levels minus values obtained in this kit.

2.6 *Statistical analysis and data processing*

Data shown in this manuscript represent mean values with standard error of the mean (SEM) values. Statistical analyses were performed by Student's t-test, paired samples Student's t-test and 2-way ANOVA. The grubbs' test was used to identify outliers. Absolute concentrations, from the ^{1}H-NMR, of both the lipophilic and hydrophilic extracts were ordered in a single data matrix, to perform the PLS-DA and PCA multivariate model. Same opportunities to enter the model were provided for every metabolite as all the identified metabolites were scaled to unit variance. MetaboAnalyst 3.0 software suite for metabolomic data analysis (http://metaboanalyst.ca). Microsoft Excel XLSTAT 2017: Data Analysis and Statistical Solution for Microsoft Excel (Addinsoft, Paris, France (2017), and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) programs were used for data processing, statistical analysis and graphical plotting, respectively. Statistically significance is shown when $p < 0,05$ and statistical tendency when $p > 0,05 - \leq 0.1$.

3. Results

3.1 *Multivariate chemometric analysis of ^{1}H-NMR data*

The PCA methodology was used to detect putative outliers. The PCA was carried out with the whole spectra removing the TSP or TMS, methanol, chloroform and water regions in aqueous and lipid phases. After alignment and normalization of the spectres, 15 and 11 in the lipid phase from

serum and iWAT, respectively (Tables 1A and 2A), and 28 and 24 metabolites in the aqueous phase from serum and iWAT, respectively, were identified and integrated (Tables 1B and 2B).

Table 1A

Summary of metabolites from the serum lipidic extraction.

	VH	MIX
Total Cholesterol	0.902 ± 0.02	0.819 ± 0.03 *
Free Cholesterol	0.361 ± 0.01	0.316 ± 0.01 *
Esterified Cholesterol	0.522 ± 0.02	0.507 ± 0.02
Triglycerides	0.903 ± 0.08	0.757 ± 0.08
Diglycerides	0.033 ± 0.004	0.026 ± 0.002
Lysophosphatidyl Choline	0.062 ± 0.002	0.05 ± 0.001 *
Phosphatidyl Choline	0.793 ± 0.03	0.682 ± 0.02 *
Linoleic acid	113.42 ± 15.6	88.2 ± 12.1
Oleic acid	48.18 ± 3.9	31.97 ± 2.97 *
ARA+EPA	21.33 ± 2.0	15.15 ± 0.8 *
DHA	2.79 ± 0.3	2.07 ± 0.2 #
Omega-3	5.11 ± 0.2	3.92 ± 0.4 *
PUFA	88.25 ± 6.4	63.75 ± 4.6 *
MUFA	159.46 ± 20.3	123.81 ± 16.2
Fatty fatty acids	56.609 ± 3.09	50.774 ± 2.6

Values (mM) are presented as the mean ± SEM of 15-16 animals per group. ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; PUFA: Polyunsaturated fatty acid; MUFA: Monounsaturated fatty acid. * denotes statistical significance on t-student: ($p \leq 0.05$). # denotes statistical tendency on t-student: ($p > 0.05$ - ≤ 0.1). ARA: arachidonic acid; EPA: eicosapentaenoic acid; PUFA: polyunsaturated fatty acid; MUFA: monounsaturated fatty acid; DHA: Docosahexaenoic acid.

Table 1B

Summary of metabolites from the serum aqueous extraction.

	VH	MIX
Formate	0.02 ± 0.001	0.02 ± 0.001
Glycerol	0.2 ± 0.007	0.21 ± 0.008
Acetate	0.06 ± 0.004	0.06 ± 0.003
3-Hidroxybutyrate	0.02 ± 0.002	0.05 ± 0.008 *
Glucose	0.68 ± 0.07	0.84 ± 0.08
Pyruvate	0.01 ± 0.002	0.01 ± 0.002
Succinate	0.004 ± 0.0004	0.006 ± 0.0008 *
Lactate	0.78 ± 0.1	0.83 ± 0.1
Citrate	0.02 ± 0.002	0.02 ± 0.002
Leucine	0.02 ± 0.001	0.03 ± 0.002 #
Threonine	0.02 ± 0.003	0.03 ± 0.004
Tryptophan	0.01 ± 0.002	0.02 ± 0.002
Tyrosine	0.01 ± 0.001	0.02 ± 0.002

Proline	0.02 ± 0.003	0.02 ± 0.003
Isoleucine	0.01 ± 0.001	0.02 ± 0.002 *
Glycine	0.1 ± 0.01	0.11 ± 0.01
Glutamate	0.01 ± 0.001	0.01 ± 0.002
Glutamine	0.09 ± 0.01	0.12 ± 0.01 #
Citrulline	0.02 ± 0.002	0.02 ± 0.002
Lysine	0.05 ± 0.004	0.07 ± 0.007 *
Valine	0.03 ± 0.002	0.04 ± 0.003
Histidine	0.01 ± 0.001	0.01 ± 0.001
Alanine	0.1 ± 0.01	0.11 ± 0.01
Phenylalanine	0.01 ± 0.001	0.01 ± 0.001
Choline	0.0063 ± 0.001	0.0083 ± 0.001 #
Creatinine	0.004 ± 0.001	0.005 ± 0.001
TMAO	0.03 ± 0.003	0.03 ± 0.003
Creatine	0.07 ± 0.01	0.09 ± 0.01 #

Values (mM) are presented as the mean ± SEM of 15-16 animals per group. TMAO: Trimethyl amino oxide. * denotes statistical significance on t-student: (P ≤ 0.05). # denotes statistical tendency on t-student: (P > 0,05 - ≤ 0.1). TMAO: Trimethylamine N-oxide

Table 2A

Summary of metabolites from the iWAT lipid extraction.

	VH	MIX
Total Cholesterol	1.27 ± 0.09	1.18 ± 0.05
Triglycerides	696.31 ± 17.5	628.22 ± 14.5 *
Diglycerides	3.01 ± 0.1	2.68 ± 0.1
Phosphatidyl Choline	0.45 ± 0.02	0.42 ± 0.02
Linoleic acid	267.59 ± 9	239.56 ± 8.3 *
Oleic acid	1040.09 ± 33.4	941.95 ± 28.7 *
Sphingomyelin	0.022 ± 0.002	0.016 ± 0.001 *
ARA+EPA	16.46 ± 0.7	16.22 ± 0.6
PUFA	44.96 ± 2.1	38.12 ± 0.9 *
MUFA	1470.95 ± 47.8	1320.94 ± 35.4 *
Total fatty acids	2169.81 ± 58.9	1949.68 ± 44.1 *

Values (mM) are presented as the mean ± SEM of 15-16 animals per group. ARA: Arachidonic acid; EPA: Eicosapentaenoic aci; PUFA: Polyunsaturated fatty acid; MUFA: Monounsaturated fatty acid. * denotes statistical significance on t-student: (P ≤ 0.05). # denotes statistical tendency on t-student: (P > 0,05 - ≤ 0.1). ARA: arachidonic acid; EPA: eicosapentaenoic acid; PUFA: polyunsaturated fatty acid; MUFA: monounsaturated fatty acid.

Table 2B

Summary of metabolites from the iWAT aqueous extraction.

	VH	MIX
AMP	0.01 ± 0.001	0.01 ± 0.001
Formate	0.017 ± 0.001	0.021 ± 0.002 #
Uracil	0.01 ± 0.001	0.01 ± 0.001
Glycerol	0.48 ± 0.03	0.58 ± 0.04 *
Acetate	0.05 ± 0.002	0.05 ± 0.002
Propionate	0.01 ± 0.001	0.01 ± 0.001
3-Hydroxybutyrate	0.006 ± 0.001	0.097 ± 0.002 *
Fumarate	0.0013 ± 0.0002	0.0008 ± 0.0001 *
Glucose	0.13 ± 0.01	0.12 ± 0.01
Succinate	0.02 ± 0.002	0.01 ± 0.001 #
Lactate	0.7 ± 0.07	0.59 ± 0.06
Leucine	0.03 ± 0.002	0.03 ± 0.003
Tyrosine	0.01 ± 0.001	0.02 ± 0.003
Isoleucine	0.01 ± 0.001	0.02 ± 0.003
Aspartate	0.02 ± 0.002	0.02 ± 0.003
Glutamate	0.09 ± 0.007	0.09 ± 0.01
Glutamine	0.37 ± 0.05	0.31 ± 0.02
Valine	0.03 ± 0.004	0.03 ± 0.004
Alanine	0.14 ± 0.01	0.14 ± 0.01
Phenylalanine	0.01 ± 0.001	0.01 ± 0.002
Taurine	0.51 ± 0.05	0.4 ± 0.02 #
Choline	0.06 ± 0.003	0.06 ± 0.005
Inosine	0.02 ± 0.002	0.01 ± 0.001 *
Creatine	0.06 ± 0.009	0.05 ± 0.005

Values (mM) are presented as the mean ± SEM of 15-16 animals per group. AMP: Adenosine monophosphate. * denotes statistical significance on t-student: (P ≤ 0.05). # denotes statistical tendency on t-student: (P > 0,05 - ≤ 0.1). AMP: adenosine monophosphate.

3.2 Metabolomic profile of serum and inguinal adipose tissue

PLS-DA was performed, comparing VH and MIX groups, to determine whether MIX administration influenced the general serum metabolism (Figure 2). The model groups together most of the rats of each group and mostly separates them. The R2 and Q2 values were 0.62424 and 0.3017 respectively, using the second component. Validation of the model was positive as the permutation testing of 1000 permutations reported a significance of lower than 0.01, implying MIX had significantly changed the spectrum of metabolites found in serum. The metabolites that were found changed in the serum of MIX group were total cholesterol, free cholesterol, lysophosphatidyl choline, phosphatidyl choline, oleic acid, ARA + EPA, omega 3, PUFA , 3-hydroxibutyrate, succinate, isoleucine and lysine. DHA showed tendency to be reduced (p = 0.054), while creatine (p = 0.06), choline (p = 0.061), glutamine (p = 0.079) and leucine (p = 0.065) showed tendency to be increased in the animals administered MIX (Tables 1A and 1B).

In addition, MIX administration induced also changes in the iWAT levels of triglycerides, linoleic acid, oleic acid, sphingomyelin, PUFA, MUFA, total fatty acids, glycerol, inosine, fumarate and 3-hydroxybutyrate, while succinate ($p = 0.08$), formate ($p = 0.06$) and taurine ($p = 0.057$) showed tendency to be lowered (Tables 2A and 2B).

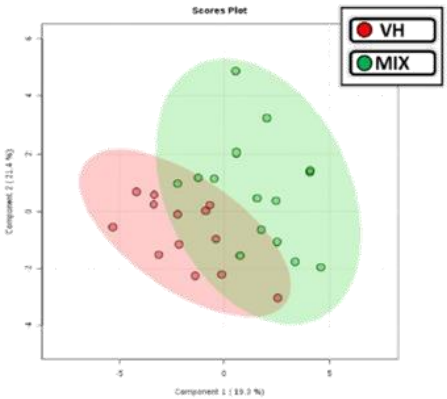


Figure 2. PLS-DA from serum including both aqueous and lipidic phases after 3 weeks of MIX or VH supplementation in CAF diet-fed rats.

3.3 Glucose and lipid metabolism

The administration of MIX did not change significantly serum glucose concentration, but MIX reduced serum insulin levels (Table 3). In addition, the insulin resistance indices HOMA-IR, HOMA-β and QUICKI showed significantly lower values in the animals administered MIX than in the rats from VH group.

Table 3

Serum biochemical values and insulin resistance 3 weeks after starting MIX administration.

	VH	MIX
Glucose (mM)	8.1 ± 0.3	7.9 ± 0.3
LDL-C (mg/dL)	48.1 ± 4	49.9 ± 4
HDL-C (mg/dL)	11.2 ± 1	9.1 ± 1
Triglycerides (mg/dL)	313 ± 29	249 ± 22
Insulin (ng/mL)	9.6 ± 0.9	5.5 ± 0.7 *
HOMA-IR	99 ± 10	57 ± 7 *
HOMA-β	1261 ± 133	709 ± 87 *
QUICKI	0.221 ± 0.003	0.234 ± 0.030 *

Values are presented as the mean ± SEM of 15-16 animals per group. Glucose, LDL-C and HDL-C and triglycerides levels are measured with an enzymatic colorimetric assay and insulin levels by ELISA kit. Blood was obtained from the sacrifice after 3 hours fasting period. * denotes statistical significance on t-student: ($P \leq 0.05$). HOMA: Homeostasis Model Assessment; QUICKI: Quantitative Insulin Sensitivity Check Index.

3.4 Lipid metabolism

Fasting total cholesterol levels were found reduced in the animals from MIX group 16 days after starting MIX administration (Figure 3A), while triglycerides concentration showed a tendency to decrease (Figure 3B). In addition, MIX administration showed a tendency to block the increase in serum of triglycerides induced by lard (Figures 3C), whereas the initial acute lipid tolerance test carried out before MIX administration showed no differences among groups prior MIX

supplementation (data not shown). However, no differences were found in serum triglycerides, HDL-C nor LDL-C between both groups of animals at the end of the experiment (Table 3).

Regarding the metabolomic analysis obtained by 1H-NMR of iWAT and serum, a summary of relevant improved metabolites is shown in Figure 4 for easier readability. MIX supplementation significantly reduced circulatory triglycerides and total cholesterol. In addition, a reduction of triglycerides was also found in iWAT. Besides, MIX supplementation reduced total fatty acids, PUFA and MUFA concentration in iWAT.

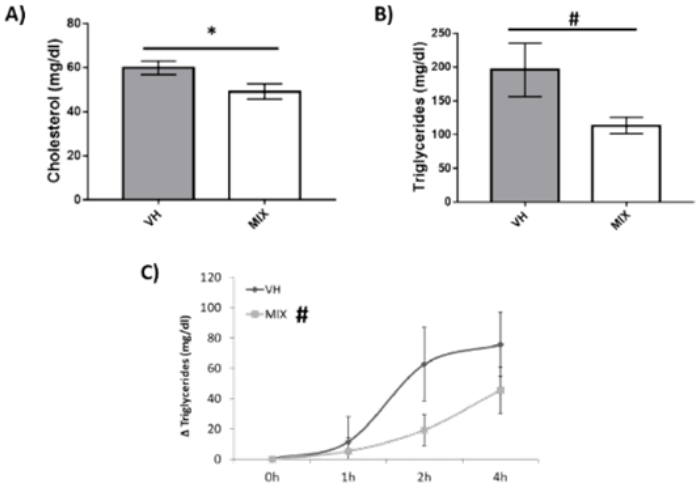


Figure 3. Summary of relevant results from the lipid tolerance test performed 16 days after starting MIX adminsitration and 16 hours of fasting. (A) Fasting cholesterol (mg/mL) concentration on serum obtained from point zero of the acute test. (B) Fasting triglycerides (mg/dL) obtained under the same circumstances. (C) Values of triglycerides concentration obtained from the test represented as increments of concentration. Data represent mean ± SEM (n=8). * denotes statistical significance ($p \leq 0.05$). # denotes statistical tendency ($p < 0.1$).

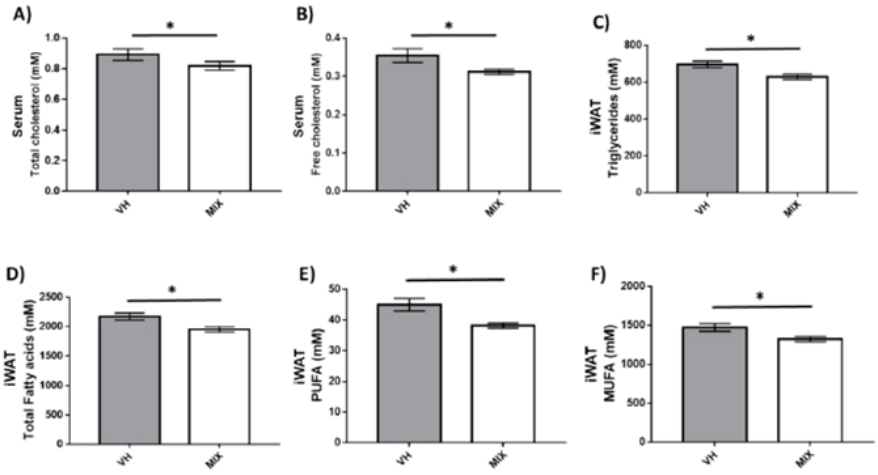


Figure 4. Summary of lipid metabolism-related metabolites from serum and iWAT. (**A**) Serum total cholesterol and (**B**) free cholesterol. iWAT (**C**) triglycerides, (**D**) total fatty acids, (**E**) PUFA (Polyunsaturated fatty acids) and (**F**) MUFA (Monounsaturated fatty acids). Data are expressed as the mean ± SEM* denotes statistical significance on t-student: ($P \leq 0.05$). iWAT: Inguinal white adipose tissue)

3.6 Blood pressure

Rats presented HTN at the 8th week of CAF diet, reaching values of SBP of 134 ± 2 mmHg. However, after 3 weeks of MIX administration, rats showed a huge decrease in SBP (-17 ± 2 mmHg), restoring values for SBP considered as normotensive (Figures 5). On the contrary, rats receiving VH did not exhibited changes in their SBP, presenting HTN at the end of the experiment.

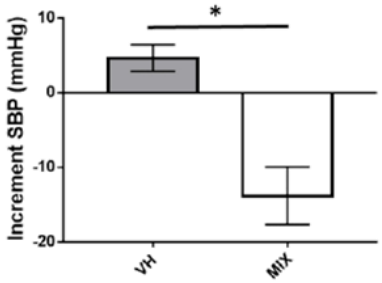


Figure 5. Increments of SBP (Systolic blood pressure) in animals administered vehicle (VH) or MIX. Increment for each group is calculated as the difference between before and 3 weeks after the start of administration. Data are expressed as the mean ± SEM. * denotes statistical significance on paired samples t-student: ($p < 0.05$).

4. Discussion

MetS is a cluster of different of conditions that involves deep changes in a healthy metabolic status and make them prone to develop other CVDs besides elevating the risk of cancer, non-alcoholic fatty liver disease NAFLD and other health complications [45]. Changes in lifestyle is the first strategy recommended in the current guidelines to treat MetS [46]. In fact, higher adherence habits related to healthy lifestyle is associated with a lower risk of developing MetS. In this sense, dietary habits are considered as crucial [47]. Nowadays, clinical management of this disease is difficult because there is no recognized method to prevent or improve the whole syndrome [48]. In fact, targeting the whole MetS alterations should not require the individually administration of drugs targeting specific components including obesity, insulin resistance, dyslipidemia or HTN.

Dietary supplements have been widely studied for their health effects on different components of MetS. In this sense, our group has recently demonstrated the efficacy of the co-administration of CLA, the flavonoids proanthocyanidins and anthocyanidins and an antihypertensive protein hydrolysate to improve the obesity related to MetS. The body weight lowering properties of the MIX were remarkable, showing the rats MIX treated a decrease of 8% in their body weight compared with VH group [38]. Nevertheless, MIX includes different beneficial compounds that could be useful in the management of different components of MetS and therefore, their administration could be a successful strategy for the treatment of this disease. In addition, tackling simultaneously different compounds of MetS might positively affect the metabolism, reverting partially damage caused by the ectopic fat accumulation. Moreover, each compound, could subsequently, help the others as they provide a healthier metabolic status on which to effect upon. In this context, Rondanelli et al. showed that the administration to obese patients of a combination of several bioactive products that individually were known to ameliorate leptin/adiponectin ratio, insulin resistance and respiratory quotient, was found to produce a greater effect when combined [49]. Other studies reported similar findings [50,51], while others did not find this synergetic effect [52,53].

The specific bioactive compounds included in MIX composition are an equal mixture of a low doses of the CLA isomers c9,t11 and t10,c12, the proanthocianidins and anthocianidins rich extracts GSPE and Medox, and the chicken feet hydrolysate Hpp11. CLA is one of the most studied dietary supplements for the body weight loss and although some studies reported potential adverse effects related to their consumption, the beneficial effects on obesity, without compromising other metabolic

parameters, of a low dose of 100mg/kg of an equal mixture of the CLA isomers c9,t11 and t10,c12 CLA in CAF diet-fed rats has been recently reported by our group [19].

Proanthocyanidins are a group of structurally similar phenolic compounds, abundant in many fruits and vegetables, among them, in grape seeds [33]. Beneficial effect of GSPE has been found to be of interest in the treatment against elevated triglyceridess in blood, as acute administration of 375 mg/kg has shown to lower hepatic and blood triglycerides [54]. Particularly, GSPE affects the lipidic metabolism by promoting fatty acid oxidation and inhibiting lipogenesis in liver [55–57], while also promoting lipolysis in WAT, with a dose of just 25 mg/kg [58], and cultured adipocytes [59]. It has also been directly linked with obesity, as experiments with obese rats have proved to improve obesity by reducing the size of visceral adipocytes, besides promoting an increase in their number, facilitating the mobilization of fats [58,60]. Besides, GSPE also improves insulin resistance in Zucker rats as it increases the expression of Glut4 (Glucose transporter 4) and Irs-1 (Insulin receptor substrate-1) in WAT [61]. Finally, GSPE have also been reported to have anti-obesogenic properties [62] and satiating effects [63].

Medox is a nutritional supplement, a highly concentrated dose of anthocyanins that would be predominantly represented by Cyanidin-3G and Delphinidin-3G. Initial studies showed the impact of anthocyanins on inflammation and oxidative stress [41,64], by lowering TNF-α [65], CRP [66] and NF-κB [41]. Others have found amelioration of insulin resistance and glucose homeostasis [67], while inducing an improvement on lipid metabolism and the symptoms of the MetS by increasing HDL-C, lowering triglycerides and fasting glucose [68].

Hpp11 is a multi-peptide product obtained after a process of enzymatic digestion of chicken feet [29]. The particular procedure that was followed, results in a combination of peptides that have been found to greatly improve HTN in CAF diet-fed rats after an acute administration of 55 mg/kg [27] and long term anti-hypertensive effects [28].

Therefore, in this study, the effects of the co-administration of all these bioactive compounds on the cardiometabolic risk factors related to MetS were investigated. In order to achieve a previous state of MetS, prior MIX administration, obesity and other related pathologies were induced in rats by cafeteria diet. These rats are considered as a model of human MetS since the animals have evidenced hyperphagia, increased body weight, HTN, insulin resistance and elevated serum glucose and triglycerides [5,69].

Hypertriglyceridemia is one of the components of MetS. To better understand the lipid metabolism after MIX administration, an oral lipid tolerance tests on overnight fasted rat was carried out. In addition to lower fasting lipids, MIX treated rats showed, after an acute dose of lard, that triglycerides levels increase in the bloodstream was smoother and did not reach the maximum peak found in the VH animals. MIX beneficial effects on triglycerides is according with previous studies carried out in high fat diet (HFD) fed rats and using the same dose of GSPE (25 mg/Kg) included in the MIX [70]. In addition, a recent meta-analysis pointed to GSPE as an effective triglycerides reducer also in humans [71]. The MIX hypotriglyceridemic effect is also in accordance with the presence of anthocyanidins in the MIX, since Medox supplementation to subjects with type 2 diabetes caused a 23 percent reduction of this lipid in serum [68].

In contrast, the triglycerides lowering MIX effect was not observed in the serum obtained from the rats at the end of the experiment, after 3 hours of fasting. It could because these animals did not present values of fasting lipid values. Therefore, although previous studies of our group have demonstrated that this short fasting time was sufficient to test the effects of some bioactive compounds, specifically GSPE, resveratrol or CLA, on glucose and lipid metabolism [19,58,72], longer food deprivation could make it easier to detect mores changes in serum lipids. According to this, no changes were observed in HDL-C or LDL-C at the end of the experimental period, although an increase in serum HDL-C was expected in the MIX treated rats, since the elevation of HDL-C has been reported after Medox and GSPE administration [68,73].

In addition, as the analysis of the symptoms of the MetS do not provide a deep understanding of lipid homeostasis, a deeper analysis using metabolomics was carried out to know lipid management after MIX administration. The serum 1H-NMR analysis showed that in addition to a

reduction of total and free cholesterol, MIX produced changes on other serum lipid metabolites. A previous study administrating the same dose of CLA included in the MIX (100 mg/Kg) to CAF diet-fed rats also reported an amelioration in serum cholesterol [19]. Specifically, in this study PUFA and ARA + EPA concentrations were found reduced in the MIX treated rats. These metabolites are associated with the inflammatory response [74], and decreasing them could suggest lowered inflammatory status in the MIX group. Phosphatidyl choline, directly related with obesity and cholesterol [75,76], was also found reduced in the animals MIX administered. Serum concentration of oleic acid was also found reduced. Low levels of this fat acid have been found under reduced caloric intake [77]. Therefore, in our study, MIX administration seems to be mimicking the effect of reduced energy reservoirs, which could be produced by an increase of energy expenditure. According to this, an increase of energy expenditure has been reported after the administration of 100 mg/Kg of the same mixture of CLA [19]. Overall, these results would suggest an amelioration of the management of lipids.

Moreover, as the white adipose tissue is the main reservoir of fat, we studied how these improvements in the serum metabolites translated to the adipose tissue. 1H-NMR results of the iWAT showed similar results to those found in serum. An overall reduction of lipids was found in this tissue in the MIX treated rats. Several lipid metabolites, among them triglycerides were found reduced after MIX administration. We also found an increase on glycerol, possibly related to an increase in breaking down triglycerides [78]. Besides, total fatty acids, PUFA and MUFA were found reduced in the animals administered MIX, suggesting increased release of lipids from the iWAT for their burn and metabolization, showing a similar effect to the reported in other studies [79,80].

Hyperglycemia is another of the components of the MetS, but no changes in fasting glucose were found after the MIX administration, which contrasts with previous studies using GSPE [31]. However, as was expected, by the presence of GSPE, Medox and CLA in the MIX [19,62,81], insulin was greatly reduced in the treated animals. According to this, HOMA-IR, HOMA-β and Quicki values showed an improvement in insulin resistance. In addition, an increase of succinate metabolite was found in the serum of the treated rats. Although high levels of succinate have been showed to be elevated in diabetes type 2 and obesity [82], recent studies have reported the importance of this metabolite as source for the glucose production in liver [83,84]. Another important metabolite related to the energy metabolism is the 3-hydroxibutyrate, and it was found increased in serum and iWAT of MIX treated animals. These results could suggest that MIX might be strongly altering the energy metabolism. The PLS-DA analysis of both serum lipidic and aqueous phases of serum confirmed that MIX administration changes the metabolic profile of serum, significantly separating both groups.
HTN is one of the principal components related to MetS, even more prevalent than obesity [85]. In this study, MIX administration for 3 weeks in CAF diet-fed rats produced an obvious decrease in the SBP of CAF diet rats compared to their counterpart animals administered VH, demonstrating the also antihypertensive properties of this multifunctional ingredient in this animal model. In fact, in only 3 weeks of MIX administration counteracted the cafeteria hypertensive effect since the MIX treated animals showed normotensive values of BP. The BP decrement is considered as promised since small reductions in BP in the hypertensive population may have an important impact on cardiovascular events (2–3% reduction in risk for each mm Hg) [86]. This result are in according with those reporting the antihypertensive effect after chronic administration of Hpp11 in CAF diet-fed rats [28] and with those reporting the attenuation of the development of HTN after chronic administration of GSPE in CAF diet-fed rats [35] or with those reporting antihypertensive effects of long-term administration of CLA and anthocyanin-rich foods in SHR [87,88]. Nevertheless, the BP lowering effect of MIX was higher than those obtained by the individual bioactive compounds, at the same doses used in the MIX, since normotensive values of SBP are recorded in the animals at the end of the experiment.

5. Conclusions

In summary, in addition to their previously reported body weight lowering effect MIX administration an improvement of other cardiometabolic risk factors in an experimental model of MetS. Specifically, an improvement in glucose and lipid homeostasis was found. In addition, MIX exhibited clear

anhithypertensive properties. In fact, the administration of MIX for only 3 weeks to these animnals counteracted the cafeteria hypertensive effect since the MIX treated animals showed normotensive values of BP. Therefore, MIX could be a good candidate to be used as nutraceutical or to be included in functional foods for the management of metabolic syndrome. Nevertheless, the quantity of MIX necessary to use in humans should be definitively established when clinical trials were conducted.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1.

Author Contributions: Conceptualization, M.S., B.M. and G.A.; methodology, M. M-G, M. I, A.G-R. and H.P.; formal analysis, M. M-G, M.I. and M.A.R.; writing-original draft preparation, M.M-G.; writing-review and editing, B.M. and G.A.; funding acquisition, B.M. and G.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministerio de Economía, Industria y Competitividad (AGL2013-40707-R and AGL2016-77105-R) and by the Universitat Rovira i Virgili (2017R2B-05). G.A. is a Serra-Hunter Fellow.

Acknowledgements: We gratefully acknowledge the aid of Niurka Dariela Llópiz and Rosa Pastor, the laboratory technicians.

Conflicts of Interest: The authors declare no conflicts of interest

References

1. Bremer, A.A.; Mietus-Snyder, M.; Lustig, R.H. Toward a Unifying Hypothesis of Metabolic Syndrome. Pediatrics 2012, 129, 557–570.
2. American, A.; Association, H.; Heart, N.; Grundy, S.M.; Cleeman, J.I.; Daniels, S.R.; Donato, K.A.; Eckel, R.H.; Franklin, B.A.; Gordon, D.J.; et al. Diagnosis and managment of the metabolic syndrome AHA / NHLBI Scientific Statement. 2006, 2735–2752.
3. Lent-Schochet, D.; McLaughlin, M.; Ramakrishnan, N.; Jialal, I. Exploratory metabolomics of metabolic syndrome: A status report. World J. Diabetes 2019, 10, 23–36.
4. Sampey, B.P.; Vanhoose, A.M.; Winfield, H.M.; Freemerman, A.J.; Muehlbauer, M.J.; Fueger, P.T.; Newgard, C.B.; Makowski, L. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: Comparison to high-fat diet. Obesity 2011, 19, 1109–1117.
5. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Acute administration of single oral dose of grape seed polyphenols restores blood pressure in a rat model of metabolic syndrome: role of nitric oxide and prostacyclin. Eur. J. Nutr. 2016, 55, 749–758.
6. De la Iglesia, R.; Loria-Kohen, V.; Zulet, A.M.; Martinez, A.J.; Reglero, G.; Ramirez de Molina, A. Dietary Strategies Implicated in the Prevention and Treatment of Metabolic Syndrome. Int. J. Mol. Sci. 2016, 17.
7. Rangel-Huerta, O.D.; Pastor-Villaescusa, B.; Aguilera, C.M.; Gil, A. A Systematic Review of the Efficacy of Bioactive Compounds in Cardiovascular Disease: Phenolic Compounds. Nutrients 2015, 7, 5177–5216.
8. Konstantinidi, M.; Koutelidakis, A.E. Functional Foods and Bioactive Compounds: A Review of Its Possible Role on Weight Management and Obesity's Metabolic Consequences. Medicines 2019, 6, 94.
9. Kim, S.K.; Mendis, E. Bioactive compounds from marine processing byproducts - A review. Food Res. Int. 2006, 39, 383–393.
10. Azmir, J.; Zaidul, I.S.M.; Rahman, M.M.; Sharif, K.M.; Mohamed, A.; Sahena, F.; Jahurul, M.H.A.; Ghafoor, K.; Norulaini, N.A.N.; Omar, A.K.M. Techniques for extraction of bioactive compounds from plant materials : A review. J. Food Eng. 2013, 117, 426–436.
11. Whigham, L.D.; Watras, A.C.; Schoeller, D.A. Efficacy of conjugated linoleic acid for reducing fat mass: a meta-analysis in humans. Am. J. Clin. Nutr. 2007, 85, 1203-1211 9p.
12. Kepler, C.R.; Hirons, K.P.; McNeill, J.J.; Tove, S.B. Intermediates and products of the biohydrogenation of linoleic acid by Butyrinvibrio fibrisolvens. J. Biol. Chem. 1966, 241, 1350–1354.
13. Pariza, M.W.; Park, Y.; Cook, M.E. The biologically active isomers of conjugated linoleic acid. Prog. Lipid Res. 2001, 40, 283–298.
14. Shen, W.; Baldwin, J.; Collins, B.; Hixson, L.; Lee, K.T.; Herberg, T.; Starnes, J.; Cooney, P.; Chuang, C.C.; Hopkins, R.; et al. Low level of trans-10, cis-12 conjugated linoleic acid decreases adiposity and increases

browning independent of inflammatory signaling in overweight Sv129 mice. J. Nutr. Biochem. 2015, 26, 616–625.
15. Tsuboyama-Kasaoka, N.; Takahashi, M.; Tanemura, K.; Kim, H.J.; Tange, T.; Okuyama, H.; Kasai, M.; Ikemoto, S.; Ezaki, O. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. Diabetes 2000, 49, 1534–42.
16. Vyas, D.; Kadegowda, A.K.G.; Erdman, R.A. Dietary conjugated linoleic acid and hepatic steatosis: Species-specific effects on liver and adipose lipid metabolism and gene expression. J. Nutr. Metab. 2012, 13.
17. Benjamin, S.; Prakasan, P.; Sreedharan, S.; Wright, A.D.; Spener, F. Pros and cons of CLA consumption: An insight from clinical evidences. Nutr. Metab. 2015.
18. Scientific Opinion on the safety of "conjugated linoleic acid (CLA)-rich oil" (Clarinol®) as a Novel Food ingredient. EFSA J. 2010, 8, 1601.
19. Martín-González, M.Z.; Palacios, H.; Rodríguez, M.A.; Arola, L.; Aragonès, G.; Muguerza, B. Beneficial Effects of a Low-dose of Conjugated Linoleic Acid on Body Weight Gain and other Cardiometabolic Risk Factors in Cafeteria Diet-fed Rats. Nutr. 2020, 12.
20. Parra, P.; Serra, F.; Palou, A. Moderate doses of conjugated linoleic acid isomers mix contribute to lowering body fat content maintaining insulin sensitivity and a noninflammatory pattern in adipose tissue in mice. J. Nutr. Biochem. 2010, 21, 107–115.
21. Sharma, S.; Singh, R.; Rana, S. Bioactive peptides: A review. Int. J. Bioautomation 2011, 15, 223–250.
22. Yoshikawa, M. Bioactive peptides derived from natural proteins with respect to diversity of their receptors and physiological effects. Peptides 2015, 72, 208–225.
23. 23. Möller, N.P.; Scholz-Ahrens, K.E.; Roos, N.; Schrezenmeir, J. Bioactive peptides and proteins from foods: Indication for health effects. Eur. J. Nutr. 2008, 47, 171–182.
24. Sánchez, A.; Vázquez, A. Bioactive peptides: A review. Food Qual. Saf. 2017, 1, 29–49.
25. Hong, F.; Ming, L.; Yi, S.; Zhanxia, L.; Yongquan, W.; Chi, L. The antihypertensive effect of peptides: A novel alternative to drugs? Peptides 2008, 29, 1062–1071.
26. Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Natural Angiotensin Converting Enzyme (ACE) Inhibitors with Antihypertensive Properties. Nat. Prod. Target. Clin. Relev. Enzym. 2017, 45–67.
27. Mas-Capdevila, A.; Pons, Z.; Aleixandre, A.; Bravo, F.I.; Muguerza, B. Dose-Related Antihypertensive Properties and the Corresponding Mechanisms of a Chicken Foot Hydrolysate in Hypertensive Rats. Nutrients 2018, 10, 1295.
28. Mas-Capdevila, A.; Iglesias-Carres, L.; Arola-Arnal, A.; Suarez, M.; Muguerza, B.; Bravo, F.I. Long-term administration of protein hydrolysate from chicken feet induces antihypertensive effect and confers vasoprotective pattern in diet-induced hypertensive rats. J. Funct. Foods 2019, 55, 28–35.
29. Bravo, F.I.; Mas-Capdevila, A.; Margalef, M.; Arola-Arnal, A.; Muguerza, B. Novel Antihypertensive Peptides Derived from Chicken Foot Proteins. Mol. Nutr. Food Res. 2019, 63, 1801176.
30. Mas-Capdevila, A.; Iglesias-Carres, L.; Arola-Arnal, A.; Aragonès, G.; Aleixandre, A.; Bravo, F.I.; Muguerza, B. Evidence that Nitric Oxide is Involved in the Blood Pressure Lowering Effect of the Peptide AVFQHNCQE in Spontaneously Hypertensive Rats. Nutrients 2019, 11, 225.
31. Bladé, C.; Aragonès, G.; Arola-Arnal, A.; Muguerza, B.; Bravo, F.I.; Salvadó, M.J.; Arola, L.; Suárez, M. Proanthocyanidins in health and disease. BioFactors 2016, 42, 5–12.
32. Naseri, R.; Farzaei, F.; Haratipour, P.; Nabavi, S.F.; Habtemariam, S.; Farzaei, M.H.; Khodarahmi, R.; Tewari, D.; Momtaz, S. Anthocyanins in the Management of Metabolic Syndrome: A Pharmacological and Biopharmaceutical Review. Front. Pharmacol. 2018, 9, 1310.
33. Gu, L.; Kelm, M.A.; Hammerstone, J.F.; Beecher, G.; Holden, J.; Haytowitz, D.; Gebhardt, S.; Prior, R.L. Concentrations of Proanthocyanidins in Common Foods and Estimations of Normal Consumption. J. Nutr. 2004, 134, 613–617.
34. Prior, R.L.; Wu, X. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. Free Radic Res 2006, 40, 1014–1028.
35. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Chronic administration of grape-seed polyphenols attenuates the development of hypertension and improves other cardiometabolic risk factors associated with the metabolic syndrome in cafeteria diet-fed rats. Br. J. Nutr. 2017, 117, 200–208.
36. Qin, Y.; Xia, M.; Ma, J.; Hao, Y.; Liu, J.; Mou, H.; Cao, L.; Ling, W. Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects. Am. J. Clin. Nutr. 2009, 90, 485–492.

37. Rodriguez-Mateos, A.; Istas, G.; Boschek, L.; Feliciano, R.P.; Mills, C.E.; Boby, C.; Gomez-Alonso, S.; Milenkovic, D.; Heiss, C. Circulating Anthocyanin Metabolites Mediate Vascular Benefits of Blueberries: Insights From Randomized Controlled Trials, Metabolomics, and Nutrigenomics. Journals Gerontol. Ser. A 2019, 74, 967–976.
38. Brazo Vázquez, F.I.; Suárez Recio, M.; Arola Arnal, A.; Muguerza Marquínez, M.B.; Ibars Serra, M.; Ardid Ruiz, A.; Aragonès Bargalló, G.; Gibert Ramos, A.; Bladé Segarra, M.C.; Mas Capdevilla, A.; et al. Composición hipoleptinémica y su uso 2019.
39. Bravo, F.I; Arola, L.; Muguerza, B. Procedure for obtaining a hydrolyzed chicken foot claws with anti-handling activity, hydrolyzed obtained and peptides contained 2017.
40. Margalef, M.; Pons, Z.; Iglesias-Carres, L.; Arola, L.; Muguerza, B.; Arola-Arnal, A. Gender-related similarities and differences in the body distribution of grape seed flavanols in rats. Mol. Nutr. Food Res. 2016, 60, 760–772.
41. Karlsen, A.; Retterstøl, L.; Laake, P.; Paur, I.; Kjølsrud-Bøhn, S.; Sandvik, L.; Blomhoff, R. Anthocyanins Inhibit Nuclear Factor-κB Activation in Monocytes and Reduce Plasma Concentrations of Pro-Inflammatory Mediators in Healthy Adults. J. Nutr. 2007, 137, 1951–1954.
42. Buñag, R.D.; Butterfield, J. Tail-cuff blood pressure measurement without external preheating in awake rats. Hypertension 1982, 4, 898–903.
43. Bunag, R.D. Validation in awake rats of a tail-cuff method for measuring systolic pressure. J. Appl. Physiol. 1973, 34, 279–282.
44. Castro, C.; Briggs, W.; Paschos, G.K.; Fitzgerald, G.A.; Griffin, J.L. A metabolomic study of adipose tissue in mice with a disruption of the circadian system. Mol. Biosyst. 2015, 11, 1897–1906.
45. Cornier, M.-A.; Dabelea, D.; Hernandez, T.L.; Lindstrom, R.C.; Steig, A.J.; Stob, N.R.; Van Pelt, R.E.; Wang, H.; Eckel, R.H. The Metabolic Syndrome. Endocr. Rev. 2008, 29, 777–822.
46. Perk, J.; De Backer, G.; Gohlke, H.; Graham, I.; Reiner, Z.; Verschuren, M.; Albus, C.; Benlian, P.; Boysen, G.; Cifkova, R.; et al. European Guidelines on cardiovascular disease prevention in clinical practice (version 2012): The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by re. Eur. Heart J. 2012, 33, 1635–1701.
47. Garralda-Del-Villar, M.; Carlos-Chillerón, S.; Diaz-Gutierrez, J.; Ruiz-Canela, M.; Gea, A.; Martínez-González, M.A.; Bes-Rastrollo, M.; Ruiz-Estigarribia, L.; Kales, S.N.; Fernández-Montero, A. Healthy Lifestyle and Incidence of Metabolic Syndrome in the SUN Cohort. Nutrients 2018, 11, 65.
48. M., G.S.; Barbara, H.; C., S.S.; I., C.J.; A., K.R.; null, null Clinical Management of Metabolic Syndrome. Circulation 2004, 109, 551–556.
49. Rondanelli, M.; Opizzi, A.; Perna, S.; Faliva, M.; Solerte, S.B.; Fioravanti, M.; Klersy, C.; Edda, C.; Maddalena, P.; Luciano, S.; et al. Improvement in insulin resistance and favourable changes in plasma inflammatory adipokines after weight loss associated with two months' consumption of a combination of bioactive food ingredients in overweight subjects. Endocrine 2013, 44, 391–401.
50. Liu, R.H. Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action. J. Nutr. 2004, 134, 3479S-3485S.
51. Wang, F.; Zhao, S.; Li, F.; Zhang, B.; Qu, Y.; Sun, T.; Luo, T.; Li, D. Investigation of antioxidant interactions between radix Astragali and Cimicifuga foetida and identification of synergistic antioxidant compounds. PLoS One 2014, 9.
52. Carpéné, C.; Gomez-Zorita, S.; Gupta, R.; Grès, S.; Rancoule, C.; Cadoudal, T.; Mercader, J.; Gomez, A.; Bertrand, C.; Iffiu-Soltész, Z. Combination of low dose of the anti-adipogenic agents resveratrol and phenelzine in drinking water is not sufficient to prevent obesity in very-high-fat diet-fed mice. Eur. J. Nutr. 2014, 1625–1635.
53. Arias, N.; Macarulla, M.T.; Aguirre, L.; Martínez-Castaño, M.G.; Gómez-Zorita, S.; Miranda, J.; Martínez, J.A.; Portillo, M.P. The combination of resveratrol and conjugated linoleic acid is not useful in preventing obesity. J. Physiol. Biochem. 2011, 67, 471–477.
54. Pons, Z.; Guerrero, L.; Margalef, M.; Arola, L.; Arola-Arnal, A.; Muguerza, B. Effect of low molecular grape seed proanthocyanidins on blood pressure and lipid homeostasis in cafeteria diet-fed rats. J. Physiol. Biochem. 2014, 70, 629–637.
55. Aragonès, G.; Suárez, M.; Ardid-Ruiz, A.; Vinaixa, M.; Rodríguez, M.A.; Correig, X.; Arola, L.; Bladé, C. Dietary proanthocyanidins boost hepatic NAD+ metabolism and SIRT1 expression and activity in a dose-dependent manner in healthy rats. Sci. Rep. 2016, 6, 24977.

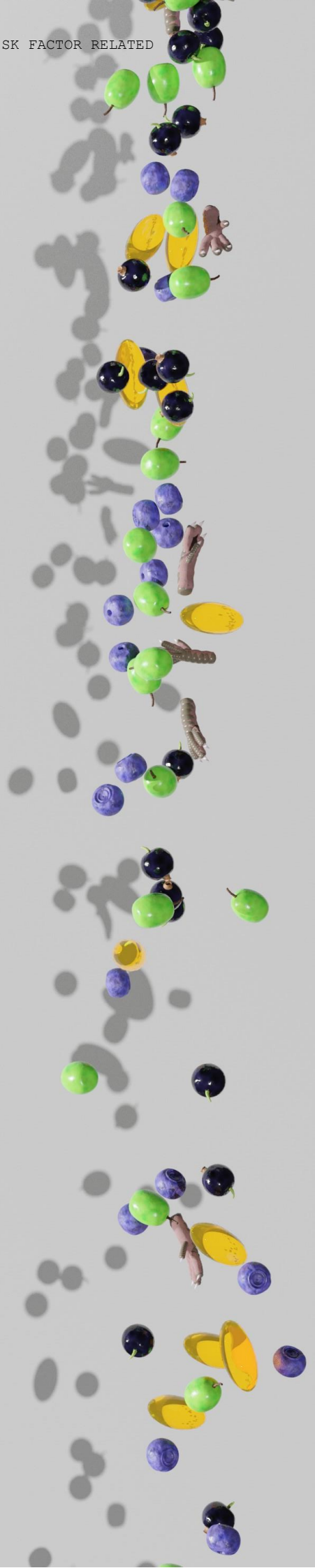
56. Del Bas, J.M.; Ricketts, M.-L.; Vaqué, M.; Sala, E.; Quesada, H.; Ardevol, A.; Salvadó, M.J.; Blay, M.; Arola, L.; Moore, D.D.; et al. Dietary procyanidins enhance transcriptional activity of bile acid-activated FXR in vitro and reduce triglyceridemia in vivo in a FXR-dependent manner. Mol. Nutr. Food Res. 2009, 53, 805–814.
57. Del Bas, J.M.; Ricketts, M.L.; Baiges, I.; Quesada, H.; Ardevol, A.; Salvadó, M.J.; Pujadas, G.; Blay, M.; Arola, L.; Bladé, C.; et al. Dietary procyanidins lower triglyceride levels signaling through the nuclear receptor small heterodimer partner. Mol. Nutr. Food Res. 2008, 52, 1172–1181.
58. Pascual-Serrano, A.; Arola-Arnal, A.; Suárez-García, S.; Bravo, F.I.; Suárez, M.; Arola, L.; Bladé, C. Grape seed proanthocyanidin supplementation reduces adipocyte size and increases adipocyte number in obese rats. Int. J. Obes. 2017, 41, 1246–1255.
59. Pinent, M.; Bladé M. Cinta; Salvadó, M.J.; Arola, L.; Ardévol, A. Intracellular Mediators of Procyanidin-Induced Lipolysis in 3T3-L1 Adipocytes. J. Agric. Food Chem. 2005, 53, 262–266.
60. Salvadó, M.J.; Casanova, E.; Fernández-Iglesias, A.; Arola, L.; Bladé, C. Roles of proanthocyanidin rich extracts in obesity. Food Funct. 2015, 6, 1053–1071.
61. Ardévol, A.; Motilva, M.J.; Serra, A.; Blay, M.; Pinent, M. Procyanidins target mesenteric adipose tissue in Wistar lean rats and subcutaneous adipose tissue in Zucker obese rat. Food Chem. 2013, 141, 160–166.
62. Ginés, I.; Gil-Cardoso, K.; Serrano, J.; Casanova-Martí, À.; Blay, Mt.; Pinent, M.; Ardévol, A.; Terra, X. Effects of an intermittent grape-seed proanthocyanidin (GSPE) treatment on a cafeteria diet obesogenic challenge in rats. Nutrients 2018, 10.
63. Serrano, J.; Casanova-Martí, À.; Gual, A.; Pérez-Vendrell, A.M.; Blay, M.T.; Terra, X.; Ardévol, A.; Pinent, M. A specific dose of grape seed-derived proanthocyanidins to inhibit body weight gain limits food intake and increases energy expenditure in rats. Eur. J. Nutr. 2017, 56, 1629–1636.
64. Cimino, F.; Speciale, A.; Anwar, S.; Canali, R.; Ricciardi, E.; Virgili, F.; Trombetta, D.; Saija, A. Anthocyanins protect human endothelial cells from mild hyperoxia damage through modulation of Nrf2 pathway. Genes Nutr. 2013, 8, 391–399.
65. Luo, X.; Fang, S.; Xiao, Y.; Song, F.; Zou, T.; Wang, M.; Xia, M.; Ling, W. Cyanidin-3-glucoside suppresses TNF-α-induced cell proliferation through the repression of Nox activator 1 in mouse vascular smooth muscle cells: involvement of the STAT3 signaling. Mol. Cell. Biochem. 2012, 362, 211–218.
66. Zhu, Y.; Ling, W.; Guo, H.; Song, F.; Ye, Q.; Zou, T.; Li, D.; Zhang, Y.; Li, G.; Xiao, Y.; et al. Anti-inflammatory effect of purified dietary anthocyanin in adults with hypercholesterolemia: A randomized controlled trial. Nutr. Metab. Cardiovasc. Dis. 2013, 23, 843–849.
67. Zhang, P.-W.; Chen, F.-X.; Li, D.; Ling, W.-H.; Guo, H.-H. A CONSORT-compliant, randomized, double-blind, placebo-controlled pilot trial of purified anthocyanin in patients with nonalcoholic fatty liver disease. Medicine (Baltimore). 2015, 94, e758.
68. Li, D.; Zhang, Y.; Liu, Y.; Sun, R.; Xia, M. Purified Anthocyanin Supplementation Reduces Dyslipidemia, Enhances Antioxidant Capacity, and Prevents Insulin Resistance in Diabetic Patients. J. Nutr. 2015, 145, 742–748.
69. Pinto Júnior, D.A.C.; Seraphim, P.M. Cafeteria diet intake for fourteen weeks can cause obesity and insulin resistance in wistar rats. Rev. Nutr. 2012, 25, 313–319.
70. Quesada, H.; del Bas, J.M.; Pajuelo, D.; Díaz, S.; Fernandez-Larrea, J.; Pinent, M.; Arola, L.; Salvadó, M.J.; Bladé, C. Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. Int. J. Obes. 2009, 33, 1007–1012.
71. Asbaghi, O.; Nazarian, B.; Reiner, Ž.; Amirani, E.; Kolahdooz, F.; Chamani, M.; Asemi, Z. The effects of grape seed extract on glycemic control, serum lipoproteins, inflammation, and body weight: A systematic review and meta-analysis of randomized controlled trials. Phyther. Res. 2019.
72. Ardid-Ruiz, A.; Ibars, M.; Mena, P.; Del Rio, D.; Muguerza, B.; Bladé, C.; Arola, L.; Aragonès, G.; Suárez, M. Potential Involvement of Peripheral Leptin/STAT3 Signaling in the Effects of Resveratrol and Its Metabolites on Reducing Body Fat Accumulation. Nutrients 2018, 10, 1757.
73. Bladé, C.; Arola, L.; Salvadó, M.-J. Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. Mol. Nutr. Food Res. 2010, 54, 37–59.
74. Pickens, C.A.; Yin, Z.; Sordillo, L.M.; Fenton, J.I. Arachidonic acid-derived hydroxyeicosatetraenoic acids are positively associated with colon polyps in adult males : a cross- sectional study. Sci. Rep. 2019, 1–10.
75. Eisinger, K.; Liebisch, G.; Schmitz, G.; Aslanidis, C.; Krautbauer, S.; Buechler, C. Lipidomic analysis of serum from high fat diet induced obese mice. Int. J. Mol. Sci. 2014, 15, 2991–3002.

76. Heilbronn, L.K.; Coster, A.C.F.; Campbell, L. V.; Greenfield, J.R.; Lange, K.; Christopher, M.J.; Meikle, P.J.; Samocha-Bonet, D. The effect of short-term overfeeding on serum lipids in healthy humans. Obesity 2013, 21.
77. Lee, Y.J.; Lee, A.; Yoo, H.J.; Kim, M.; Kim, M.; Jee, S.H.; Shin, D.Y.; Lee, J.H. Effect of weight loss on circulating fatty acid profiles in overweight subjects with high visceral fat area: A 12-week randomized controlled trial. Nutr. J. 2018, 17, 1–14.
78. Duncan, R.E.; Ahmadian, M.; Jaworski, K.; Sarkadi-Nagy, E.; Sul, H.S. Regulation of lipolysis in adipocytes. Annu. Rev. Nutr. 2007, 27, 79–101.
79. Martins, S. V; Lopes, P.A.; Alves, S.P.; Alfaia, C.M.; Castro, M.F.; Bessa, R.J.B.; Prates, J.A.M. Dietary CLA Combined with Palm Oil or Ovine Fat Differentially Influences Fatty Acid Deposition in Tissues of Obese Zucker Rats. Lipids 2012, 47, 47–58.
80. Demaree, S.R.; Gilbert, C.D.; Mersmann, H.J.; Smith, S.B. Conjugated Linoleic Acid Differentially Modifies Fatty Acid Composition in Subcellular Fractions of Muscle and Adipose Tissue but Not Adiposity of Postweanling Pigs. J. Nutr. 2002, 132, 3272–3279.
81. Cho, K.; Song, Y.; Kwon, D. Conjugated linoleic acid supplementation enhances insulin sensitivity and peroxisome proliferator-activated receptor gamma and glucose transporter type 4 protein expression in the skeletal muscles of rats during endurance exercise. Iran. J. Basic Med. Sci. 2016, 19, 20–27.
82. Serena, C.; Ceperuelo-Mallafré, V.; Keiran, N.; Queipo-Ortuño, M.I.; Bernal, R.; Gomez-Huelgas, R.; Urpi-Sarda, M.; Sabater, M.; Pérez-Brocal, V.; Andrés-Lacueva, C.; et al. Elevated circulating levels of succinate in human obesity are linked to specific gut microbiota. ISME J. 2018, 12, 1642–1657.
83. Wang, K.; Liao, M.; Zhou, N.; Bao, L.; Ma, K.; Zheng, Z.; Wang, Y.; Liu, C.; Wang, W.; Wang, J.; et al. Parabacteroides distasonis Alleviates Obesity and Metabolic Dysfunctions via Production of Succinate and Secondary Bile Acids. Cell Rep. 2019, 26, 222-235.e5.
84. de Vadder, F.; Mithieux, G. Gut-brain signaling in energy homeostasis: The unexpected role of microbiota-derived succinate. J. Endocrinol. 2018, 236, R105–R108.
85. Moreno, J.; Alegría, E.; Cordero Fort, A. Hipertensión arterial y síndrome metabólico. Rev. Española Cardiol. 2005, 5, 38–45.
86. Collaboration, B.P.L.T.T. Effects of Different Blood Pressure–Lowering Regimens on Major Cardiovascular Events in Individuals With and Without Diabetes Mellitus: Results of Prospectively Designed Overviews of Randomized Trials. Arch. Intern. Med. 2005, 165, 1410–1419.
87. Inoue, N.; Nagao, K.; Hirata, J.; Wang, Y.-M.; Yanagita, T. Conjugated linoleic acid prevents the development of essential hypertension in spontaneously hypertensive rats. Biochem. Biophys. Res. Commun. 2004, 323, 679–684.
88. SHINDO, M.; KASAI, T.; ABE, A.; KONDO, Y. Effects of Dietary Administration of Plant-Derived Anthocyanin-Rich Colors to Spontaneously Hypertensive Rats. J. Nutr. Sci. Vitaminol. (Tokyo). 2007, 53, 90–93.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

GENERAL DISCUSSION



UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

General discussion

The current spread of obesity in western countries has raised alarms and led to its classification as an epidemic by the World Health Organization [1]. While obesity is usually seen as a sole pathology, in reality, the excess of body weight correlates with other diseases such as type 2 diabetes, inflammation, MetS or NAFLD [2]. Regarding obesity, concretely, the abnormal growth of the abdominal WAT is considered a symptom of what is known as MetS, another disease of widespread and increasing occurrence [3]. Other components related to this disease are hypertriglyceridemia, reduced HDL-C, hyperglycemia and hypertension. MetS is considered when at least three of these symptoms are present [4].

The origins and development of obesity can be understood as an imbalance between energy intake and energy expenditure, being fat, the main form the body has to store this excess of energy, but this pathology has a more complex basis. The ectopic accumulation of fat, once the disease of obesity starts, predominantly occurs in the abdominal WAT, thus, why waist circumference is measured to assess the significance of its development [5]. Excess of energy in the WAT is mainly stored in the form of triglycerides, which, correlating with the hypertrophy the tissue develops, tend to be raised in the bloodstream [6]. This hypertriglyceridemia has also been linked with ectopic fat accumulations in other organs such as liver, which might promote the development of NAFLD and further worsening with inflammation or fibrosis [7]. Furthermore, obesity-induce dyslipidaemia alters the modulation of a wide set of metabolites [8]. Among the alterations, HDL-C concentration is lowered in MetS [9]. HDL-C is responsible for the transportation of cholesterol, from peripheral organs, to the liver for its removal and metabolization. HDL-C dysregulated concentration in the MetS has been reported to be positively correlated with the incidence of CVD and endothelial dysfunction [10,11]. In turn, the impaired endothelial function is closely related to HTN, which, although also can be present in lean individuals, has been correlated with elevated waist WAT and the chronic low-level state of inflammation that tends to be present in obesity [12]. The study of HTN in the MetS has found that the hyperphagia associated with obesity over-activates the RAAS, an important regulator of BP [13]. Besides, chronic inflammation, frequent in MetS and, thus, elevated circulatory concentration of inflammatory cytokines such as TNF-α or IL-6, has also been associated with an increment in the production of vasoconstrictor factors [14]. Finally, elevated glucose in MetS has also been associated with type II diabetes and insulin resistance [15]. The development of insulin resistance is reported to be closely related with elevated waist circumference as WAT will release NEFA, which promotes the formation of diglycerides in organs such as liver or skeletal muscle [16]. In turn, diglycerides accumulation have been reported to disrupt an adequate insulin signalling, inducing insulin resistance [17].

Lifestyle modifications and improvements in healthier food consumption are considered the first and most needed step to combat obesity and MetS [18]. Studies in humans have shown the importance of an adequate diet showing improvements with diets low on carbohydrates or lipids [19]. However, persistence in the long-term is key for amelioration of obesity and patients tend to not follow procedures for long [20]. Of particular success is the Mediterranean diet that has shown to improve factors of the MetS [21]. In addition, several pharmacological approaches and bioactive compounds have been investigated to address MetS, all of them aimed to tackle specific symptoms of this disease. Flavonoids anthocyanidins and proanthocyanidins, protein hydrolysates and CLA are some of the bioactive compounds that have shown beneficial effects in some of the components associated with MetS.

Medox is a berry-derived supplement rich in anthocyanidins, which has been demonstrated to modulate different symptoms related to MetS, increasing the HDL-C levels and improving inflammation [22,23]. Several other beneficial properties have also been associated with Medox. Studies with diabetic patients have related this extract to a reduction of insulin resistance and fasting glucose [24]. In addition, other studies have found reductions in markers of NAFLD and confirmed improvements of glucose homeostasis [25].

The grape seed extract GSPE is rich in proanthocyanidins, which has been reported to reduce serum and hepatic triglyceride levels [26] and to module other of the cardiometabolic risk factors related to MetS. Specifically, the grape seed extract improves the lipid metabolism by promoting hepatic fatty acid oxidation and reducing lipogenesis [27], while also inducing lipolysis in WAT [28]. GSPE is also known to improve other symptoms of the MetS such as reduced HDL-C concentration, elevated circulatory glucose and hypertension. HDL-C has been found to increase after GSPE administration, via increasing the expression of *Abca1* [29]. In addition, GSPE was found to lower glucose in acute studies with diabetic rats [30] and to increase the expression of *Glut4* in obese rats with chronic supplementation [31]. Finally, GSPE has been proven to exhibit antihypertensive activity in short and long-term administration in both SHR and CAF-induced hypertensive rats [32–34]. In this regard, the chicken feet hydrolysate Hpp11 has also been reported as a potent vasodilator and effective to combat hypertension [35]. In this sense, Hpp11 has been demonstrated to display ACE inhibitory activity, [36] and to increase the release of the vasodilator factor NO [37].

CLA is a dietary supplement of proven efficacy to combat obesity and lower fat mass, although its effects vary depending on species [38,39]. However, a meta-analysis of human trials concluded that at an average dose of 3.2 g per day of CLA reduces body fat mass [40]. The CLA effect on body weight has been mostly linked with the isomer *t10,c12* [41], whereas *c9,t11* have shown to ameliorate insulin sensitivity and inflammation [42]. Several molecular mechanisms have been associated with the

reduction of fat mass. An activation of basal metabolism via an increase of thermogenesis has been reported with *t10,c12* supplementation [43]. This has also been linked with modulation of the lipid metabolism by activation fatty acid oxidation and promoting the release of lipids from WAT [44]. However, some studies have linked CLA supplementation with an increase in the incidence of developing NAFLD [45]. This adverse effect of CLA has been associated with the isomer *t10,c12*, which have been found to increase the expression of channels like *Cd36* in mice [46] and promote lipogenesis [47]. Nevertheless, most animal studies have been performed with high doses of approximately 1% CLA in the diet [48]. Consequently, the results in animal studies might not be comparable with those obtained in human studies since much higher CLA doses are used in preclinical trials than the doses in clinical trials [49]. Then, a study with low doses of an equal ratio of both *t10,c12* and *c9,t11* CLA isomers was performed to assess their effect on body weight reduction and rule out any potential adverse effect. Rats fed CAF were used as an experimental model of human MetS. The results indicated that the lowest dose of 100 mg/kg was able to achieve a reduction on body weight gain **[Manuscript 1]**. In addition, other serum and liver metabolites related to the glucose homeostasis were found improved in the CLA treated group. In this sense, insulin levels were reduced, reaching the values of STD rats. The metabolomics analysis of liver and serum by ^{1}H-NMR revealed also a normalization of the serum and hepatic glucose levels. Hepatic alanine and lactate were reduced to the STD levels as well, suggesting a reduction of gluconeogenesis [50]. In addition, lower levels of diglycerides in liver, which have been associated with improvements in insulin resistance, were found in the animals treated with CLA, compared with those administered VH [51].

Besides, the lipid metabolism of the animals treated showed an improvement of lipid homeostasis. The study of serum by ^{1}H-NMR found reductions of various serum lipids such as triglycerides, diglycerides, total phospholipid and oleic acid, among others, agreeing with previous studies with using higher CLA doses [52]. Remarkably, although enhanced hepatic cholesterol accumulation was also observed in CLA treated group, metabolomics and histopathological studies of liver showed no worsening of hepatic steatosis. In fact, hepatic triglycerides levels were not increased in these animals and histologically hepatic steatosis were equivalent to the CAF group. In addition, quantification of GOT and GPT showed a normalization of the values found in the CLA treated rats with respect to STD animals, indicating there was no increased hepatic damage with CLA supplementation. Thus, the administration of low doses of a mixture of CLA isomers for 3 weeks produced a decrease in the body weight gain in animals fed a CAF diet. In addition, an improvement in glucose and lipid metabolisms were found.

As mentioned, many dietary supplements have been investigated as strategies to prevent the development and onset of obesity and MetS. These compounds have gained interest in recent years

because, given their natural food origins, they are usually considered as harmless and are safer in their supplementation than classical pharmaceutical compounds [53–55]. This has prompted scientific interest as an alternative medicinal approach to prevent or ameliorate some of the cardiometabolic risk factors related to MetS [56,57]. However, several studies have evidenced that the use of a single family of bioactive compounds is usually not sufficient to correct complex, multisystemic and highly regulated processes such as body weight and all its metabolic associated pathologies. Thus, it is plausible to hypothesize that the simultaneous administration of different bioactive compounds could promote the management of body weight and other cardiometabolic risk factors of MetS in a much more effective way than its individual administration.

In this context, we developed a multifunctional ingredient or MIX consisting in different natural dietary bioactive compounds that have individually shown to exert certain beneficial effects against different targets associated with MetS. In particular, MIX was based on the co-administration of the same low dose of CLA that we had previously studied (100 mg/Kg), together with the protein hydrolysate from chicken feet Hpp11 (55 mg/Kg) [37], and the flavonoids proanthocyanidins extracted from grape seeds (25 mg/Kg) [58,59] and anthocyanins from berries (100 mg/Kg) [60,61]. Thus, the purpose of our next study was to determine whether the co-administration of this dietary multifunctional ingredient was able to greater manage the body weight in animals fed a CAF diet. The results showed that the co-administration of different bioactive compounds in a single multifunctional ingredient promoted a much more effective body weight management than the individual administration of CLA at the same dose than the used in the MIX [**Manuscript 2**] and [**Patent 1**]. In particular, the administration of the MIX for three weeks resulted in a marked reduction in both body weight and fat mass without any impact on lean body mass. This protection against lean body mass loss, is of great value since several studies reported that loss in lean body mass could have multiple negative health implications [62]. The results also showed that the administration of the MIX did not exacerbate the liver injury and hepatic triglycerides accumulation of CAF diet fed rats, although some hepatic lipid concentrations were affected by MIX administration, most notably total and esterified cholesterol concentrations. However, the hepatic histopathological analyses confirmed no signs of liver damage aggravation in animals supplemented with the MIX, and even a lower serum transaminases activity was observed with respect to non-supplemented animals.

The results also showed an increase in energy expenditure and fat utilization as energetic substrate in animals administered MIX, resulting in a reduced energy balance compared to the control animals. Remarkably, food intake and intestinal lipid absorption were not affected by the administration of the MIX. Supporting these results, different studies with CLA, anthocyanins and GSPE demonstrated the ability of these bioactive compounds in inducing fat oxidation in obese animals [63–

65]. Conversely, only CLA supplementation showed an increased energy expenditure in these animals [65,66]. Thus, it is plausible to mainly attribute the observed differences in energy expenditure to the CLA although the effects on fat oxidation could be probably caused by a synergic effect of the co-administration of CLA with the other functional ingredients.

Finally, the impact of MIX administration on leptin system was also examined since this hormone is reported to maintain energy balance in mammals. Our results showed that MIX administration restored serum leptin values and increased the leptin sensitivity of the first-order neurons in the hypothalamus of CAF fed animals. This improvement of central leptin sensitivity could be behind the decreased body weight and fat mass as well as the increased energy expenditure observed in animals supplemented with the MIX. The induction of central leptin resistance in diet-induced obesity has been mainly attributed to hypothalamic inflammation as a result of the induction of pro-inflammatory signalling molecules such as JNK, NF-κB [67,68]. Remarkably, previous results demonstrated that GSPE supplementation reduced the hypothalamic inflammation [69], suggesting that this local anti-inflammatory activity of proanthocyanidins in this tissue could be one of the mechanisms by which MIX administration could restore hypothalamic leptin signalling. In addition, Sirtuin-1 activity has been also highlighted as mediator of hypothalamic leptin action [70,71], thus, the up-regulation of the of Sirtuin-1 activity reported for GSPE supplementation at 25 mg/Kg [27] could be another mechanism by which MIX administration reduced hypothalamic leptin resistance. However, further studies are needed in order to elucidate the molecular mechanism by which MIX administration re-establishes central leptin sensitivity in CAF diet fed animals.

Besides obesity, the effect of MIX over other cardiometabolic risk factors related to MetS were investigated. The results showed beneficial effects of MIX administration on glucose and lipid metabolisms. In addition, MIX exhibited BP lowering properties **[Manuscript 3].** The oral lipid tolerance test carried out 16 days after starting MIX administration showed that the animals presented lower initial triglycerides levels. In addition, MIX treated rats displayed an increase of triglycerides in the bloodstream smoother after lard administration. These findings, were in accordance with the results reported by previous studies administrating GSPE to animals [72] and humans [73]. The MIX triglycerides lowering effect is also in accordance with the presence of anthocyanidins in the MIX, since Medox supplementation to subjects with type 2 diabetes caused a 23 percent reduction of this lipid in serum [24]. The metabolomics study carried out in serum and liver also showed differences between animals administered VH and MIX. The animals MIX treated showed a decrease in many metabolites such as serum PUFAs, oleic acid and phosphatidylcholine and iWAT levels of triglycerides, oleic and linoleic acids and total fatty acids, among others, suggesting an improvement in lipid management. Serum total and free cholesterol measured by metabolomics were

also found decreased in the MIX group. However, no changes were observed in HDL-C or LDL-C at the end of the experimental period, although an increase in serum HDL-C was expected in the MIX treated rats, since high levels of HDL-C has been reported after Medox and GSPE administration [24,74]. A short fasting time of the animals before their sacrifice could explain these findings, since no changes were also found in triglycerides at the end of the experiment. Further analysis of the aqueous phase of serum provided us with a stronger picture of the changes MIX administration produced as statistical analysis by PLS-DA showed that MIX was significantly changing the overall profile of serum, hence, putatively inducing major changes and corrections on the whole-body metabolism.

The study of the glucose metabolism showed that MIX administration did not alter fasting glucose concentration, but insulin levels were significantly reduced and the insulin resistance indices HOMA-IR, HOMA-β and QUICKI were also lower, showing that MIX administration was improving insulin sensitivity [75,76]. In addition, to the observed effect on lipid and glucose metabolisms, MIX exhibited a clear antihypertensive effect, achieving the treated rats normotensive values of BP. These results are in accordance with the BP lowering properties of CLA, GSPE and Hpp11 [34,37,77], and the improvement of endothelial function reported by anthocyanidins [60,78].

Therefore, the results of this Thesis demonstrated that the administration of an equal ratio of the CLA isomers *c9,t11* and *t10,c12* at low doses decreased the body weight gain, induced by a cafeteria diet, and improved other cardiometabolic risk factors, without presenting any CLA-related detrimental effects. Moreover, the body weight lowering effect of CLA was higher when it was co-administered with the chicken feet hydrolysate Hpp11 and the flavonoids proanthocyanidins and anthocyanidins as a multifunctional ingredient. In addition, this body weight lowering effect on body weight, which could be mediated by an improvement of hypothalamic leptin sensitivity, was accompanied by an improvement on glucose and lipid metabolism and antihypertensive activity. Thus, MIX could be a good candidate to be used as nutraceutical or to be included in functional foods for the management of metabolic syndrome

References

1. James, W.P.T. WHO recognition of the global obesity epidemic. *Int. J. Obes.* **2008**, *32*, S120–S126.
2. Martínez-Larrad, M.T.; Corbatón Anchuelo, A.; Del Prado, N.; Ibarra Rueda, J.M.; Gabriel, R.; Serrano-Ríos, M. Profile of Individuals Who Are Metabolically Healthy Obese Using Different Definition Criteria. A Population-Based Analysis in the Spanish Population. *PLoS One* **2014**, *9*, e106641.
3. Saklayen, M.G. The Global Epidemic of the Metabolic Syndrome. *Curr. Hypertens. Rep.* **2018**, *20*, 12.
4. Eckel, R.H.; Grundy, S.M.; Zimmet, P.Z. The metabolic syndrome. *Lancet* **2005**, *365*, 1415–1428.
5. Cameron, A.J.; Magliano, D.J.; Söderberg, S. A systematic review of the impact of including both waist and hip circumference in risk models for cardiovascular diseases, diabetes and mortality. *Obes. Rev.* **2013**, *14*, 86–94.
6. Klop, B.; Wouter Jukema, J.; Rabelink, T.J.; Castro Cabezas, M. A physician's guide for the management of hypertriglyceridemia: the etiology of hypertriglyceridemia determines treatment strategy. *Panminerva Med.* **2012**, *54*, 91–103.
7. Kanwar, P.; Kowdley, K. V The Metabolic Syndrome and Its Influence on Nonalcoholic Steatohepatitis. *Clin. Liver Dis.* **2016**, *20*, 225–243.
8. Libert, D.M.; Nowacki, A.S.; Natowicz, M.R. Metabolomic analysis of obesity, metabolic syndrome, and type 2 diabetes: Amino acid and acylcarnitine levels change along a spectrum of metabolic wellness. *PeerJ* **2018**, *2018*.
9. Chen, S.Y.; Li, N.; Jin, T.L.; Gou, L.; Hao, D.X.; Tian, Z.Q.; Zhang, S.L.; Zhang, L. Lipoprotein in cholesterol transport: Highlights and recent insights into its structural basis and functional mechanism. *Chinese Phys. B* **2018**, *27*.
10. Gordon, D.J.; Probstfield, J.L.; Garrison, R.J.; Neaton, J.D.; Castelli, W.P.; Knoke, J.D.; Jacobs, D.R.; Bangdiwala, S.; Tyroler, H.A. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation* **1989**, *79*, 8–15.
11. Mineo, C.; Deguchi, H.; Griffin, J.H.; Shaul, P.W. Endothelial and antithrombotic actions of HDL. *Circ. Res.* **2006**, *98*, 1352–1364.
12. Hideki, K.; Tetsuya, Y.; Yoshitomo, O. Adiposity and Cardiovascular Disorders. *Circ. Res.* **2007**, *101*, 27–39.
13. Engeli, S.; Schling, P.; Gorzelniak, K.; Boschmann, M.; Janke, J.; Ailhaud, G.; Teboul, M.; Massiéra, F.; Sharma, A.M. The adipose-tissue renin–angiotensin–aldosterone system: role in the metabolic syndrome? *Int. J. Biochem. Cell Biol.* **2003**, *35*, 807–825.
14. Molet, S.; Furukawa, K.; Maghazechi, A.; Hamid, Q.; Giaid, A. Chemokine- and cytokine-induced expression of endothelin 1 and endothelin-converting enzyme 1 in endothelial cells. *J. Allergy Clin. Immunol.* **2000**, *105*, 333–338.
15. Leitner, D.R.; Frühbeck, G.; Yumuk, V.; Schindler, K.; Micic, D.; Woodward, E.; Toplak, H. Obesity and type 2 diabetes: Two diseases with a need for combined treatment strategies - EASO can lead the way. *Obes. Facts* **2017**, *10*, 483–492.
16. Després, J.-P.; Lemieux, I. Abdominal obesity and metabolic syndrome. *Nature* **2006**, *444*, 881–887.
17. Bjorntorp, P. "Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. *Arteriosclerosis* **1990**, *10*, 493–496.
18. Warburton, D.E.R.; Nicol, C.W.; Bredin, S.S.D. Health benefits of physical activity: the evidence. *CMAJ* **2006**, *174*, 801–809.
19. Pérez-Martínez, P.; Mikhailidis, D.P.; Athyros, V.G.; Bullo, M.; Couture, P.; Covas, M.I.; de Koning, L.; Delgado-Lista, J.; Díaz-López, A.; Drevon, C.A.; et al. Lifestyle recommendations for the prevention and management of metabolic syndrome: an international panel recommendation. *Nutr. Rev.* **2017**, *75*, 307–326.
20. Expert Panel on the Identification and Treatment of Overweight and Obesity in Adults, E. Executive Summary of the Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and

Obesity in Adults. *Arch. Intern. Med.* **1998**, *158*, 1855–1867.

21. Steffen, L.M.; Van Horn, L.; Daviglus, M.L.; Zhou, X.; Reis, J.P.; Loria, C.M.; Jacobs, D.R.; Duffey, K.J. A modified Mediterranean diet score is associated with a lower risk of incident metabolic syndrome over 25 years among young adults: the CARDIA (Coronary Artery Risk Development in Young Adults) study. *Br. J. Nutr.* **2014**, *112*, 1654–1661.
22. Qin, Y.; Xia, M.; Ma, J.; Hao, Y.; Liu, J.; Mou, H.; Cao, L.; Ling, W. Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects. *Am. J. Clin. Nutr.* **2009**, *90*, 485–492.
23. Karlsen, A.; Retterstøl, L.; Laake, P.; Paur, I.; Kjølsrud-Bøhn, S.; Sandvik, L.; Blomhoff, R. Anthocyanins Inhibit Nuclear Factor-κB Activation in Monocytes and Reduce Plasma Concentrations of Pro-Inflammatory Mediators in Healthy Adults. *J. Nutr.* **2007**, *137*, 1951–1954.
24. Li, D.; Zhang, Y.; Liu, Y.; Sun, R.; Xia, M. Purified Anthocyanin Supplementation Reduces Dyslipidemia, Enhances Antioxidant Capacity, and Prevents Insulin Resistance in Diabetic Patients. *J. Nutr.* **2015**, *145*, 742–748.
25. Zhang, P.-W.; Chen, F.-X.; Li, D.; Ling, W.-H.; Guo, H.-H. A CONSORT-compliant, randomized, double-blind, placebo-controlled pilot trial of purified anthocyanin in patients with nonalcoholic fatty liver disease. *Medicine (Baltimore).* **2015**, *94*, e758.
26. Pons, Z.; Guerrero, L.; Margalef, M.; Arola, L.; Arola-Arnal, A.; Muguerza, B. Effect of low molecular grape seed proanthocyanidins on blood pressure and lipid homeostasis in cafeteria diet-fed rats. *J. Physiol. Biochem.* **2014**, *70*, 629–637.
27. Aragonès, G.; Suárez, M.; Ardid-Ruiz, A.; Vinaixa, M.; Rodríguez, M.A.; Correig, X.; Arola, L.; Bladé, C. Dietary proanthocyanidins boost hepatic NAD+ metabolism and SIRT1 expression and activity in a dose-dependent manner in healthy rats. *Sci. Rep.* **2016**, *6*, 24977.
28. Pascual-Serrano, A.; Arola-Arnal, A.; Suárez-García, S.; Bravo, F.I.; Suárez, M.; Arola, L.; Bladé, C. Grape seed proanthocyanidin supplementation reduces adipocyte size and increases adipocyte number in obese rats. *Int. J. Obes.* **2017**, *41*, 1246–1255.
29. Fernández-Hernando, C.; Moore, K.J. MicroRNA modulation of cholesterol homeostasis. *Arterioscler. Thromb. Vasc. Biol.* **2011**, *31*, 2378–2382.
30. Pinent, M.; Blay, M.; Bladé, M.C.; Salvadó, M.J.; Arola, L.; Ardévol, A. Grape Seed-Derived Procyanidins Have an Antihyperglycemic Effect in Streptozotocin-Induced Diabetic Rats and Insulinomimetic Activity in Insulin-Sensitive Cell Lines. *Endocrinology* **2004**, *145*, 4985–4990.
31. Ardévol, A.; Motilva, M.J.; Serra, A.; Blay, M.; Pinent, M. Procyanidins target mesenteric adipose tissue in Wistar lean rats and subcutaneous adipose tissue in Zucker obese rat. *Food Chem.* **2013**, *141*, 160–166.
32. Quiñones, M.; Guerrero, L.; Suarez, M.; Pons, Z.; Aleixandre, A.; Arola, L.; Muguerza, B. Low-molecular procyanidin rich grape seed extract exerts antihypertensive effect in males spontaneously hypertensive rats. *Food Res. Int.* **2013**, *51*, 587–595.
33. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Acute administration of single oral dose of grape seed polyphenols restores blood pressure in a rat model of metabolic syndrome: role of nitric oxide and prostacyclin. *Eur. J. Nutr.* **2016**, *55*, 749–758.
34. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Chronic administration of grape-seed polyphenols attenuates the development of hypertension and improves other cardiometabolic risk factors associated with the metabolic syndrome in cafeteria diet-fed rats. *Br. J. Nutr.* **2017**, *117*, 200–208.
35. Mas-Capdevila, A.; Pons, Z.; Aleixandre, A.; Bravo, F.I.; Muguerza, B. Dose-Related Antihypertensive Properties and the Corresponding Mechanisms of a Chicken Foot Hydrolysate in Hypertensive Rats. *Nutrients* **2018**, *10*, 1295.
36. Bravo, F.I.; Mas-Capdevila, A.; Margalef, M.; Arola-Arnal, A.; Muguerza, B. Novel Antihypertensive Peptides Derived from Chicken Foot Proteins. *Mol. Nutr. Food Res.* **2019**, *63*, 1801176.
37. Mas-Capdevila, A.; Iglesias-Carres, L.; Arola-Arnal, A.; Suarez, M.; Muguerza, B.; Bravo, F.I. Long-term

administration of protein hydrolysate from chicken feet induces antihypertensive effect and confers vasoprotective pattern in diet-induced hypertensive rats. *J. Funct. Foods* **2019**, *55*, 28–35.

38. Takahashi, Y.; Kushiro, M.; Shinohara, K.; Ide, T. Dietary conjugated linoleic acid reduces body fat mass and affects gene expression of proteins regulating energy metabolism in mice. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* **2002**, *133*, 395–404.
39. Jobgen, W.; Meininger, C.J.; Jobgen, S.C.; Li, P.; Lee, M.-J.; Smith, S.B.; Spencer, T.E.; Fried, S.K.; Wu, G. Dietary l-Arginine Supplementation Reduces White Fat Gain and Enhances Skeletal Muscle and Brown Fat Masses in Diet-Induced Obese Rats. *J. Nutr.* **2008**, *139*, 230–237.
40. Whigham, L.D.; Watras, A.C.; Schoeller, D.A. Efficacy of conjugated linoleic acid for reducing fat mass: a meta-analysis in humans. *Am. J. Clin. Nutr.* **2007**, *85*, 1203-1211 9p.
41. Pariza, M.W.; Park, Y.; Cook, M.E. The biologically active isomers of conjugated linoleic acid. *Prog. Lipid Res.* **2001**, *40*, 283–298.
42. Taylor, C.G.; Zahradka, P. Dietary conjugated linoleic acid and insulin sensitivity and resistance in rodent models. *Am. J. Clin. Nutr.* **2004**, *79*, 1164–1168.
43. Terpstra, A.H.M.; Javadi, M.; Beynen, A.C.; Kocsis, S.; Lankhorst, A.E.; Lemmens, A.G.; Mohede, I.C.M. Dietary Conjugated Linoleic Acids as Free Fatty Acids and Triacylglycerols Similarly Affect Body Composition and Energy Balance in Mice. *J. Nutr.* **2003**, *133*, 3181–3186.
44. Ma, L.; Lengi, A.J.; McGilliard, M.L.; Bauman, D.E.; Corl, B.A. Short communication: Effect of trans-10,cis-12 conjugated linoleic acid on activation of lipogenic transcription factors in bovine mammary epithelial cells. *J. Dairy Sci.* **2014**, *97*, 5001–5006.
45. Vyas, D.; Kadegowda, A.K.G.; Erdman, R.A. Dietary conjugated linoleic acid and hepatic steatosis: Species-specific effects on liver and adipose lipid metabolism and gene expression. *J. Nutr. Metab.* **2012**, 13.
46. Degrace, P.; Moindrot, B.; Mohamed, I.; Gresti, J.; Du, Z.Y.; Chardigny, J.M.; Sébédio, J.L.; Clouet, P. Upregulation of liver VLDL receptor and FAT/CD36 expression in LDLR -/- apoB100/100 mice fed trans-10,cis-12 conjugated linoleic acid. *J. Lipid Res.* **2006**, *47*, 2647–2655.
47. Takahashi, Y.; Kushiro, M.; Shinohara, K.; Ide, T. Activity and mRNA levels of enzymes involved in hepatic fatty acid synthesis and oxidation in mice fed conjugated linoleic acid. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **2003**, *1631*, 265–273.
48. Lada, A.T.; Rudel, L.L.; St. Clair, R.W. Effects of LDL enriched with different dietary fatty acids on cholesteryl ester accumulation and turnover in THP-1 macrophages. *J. Lipid Res.* **2003**, *44*, 770–779.
49. Benjamin, S.; Prakasan, P.; Sreedharan, S.; Wright, A.D.; Spener, F. Pros and cons of CLA consumption: An insight from clinical evidences. *Nutr. Metab.* **2015**.
50. Petersen, M.C.; Vatner, D.F.; Shulman, G.I. Regulation of hepatic glucose metabolism in health and disease. *Nat. Rev. Endocrinol.* **2017**, *13*, 572–587.
51. Arnold, R.S.; Newton, A.C. Diacylglycerol directly stimulates the insulin receptor tyrosine kinase. *FEBS Lett.* **1996**, *380*, 58–62.
52. Kloss, R.; Linscheid, J.; Johnson, A.; Lawson, B.; Edwards, K.; Linder, T.; Stocker, K.; Petitte, J.; Kern, M. Effects of conjugated linoleic acid supplementation on blood lipids and adiposity of rats fed diets rich in saturated versus unsaturated fat. *Pharmacol. Res.* **2005**, *51*, 503–507.
53. De la Iglesia, R.; Loria-Kohen, V.; Zulet, A.M.; Martinez, A.J.; Reglero, G.; Ramirez de Molina, A. Dietary Strategies Implicated in the Prevention and Treatment of Metabolic Syndrome. *Int. J. Mol. Sci.* 2016, *17*.
54. Rangel-Huerta, O.D.; Pastor-Villaescusa, B.; Aguilera, C.M.; Gil, A. A Systematic Review of the Efficacy of Bioactive Compounds in Cardiovascular Disease: Phenolic Compounds. *Nutrients* **2015**, *7*, 5177–5216.
55. Konstantinidi, M.; Koutelidakis, A.E. Functional Foods and Bioactive Compounds: A Review of Its Possible Role on Weight Management and Obesity's Metabolic Consequences. *Medicines* **2019**, *6*, 94.
56. Kim, S.K.; Mendis, E. Bioactive compounds from marine processing byproducts - A review. *Food Res.*

Int. 2006, *39*, 383–393.

57. Azmir, J.; Zaidul, I.S.M.; Rahman, M.M.; Sharif, K.M.; Mohamed, A.; Sahena, F.; Jahurul, M.H.A.; Ghafoor, K.; Norulaini, N.A.N.; Omar, A.K.M. Techniques for extraction of bioactive compounds from plant materials : A review. *J. Food Eng.* **2013**, *117*, 426–436.

58. Serrano, J.; Casanova-Martí, À.; Gual, A.; Pérez-Vendrell, A.M.; Blay, M.T.; Terra, X.; Ardévol, A.; Pinent, M. A specific dose of grape seed-derived proanthocyanidins to inhibit body weight gain limits food intake and increases energy expenditure in rats. *Eur. J. Nutr.* **2017**, *56*, 1629–1636.

59. Ginés, I.; Gil-Cardoso, K.; Serrano, J.; Casanova-Martí, À.; Blay, Mt.; Pinent, M.; Ardévol, A.; Terra, X. Effects of an intermittent grape-seed proanthocyanidin (GSPE) treatment on a cafeteria diet obesogenic challenge in rats. *Nutrients* **2018**, *10*.

60. Rodriguez-Mateos, A.; Istas, G.; Boschek, L.; Feliciano, R.P.; Mills, C.E.; Boby, C.; Gomez-Alonso, S.; Milenkovic, D.; Heiss, C. Circulating Anthocyanin Metabolites Mediate Vascular Benefits of Blueberries: Insights From Randomized Controlled Trials, Metabolomics, and Nutrigenomics. *Journals Gerontol. Ser. A* **2019**, *74*, 967–976.

61. Cimino, F.; Speciale, A.; Anwar, S.; Canali, R.; Ricciardi, E.; Virgili, F.; Trombetta, D.; Saija, A. Anthocyanins protect human endothelial cells from mild hyperoxia damage through modulation of Nrf2 pathway. *Genes Nutr.* **2013**, *8*, 391–399.

62. Willoughby, D.; Hewlings, S.; Kalman, D. Body Composition Changes in Weight Loss: Strategies and Supplementation for Maintaining Lean Body Mass, a Brief Review. *Nutrients* **2018**, *10*, 1876.

63. Guo, H.; Ling, W. The update of anthocyanins on obesity and type 2 diabetes: Experimental evidence and clinical perspectives. *Rev. Endocr. Metab. Disord.* **2015**, *16*, 1–13.

64. Casanova, E.; Baselga-Escudero, L.; Ribas-Latre, A.; Cedó, L.; Arola-Arnal, A.; Pinent, M.; Bladé, C.; Arola, L.; Salvadó, M.J. Chronic intake of proanthocyanidins and docosahexaenoic acid improves skeletal muscle oxidative capacity in diet-obese rats. *J. Nutr. Biochem.* **2014**, *25*, 1003–1010.

65. Terpstra, A.H.M.; Beynen, A.C.; Everts, H.; Kocsis, S.; Katan, M.B.; Zock, P.L. The Decrease in Body Fat in Mice Fed Conjugated Linoleic Acid Is Due to Increases in Energy Expenditure and Energy Loss in the Excreta. *J. Nutr.* **2002**, *132*, 940–945.

66. Den Hartigh, L.J.; Wang, S.; Goodspeed, L.; Wietecha, T.; Houston, B.; Omer, M.; Ogimoto, K.; Subramanian, S.; Gowda, G.A.N.; O'Brien, K.D.; et al. Metabolically distinct weight loss by 10,12 CLA and caloric restriction highlight the importance of subcutaneous white adipose tissue for glucose homeostasis in mice. *PLoS One* **2017**, *12*, 1–25.

67. Benzler, J.; Ganjam, G.K.; Pretz, D.; Oelkrug, R.; Koch, C.E.; Legler, K.; Stöhr, S.; Culmsee, C.; Williams, L.M.; Tups, A. Central Inhibition of IKKβ/NF-κB Signaling Attenuates High-Fat Diet–Induced Obesity and Glucose Intolerance. *Diabetes* **2015**, *64*, 2015 LP – 2027.

68. de Git, K.C.G.; Adan, R.A.H. Leptin resistance in diet-induced obesity: The role of hypothalamic inflammation. *Obes. Rev.* 2015, *16*, 207–224.

69. Ibars, M.; Ardid-Ruiz, A.; Suárez, M.; Muguerza, B.; Bladé, C.; Aragonès, G. Proanthocyanidins potentiate hypothalamic leptin/STAT3 signalling and Pomc gene expression in rats with diet-induced obesity. *Int. J. Obes.* **2017**, *41*, 129–136.

70. Sasaki, T.; Kikuchi, O.; Shimpuku, M.; Susanti, V.Y.; Yokota-Hashimoto, H.; Taguchi, R.; Shibusawa, N.; Sato, T.; Tang, L.; Amano, K.; et al. Hypothalamic SIRT1 prevents age-associated weight gain by improving leptin sensitivity in mice. *Diabetologia* **2014**, *57*, 819–831.

71. Sasaki, T. Age-Associated Weight Gain, Leptin, and SIRT1: A Possible Role for Hypothalamic SIRT1 in the Prevention of Weight Gain and Aging through Modulation of Leptin Sensitivity . *Front. Endocrinol.* 2015, *6*, 109.

72. Quesada, H.; del Bas, J.M.; Pajuelo, D.; Díaz, S.; Fernandez-Larrea, J.; Pinent, M.; Arola, L.; Salvadó, M.J.; Bladé, C. Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. *Int. J. Obes.* **2009**, *33*, 1007–1012.

73. Asbaghi, O.; Nazarian, B.; Reiner, Ž.; Amirani, E.; Kolahdooz, F.; Chamani, M.; Asemi, Z. The effects of

grape seed extract on glycemic control, serum lipoproteins, inflammation, and body weight: A systematic review and meta-analysis of randomized controlled trials. *Phyther. Res.* **2019**.

74. Bladé, C.; Arola, L.; Salvadó, M.-J. Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. *Mol. Nutr. Food Res.* **2010**, *54*, 37–59.

75. Matthews, D.R.; Hosker, J.P.; Rudenski, A.S.; Naylor, B.A.; Treacher, D.F.; Turner, R.C. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **1985**, *28*, 412–419.

76. Henríquez, S.; Jara, N.; Bunout, D.; Hirsch, S.; de la Maza, M.P.; Leiva, L.; Barrera, G. Variability of formulas to assess insulin sensitivity and their association with the Matsuda index. *Nutr. Hosp.* **2013**, *28*, 1594–1598.

77. Inoue, N.; Nagao, K.; Hirata, J.; Wang, Y.-M.; Yanagita, T. Conjugated linoleic acid prevents the development of essential hypertension in spontaneously hypertensive rats. *Biochem. Biophys. Res. Commun.* **2004**, *323*, 679–684.

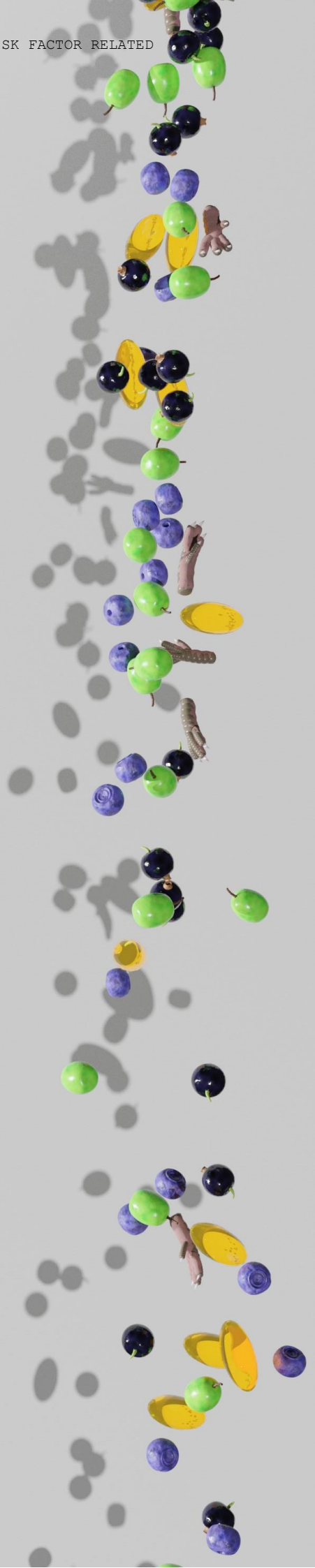
78. Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J.P.E.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signal.* **2013**, *18*, 1818–1892.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

CONCLUSIONS



UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Conclusions

The main conclusions of the present Doctoral Thesis are:

1. **A low dose of 100 mg/kg of CLA, but not 200 and 300 mg/kg, promoted a decrease in body weight gain in animals fed a cafeteria diet**. The most effective dose of CLA corresponds to approximately 0.036% of CLA in the diet. This dose is significantly lower than those previously used in preclinical studies (0.5-1.5%) and that the maximum dose recommended for humans.

2. **This low dose of CLA did not result in any metabolic adverse effect.** The daily administration of 100 mg/kg of CLA did not cause a reduction of lean mass, insulin sensitivity or liver functionality, which are the major adverse effects reported for CLA consumption. The use of an equal ratio of the CLA isomers c9-t11 and t10-c12 used in this study could also be decisive.

3. **This low dose of CLA improved other important cardiometabolic risk factors associated with obesity.** The increase in serum concentrations of glucose, insulin, cholesterol, triglyceride, diglyceride and total phospholipid induced by cafeteria diet were reverted by the administration of this dose of CLA.

4. **The co-administration of different bioactive compounds in a single multifunctional ingredient (MIX) promoted a much more effective body weight management than the individual administration of CLA**. The co-administration of a low dose of CLA (100 mg/Kg) with the protein hydrolysate from chicken feet Hpp11 (55 mg/Kg) and of the flavonoid grape-seed proanthocyanidins (25 mg/Kg) and berry anthocyanidins (100 mg/Kg) resulted in a marked decrease of body weight and fat mass in cafeteria diet fed rats.

5. **MIX did not cause liver injury and hepatic fat accumulation.** This marked decrease in body weight did not increase the hepatic fatty acid and triglyceride content, which is one of the major observed undesirable effects of fat-burning strategies for weight loss. However, a note of caution should be sounded concerning the increased values of esterified cholesterol observed in the liver. Although biomarkers of hepatic damage were not affected.

6. **MIX restored serum leptin values and increased the sensitivity of the first-order neurons to leptin**. This improvement of central leptin sensitivity could be behind the decreased body weight and fat mass as well as the increased energy expenditure observed in cafeteria diet fed rats supplemented with the MIX. Remarkably, energy intake and intestinal lipid absorption were not affected by the dietary incorporation of the MIX.

7. **MIX changed the metabolic profile of serum**. A PLS-DA analysis of serum aqueous and lipid phase confirmed changes in the metabolic profile of serum between the animals administered vehicle and the rats treated with MIX.

8. **MIX administration produced an improvement of carbohydrates and lipids homeostasis.** Insulin sensibility improved in the treated animals. Besides, triglycerides, total and free cholesterol, oleic acid and polyunsaturated fatty acids in serum and triglycerides, diglycerides, linoleic acid and total fatty acid in inguinal adipose tissue were found decreased after MIX administration. All these results suggest an improvement in carbohydrates and lipid management in the animals administered MIX.

9. **MIX administration exhibited a marked antihypertensive effect.** The administration of MIX for 3 weeks to the animals fed cafeteria diet counteracted the cafeteria hypertensive effect since the MIX treated animals showed normotensive values of blood pressure.

The administration of an equal ratio of the CLA isomers c9-t11 and t10-c12 at low doses to cafeteria diet fed rats caused a decrease in body weight gain and improved other cardiometabolic risk factors, without presenting any CLA related adverse effects. The body weight lowering effect of CLA was higher when it was co-administered with the chicken feet hydrolysate Hpp11 and a mixture of grape-seed proanthocyanidins and berry anthocyanidins. The effect of MIX on body weight, which could be mediated by an improvement of leptin sensitivity, was also not accompanied by any adverse effect. On the contrary, MIX produced an improvement on carbohydrate and lipid metabolism and exhibited antihypertensive properties.

Thus, MIX could be a good candidate to be used as nutraceutical or to be included in functional foods for the management of metabolic syndrome.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Funding

This PhD thesis has been developed in the Nutrigenomics Group of the University Rovira I Virgili, Tarragona, Spain, and was funded by the Ministerio de Educación y Ciencia of the Spanish Government, from the State program of Research, Development and Innovation oriented to the Challenges of Society (R + D + I projects) (AGL2013-40707-R and AGL2016-77105-R). This thesis has been carried out thanks to the initial support from a Project-associated scholarship from the University Rovira I Virgili and a subsequent Martí-Franquès scholarship (2016PMF-PIPF-76) provided by the same university. Besides the work performed in the mentioned university, an international short-term stay was done in Dr. Hani El-Nezami's laboratory at the University of Hong Kong, thanks to the support from a MOU scholarship, provided by the university Rovira I Virgili, and a grant from the Spanish Society of Genetics.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

Acknowledgment

This doctoral thesis has not been the sole effort of one person. This doctoral thesis was only possible thanks to the effort of many people around me, supporting, helping and guiding along the way. I would like to thank:

The university Autonoma de Madrid where everything started.

Professor Pilar Herrero and Dr. Jimenez Benjumea for providing the first step to climb this ladder.

The University of Glasgow and Pr. Marshall Stark and Dr. Femi Olorunniji for their support with my master's thesis.

The University of Hong Kong and Dr. Hani El-Nezami for the opportunity, teaching and amazing experience.

The University Rovira I Virgili and the department of Biochemistry and Biotechnology nutrigenomics research group for economic support.

The Spanish Society of Genetics for their economic support.

Dr. Begoña Muguerza and Dr. Gerard Aragonès for these years of guidance and the opportunity to develop my scientific career.

My fellow PhD students in this crazy journey: Lisard, Anna, Carlos, Aida, Cristina, Kathe, Carolina, Albert, Margalef, Angela, Carme, Iris, Alvaro, Hector, Andreu and many more.

Marina for helping with those never-ending RNA extractions and the thousand PCRs.

Rosa and Niurka, for the fun days and tireless help when needed, always easing the journey.

My friend Andres, always having an ear for my nagging.

Every friend I have had these years for coping with me during the bad days.

My parents, Miguel and Carme and my sister, Aurora "bicho bola", for their undying support and care, this could not have been possible without them.

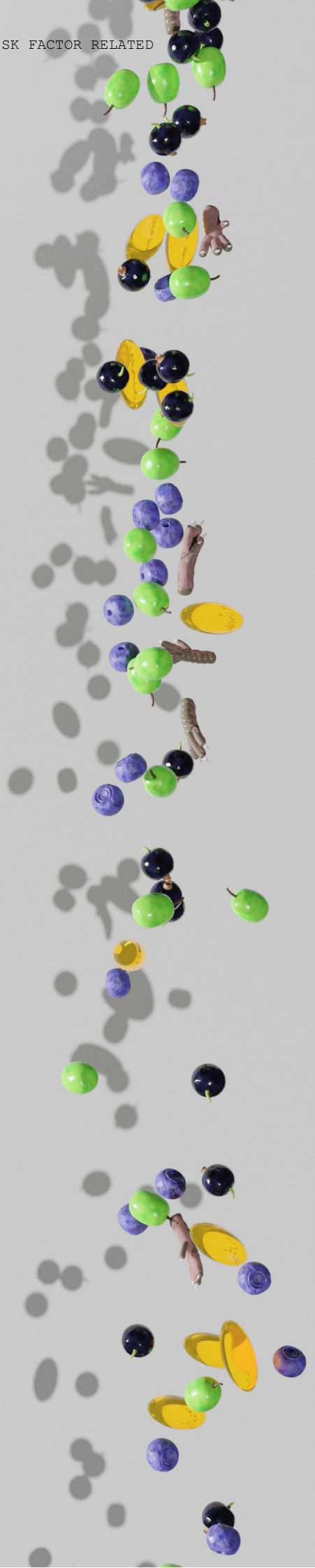
My aunt Pilar for all that help, care and ham; so needed abroad.

And Nessy, he knows why, he is fun.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González



ANNEXES

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

List of conferences

Low dose of conjugated linolenic acid induces thermogenesis, without generating a pathological inflammation status in cafeteria diet-fed rats
Miguel Martín, Héctor Palacios, Cinta Bladé, Begoña Muguerza, Gerard Aragonès.

NuegoWeek2018, Mitochondria, Nutrition and Health. New Castle, United Kingdom.
Poster presentation.

Low dose of conjugated linoleic acid supplementation improves serum lipid profile of cafeteria- diet induced obese rats without inducing hepatic steatosis
Miguel Martín, Héctor Palacios, Miguel A Rodríguez, Cinta Bladé, Begoña Muguerza, Gerard Aragonès.

X Seminario sobre Alimentación y Estilos de Vida Saludables 1r curso de actualización sobre NUTRIGENÓMICA Y NUTRICIÓN PERSONALIZADA Reuniones colaterares: CIBEROBN y ESFRI-ES. Palma, Spain.
Poster presentation.

Low dose of conjugated linolenic acid reduces weight gain, increasing energy expenditure without enhancing hepatic steatosis in cafeteria diet-fed rats
Miguel Martín-González, Héctor Palacios, Gerard Aragonès, Begoña Muguerza.

XVII Congreso de la Sociedad Española de Nutrición (SEÑ) y X Jornada de l'Associació Catalana de Ciències de l'Alimentació (ACCA). Barcelona, Spain.
Poster presentation.

Reduction of hypertriglyceridemia and improvement of triglycerides homeostasis in cafeteria diet-fed rats by chronic administration of a natural extract
Miguel Martín-González, Anna Mas-Capdevila, Albert Gibert, Andrea Ardid-Ruiz, Gerard Aragonès, Begoña Muguerza.

XL SEBBM Congress, The annual Congress of the SFBBM FEBS3+. Biochemical and Molecular Biology Societies. Barcelona, Spain.
Poster presentation.

Efecto de la hora del día en la que se administran las proantocianidinas o un hidrolizado de pata de pollo sobre la homeostasis de la glucosa
Héctor Palacios, **Miguel Martín-González**, Andreu Gual, Miguel Ángel Rodríguez, Cinta Bladé.

IX Seminario sobre Alimentación y Estilos de Vida Saludables. Tarragona, Spain.
Poster presentation.

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

MINISTERIO DE INDUSTRIA, ENERGÍA Y TURISMO

Oficina Española de Patentes y Marcas

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica utilizando la conexión segura de la O.E.P.M. De acuerdo con lo dispuesto en el art. 16.1 del Reglamento de ejecución de la Ley 24/2015 de Patentes, se han asignado a su solicitud un número de expediente y una fecha de recepción de forma automática. La fecha de presentación de la solicitud a la que se refiere el art. 24 de la Ley le será comunicada posteriormente.

Número de solicitud:	P201930732	
Fecha de recepción:	06 agosto 2019, 15:21 (CEST)	
Oficina receptora:	OEPM Madrid	
Su referencia:	132-19	
Solicitante:	Universitat Rovira i Virgili	
Número de solicitantes:	2	
País:	ES	
Título:	Composición hipoleptinémica y su uso	
Documentos enviados:	Descripcion-1.pdf (15 p.) Reivindicaciones-1.pdf (2 p.) Resumen-1.pdf (1 p.) Dibujos-1.pdf (3 p.) OLF-ARCHIVE.zip FEERCPT-1.pdf (1 p.) FEERCPT-2.pdf (1 p.) SEQLPDF.pdf (1 p.) SEQLTXT.txt	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
Enviados por:	C=ES,O=HERREROS & ASOCIADOS,2.5.4.97=#0C0F56415445532D423238383635323336,CN=00681552Q MARIO CARPINTERO (R: B28865236),SN=CARPINTERO LOPEZ,givenName=MARIO,serialNumber=IDCES-00681552Q,description=Ref:AEAT/AEAT0307/PUESTO 1/37016/02082018111613	
Fecha y hora de recepción:	06 agosto 2019, 15:21 (CEST)	
Codificación del envío:	FC:83:99:B3:EB:DB:3C:D5:4C:15:08:EF:56:81:FF:AB:8D:94:4E:39	

AVISO IMPORTANTE

Las tasas pagaderas al solicitar y durante la tramitación de una patente o un modelo de utilidad son las que se recogen en el Apartado "Tasas y precios públicos" de la página web de la OEPM (http://www.oepm.es/es/propiedad_industrial/tasas/). Consecuentemente, si recibe una comunicación informándole de la necesidad de hacer un pago por la inscripción de su patente o su modelo de utilidad en un "registro central" o en un "registro de internet" posiblemente se trate de un fraude.

La anotación en este tipo de autodenominados "registros" no despliega ningún tipo de eficacia jurídica ni tiene carácter oficial.

En estos casos le aconsejamos que se ponga en contacto con la Oficina Española de Patentes y Marcas en el correo electrónico informacion@oepm.es.

ADVERTENCIA: POR DISPOSICIÓN LEGAL LOS DATOS CONTENIDOS EN ESTA SOLICITUD PODRÁN SER PUBLICADOS EN EL BOLETÍN OFICIAL DE LA PROPIEDAD INDUSTRIAL E INSCRITOS EN EL REGISTRO DE PATENTES DE LA OEPM, SIENDO AMBAS BASES DE DATOS DE CARÁCTER PÚBLICO Y ACCESIBLES VÍA REDES MUNDIALES DE INFORMÁTICA.

Para cualquier aclaración puede contactar con la O.E.P.M.

/Madrid, Oficina Receptora/

MINISTERIO DE INDUSTRIA, ENERGÍA Y TURISMO

Oficina Española de Patentes y Marcas

(1) MODALIDAD:	
PATENTE DE INVENCIÓN	[✓]
MODELO DE UTILIDAD	[ ]
(2) FORMULARIO 5101. TIPO DE SOLICITUD:	
PRIMERA PRESENTACIÓN	[✓]
SOLICITUD DIVISIONAL	[ ]
CAMBIO DE MODALIDAD	[ ]
TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA	[ ]
PCT: ENTRADA FASE NACIONAL	[ ]
(3) EXP. PRINCIPAL O DE ORIGEN:	
MODALIDAD:	
N.º SOLICITUD:	
FECHA SOLICITUD:	
4) LUGAR DE PRESENTACIÓN:	OEPM, Presentación Electrónica
(5-1) SOLICITANTE 1:	
DENOMINACIÓN SOCIAL:	Universitat Rovira i Virgili
UNIVERSIDAD PÚBLICA	[✓]
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	Q9350003A
CNAE:	
PYME:	
DOMICILIO:	C/ Escorxador s/n
LOCALIDAD:	TARRAGONA
PROVINCIA:	43 Tarragona
CÓDIGO POSTAL:	43003
PAÍS RESIDENCIA:	España
CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EMPRENDEDOR:	[ ]
PERSONA DE CONTACTO:	
MODO DE OBTENCIÓN DEL DERECHO:	
INVENCIÓN LABORAL:	[✓]
CONTRATO:	[ ]
SUCESIÓN:	[ ]
PORCENTAJE DE TITULARIDAD:	090,23 %
(5-2) SOLICITANTE 2:	
DENOMINACIÓN SOCIAL:	FUNDACIÓ EURECAT
UNIVERSIDAD PÚBLICA	[ ]
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	G66210345
CNAE:	
PYME:	
DOMICILIO:	Parc Tecnològic del Vallès
LOCALIDAD:	CERDANYOLA DEL VALLES
PROVINCIA:	08 Barcelona
CÓDIGO POSTAL:	08290
PAÍS RESIDENCIA:	España

CÓDIGO PAÍS:	ES
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EMPRENDEDOR:	[ ]
PERSONA DE CONTACTO:	
MODO DE OBTENCIÓN DEL DERECHO:	
INVENCIÓN LABORAL:	[✓]
CONTRATO:	[ ]
SUCESIÓN:	[ ]
PORCENTAJE DE TITULARIDAD:	009,77 %
(6-1) INVENTOR 1:	
APELLIDOS:	Bravo Vázquez
NOMBRE:	Francisca Isabel
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	44784222-W
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-2) INVENTOR 2:	
APELLIDOS:	Suárez Recio
NOMBRE:	Manuel
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	47681287-X
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-3) INVENTOR 3:	
APELLIDOS:	Arola Arnal
NOMBRE:	Anna
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	39736329-B
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]

(6-4) INVENTOR 4:	
APELLIDOS:	Muguerza Marquínez
NOMBRE:	María Begoña
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	33420551-X
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-5) INVENTOR 5:	
APELLIDOS:	Salvadó Rovira
NOMBRE:	María Josepa
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	39645746-W
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-6) INVENTOR 6:	
APELLIDOS:	Arola Ferrer
NOMBRE:	Luis María
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	39642182-A
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-7) INVENTOR 7:	
APELLIDOS:	Crescenti Savall
NOMBRE:	Anna
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	77835455-M
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	

EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-8) INVENTOR 8:	
APELLIDOS:	Pascual Serrano
NOMBRE:	Aida
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	39904088-P
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-9) INVENTOR 9:	
APELLIDOS:	Ibars Serra
NOMBRE:	María
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	47172334-R
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-10) INVENTOR 10:	
APELLIDOS:	Ardid Ruiz
NOMBRE:	Andrea
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	48010319-G
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-11) INVENTOR 11:	
APELLIDOS:	Gibert Ramos
NOMBRE:	Albert
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	39923057-W
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	

FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-12) INVENTOR 12:	
APELLIDOS:	Mas Capdevila
NOMBRE:	Anna
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	39470377-P
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-13) INVENTOR 13:	
APELLIDOS:	Martín González
NOMBRE:	Miguel
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	52901737-N
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-14) INVENTOR 14:	
APELLIDOS:	Bladé Segarra
NOMBRE:	María Cinta
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	39659713-P
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	
CÓDIGO PAÍS:	
TELÉFONO:	
FAX:	
CORREO ELECTRÓNICO:	
EL INVENTOR RENUNCIA A SER MENCIONADO:	[ ]
(6-15) INVENTOR 15:	
APELLIDOS:	Aragonès Bargalló
NOMBRE:	Gerard
NACIONALIDAD:	España
CÓDIGO PAÍS:	ES
NIF/NIE/PASAPORTE:	39732667-Y
DOMICILIO:	
LOCALIDAD:	
PROVINCIA:	
CÓDIGO POSTAL:	
PAÍS RESIDENCIA:	

CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: EL INVENTOR RENUNCIA A SER MENCIONADO:	 [ ]
(7) TÍTULO DE LA INVENCIÓN:	Composición hipoleptinémica y su uso
(8) NÚMERO DE INFORME TECNOLÓGICO DE PATENTES (ITP):	
(9) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESIÓN SI NO	 [ ] [✓]
(10) EFECTUADO DEPÓSITO DE MATERIA BIOLÓGICA: SI NO	 [ ] [✓]
(11) DEPÓSITO: REFERENCIA DE IDENTIFICACIÓN: INSTITUCIÓN DE DEPÓSITO: NÚMERO DE DEPÓSITO: ORÍGEN BIOLÓGICO:	
(12) RECURSO GENÉTICO: NÚMERO DE REGISTRO: NÚMERO DE CERTIFICADO DE ACCESO AL RECURSO: UTILIZACIÓN DEL RECURSO GENÉTICO: CONOCIMIENTO TRADICIONAL ASOCIADO A UN RECURSO GENÉTICO:	
(13) DECLARACIONES RELATIVAS A LA LISTA DE SECUENCIAS: LA LISTA DE SECUENCIAS NO VA MÁS ALLÁ DEL CONTENIDO DE LA SOLICITUD LA LISTA DE SECUENCIAS EN FORMATO PDF Y ASCII SON IDENTICOS	 [✓] [✓]
(14) EXPOSICIONES OFICIALES: NOMBRE: LUGAR: FECHA:	
(15) DECLARACIONES DE PRIORIDAD: PAÍS DE ORIGEN: CÓDIGO PAÍS: NÚMERO: FECHA:	
(16) REMISIÓN A UNA SOLICITUD ANTERIOR: PAÍS DE ORIGEN: CÓDIGO PAÍS: NÚMERO: FECHA:	
(17) AGENTE DE PROPIEDAD INDUSTRIAL: APELLIDOS: NOMBRE: CÓDIGO DE AGENTE: NÚMERO DE PODER:	 MARIO CARPINTERO LOPEZ 0953/9
(18) DIRECCIÓN A EFECTOS DE COMUNICACIONES: DIRECCIÓN ASOCIADA AL PRIMER SOLICITANTE DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO:	

MEDIO PREFERENTE DE COMUNICACIÓN	
(19) RELACIÓN DE DOCUMENTOS QUE SE ACOMPAÑAN:	
DESCRIPCIÓN:	[✓] N.º de páginas: 15
REIVINDICACIONES:	[✓] N.º reivindicaciones: 18
DIBUJOS:	[✓] N.º de dibujos: 3
RESUMEN:	[✓] N.º de páginas: 1
FIGURA(S) A PUBLICAR CON EL RESUMEN:	[✓] N.º de figura(s):
ARCHIVO DE PRECONVERSION:	[✓]
DOCUMENTO DE REPRESENTACIÓN:	[ ] N.º de páginas:
JUSTIFICANTE DE PAGO (1):	[✓] N.º de páginas: 1
JUSTIFICANTE DE PAGO (2):	[✓] N.º de páginas: 1
LISTA DE SECUENCIAS PDF:	[✓] N.º de páginas: 1
ARCHIVO PARA LA BUSQUEDA DE LS:	[✓]
OTROS (Aparecerán detallados):	
(20) EL SOLICITANTE SE ACOGE A LA REDUCCIÓN DE TASAS PARA EMPRENDEDORES PREVISTA EN EL ART. 186 DE LA LEY 24/2015 DE PATENTES Y, A TAL EFECTO, APORTA LA SIGUIENTE DOCUMENTACIÓN ADJUNTA:	[ ]
(21) NOTAS:	
(22) FIRMA:	
FIRMA DEL SOLICITANTE O REPRESENTANTE:	00681552Q MARIO CARPINTERO (R: B28865236)
LUGAR DE FIRMA:	MADRID
FECHA DE FIRMA:	06 Agosto 2019

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martin González

UNIVERSITAT ROVIRA I VIRGILI
A MULTIFUNCTIONAL INGREDIENT FOR THE MANAGEMENT OF OBESITY AND OTHER CARDIOVASCULAR RISK FACTOR RELATED TO METABOLIC SYNDROME
Miguel Martínez Clemente

Obesity is defined as an excess of fat accumulation that represents a risk to health. It frequently occurs concurrently with other metabolic risk factors related to metabolic syndrome (MetS), such as dyslipidaemia, insulin resistance or non-alcoholic fatty liver disease (NAFLD). Many food bioactive compounds have been identified and further investigated for their ability to prevent obesity and its metabolic associated pathologies. Among them, conjugated linoleic acid (CLA) is one of the dietary bioactive compounds most investigated for weight management, although controversial metabolic results have been reported. However, the use of a single family of bioactive compounds could not be sufficient to correct multisystemic and highly regulated situations such as obesity and its associated metabolic pathologies. Thus, the aim of this Thesis was to evaluate whether the simultaneous co-administration of different bioactive compounds, as a multifunctional ingredient (MIX), including CLA, a mixture of grape-seed proanthocyanidins and berry anthocyanins, and the protein hydrolysate from chicken feet Hpp11, could reduce obesity and its associated cardiometabolic risk factors in a much more effective way than its individual administration. Our results demonstrated that the administration of an equal ratio of the CLA isomers c9,t11 and t10,c12 at low doses caused a decrease in the body weight gain induced by a cafeteria diet, and improved other cardiometabolic risk factors, without presenting any CLA-related adverse effects. In addition, the body weight lowering effect of CLA was higher when it was co-administered with the chicken feet hydrolysate Hpp11 and a mixture of proanthocyanidins and anthocyanidins. This anti-obesity effect, which could be mediated by an improvement of hypothalamic leptin sensitivity, was also not accompanied by any adverse effect of weight loss. On the contrary, MIX produced an improvement on glucose and lipid metabolism and exhibited antihypertensive properties. Thus, MIX could be a good candidate to be used as nutraceutical or to be included in functional foods for the management of metabolic syndrome.