Universidad de Navarra

Facultad de Farmacia y Nutrición

Effects of Maresin 1, an omega-3 fatty acid-derived lipid mediator, on adipose tissue and liver function in obesity

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Efectos de Maresina 1, un mediador lipídico derivado de los ácidos grasos omega-3, en la función del tejido adiposo y el hígado en obesidad

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Fdo. Laura Laiglesia González

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-When the going gets tough, the tough get going.

Popular proverb

-Happiness can be found even in the darkest of times, if one only remembers to turn on the light.

J.K. Rowling

-All truths are easy to understand once they are discovered; the point is to discover them.

Galileo Galilei

A mi Padre

A mi Madre, hermanos y yaya

A Gorka

A

AC:	Adenylate cyclase
ACC:	Acetyl CoA carboxylase
ACOX:	Acyl-coenzyme A oxidase
ADIPOQ:	Adiponectin
AMPK:	Adenosine monophosphate-activated protein kinase
ATG:	Autophagy related protein
ATGL:	Adipose triglyceride lipase
ATP:	Adenosin triphosphate
AUC:	Area under the curve

B

BAT:	Brown adipose tissue
BMI:	Body mass index
BW:	Body weight

C

C/EBP:	CCAAT-enhancer-binding proteins
cAMP:	Cyclic adenosine monophosphate
CBS:	Calf bovine serum
CCL2:	C-C motif chemokine ligand 2
CGI-58:	Comparative gene identification-58
CIDEA:	Cell death-inducing DFFA-like effector A
CPT1:	Carnitine palmitoyltransferase
COX IV:	Cytochrome c oxidase IV
CTF1:	Cardiotrophin-1

D

2DG:	2-Deoxy-D-glucose
DAG:	Diacylglycerol
DGAT:	Diacylglycerol acyltransferase
DHA:	Docosahexaenoic acid
DIO:	Diet induced obesity
DIO2:	Iodothyronine deiodinase 2

DMEM: Dulbecco's Modified Eagle's Medium

E

ERK: Extracellular signal-regulated kinase 1/2

EPA: Eicosapentaenoic acid

F

^{18}F-FDG : ^{18}F-fluorodeoxyglucose

FA: Fatty acids

FAO: Fatty acid oxidation

FAS: Fatty acid synthase

FBS: Fetal bovine serum

FFA: Free fatty acids

FGF21: Fibroblast growth factor 21

G

G0S2: G0/G1 switch gene 2

GLUT: Glucose transporter

GPR120: G-protein coupled receptor 120/Free fatty acid receptor 4

H

HDL-Chol: High-density lipoprotein-cholesterol

HFD: High fat diet

hMSC: Human mesenchymal stem cells

HOMA: Homeostasis model assessment

HSL: Hormone sensitive lipase

I

IBMX: Isobutylmethylxanthine

IL: Interleukin

ITT: Insulin tolerance test

L

LC3:	Microtubule-associated protein 1A/1B-light chain 3
LD:	Lipid droplet
LDL-Chol:	Low-density lipoprotein-cholesterol
LPL:	Lipoprotein lipase
LX:	Lipoxin

M

MAG:	Monoacylglycerol
MaR:	Maresin
MetS:	Metabolic syndrome
MGL:	Monoacylglycerol lipase
M-MLV:	Moloney Murine Leukemia Virus
MUFA:	Monounsaturated fatty acids
Myf5:	Myogenic factor 5

N

NAFLD:	Non-alcoholic fatty liver disease
NASH:	Non-alcoholic steatohepatitis
NEFA:	Non-esterified fatty acids
NPs:	Natriuretic peptides
NRF1:	Nuclear respiratory factor-1

P

PA:	Palmitic acid
PBS:	Phosphate buffered saline
PD:	Protectin
PET:	Positron emission tomography
PGC-1α:	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PI3K:	Phosphatidylinositol 3-kinase
PK:	Protein kinase
PLIN:	Perilipin
PPARγ:	Peroxisome proliferator-activated receptor γ

PPIA:	Cyclophilin A
PRDM16	PR domain containing 16

R

Rv:	Resolvin

S

SCD1:	Stearoyl-CoA desaturase-1
SFA:	Saturated fatty acid
SIRT1:	Sirtuin 1
SPMs:	Specialized pro-resolving mediators
SREBP-1c:	Sterol regulatory element-binding protein 1
SUV:	Standardized uptake value

T

T3:	Triiodothyronine
TBX:	T-box 1 transcription factor
TG:	Triglycerides
TFAM:	Mitochondrial transcription factor A
TMEM26:	Transmembrane Protein 26
TNF-α:	Tumor necrosis factor alpha
TZD:	Thiazolinediones

U

UCP1:	Uncoupling protein 1

V

VLDL:	Very low density lipoproteins

W

WAT:	White adipose tissue
WHO:	World Health Organization

I. INTRODUCTION

1. OBESITY

Obesity is a disease considered as an excess of body fat accumulated that can have a negative effect on health. According to the World Health Organization (WHO), people are considered obese when their body mass index (BMI) is ≥ 30 kg/m^2, while a BMI ≥ 25 kg/m^2 is considered as overweight (WHO 2011). Obesity is a common, serious and growing problem, which has reached worldwide epidemic proportions (WHO 2011). The rising prevalence of obesity has been defined as a global pandemic responsible of at least 2.8 millions deaths every year (Barcelo-Batllori and Gomis 2009, Popkin *et al.* 2012). The most worrisome part of this "globesity" is the estimated trends. Its incidence has almost double since 1980. In this line, the WHO has identified that 39% of adults (aged 18 years and over) were overweight and 11% were obese in 2014 (Ng *et al.* 2014)

Furthermore, obesity is characterized by excessive fat accumulation not only within adipose tissue but also in other metabolic organs, which lead to increased body weight. It is caused by a complex interaction of several factors including genomic, epigenomic and environmental conditions, which favour a positive imbalance between energy intake and expenditure. This imbalance promotes the accumulation of fat over time (**Figure 1)** (Prieto-Hontoria *et al.* 2011, Manco and Dallapiccola 2012, Apalasamy and Mohamed 2015).

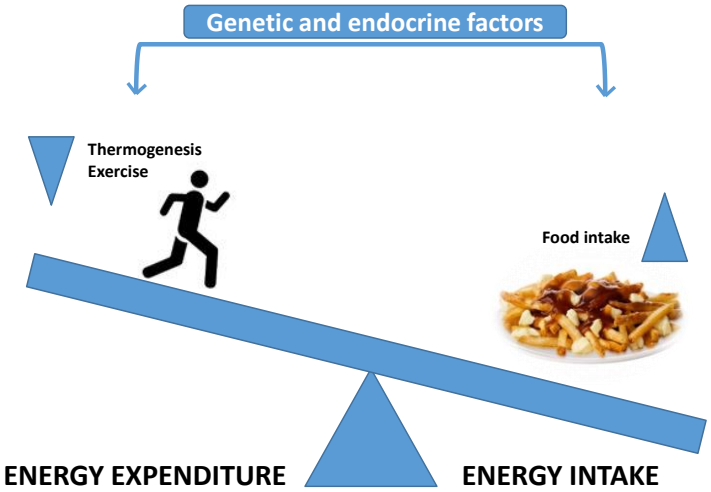

Figure 1. Causes of obesity.

In this context, it is well known that adipose tissue is crucial for systemic energy homeostasis (Sam and Mazzone 2014, Rutkowski *et al.* 2015). Indeed, adipose tissue is the main source of fatty acids (FA) to accomplish energy requirements and heat production during postprandial periods (Rutkowski *et al.* 2015). However, this excess of fat accumulated in adipose tissue is also accompanied by subacute chronic inflammation (Gregor and Hotamisligil 2011). This inflammatory state has been associated with the development of different obesity-related complications such as non-alcoholic fatty liver disease (NAFLD), type 2 diabetes, cardiovascular diseases, hypertension, dyslipidaemia and particular types of cancer **(Figure 2)** (Prieto-Hontoria *et al.* 2011, Saltiel and Olefsky 2017).

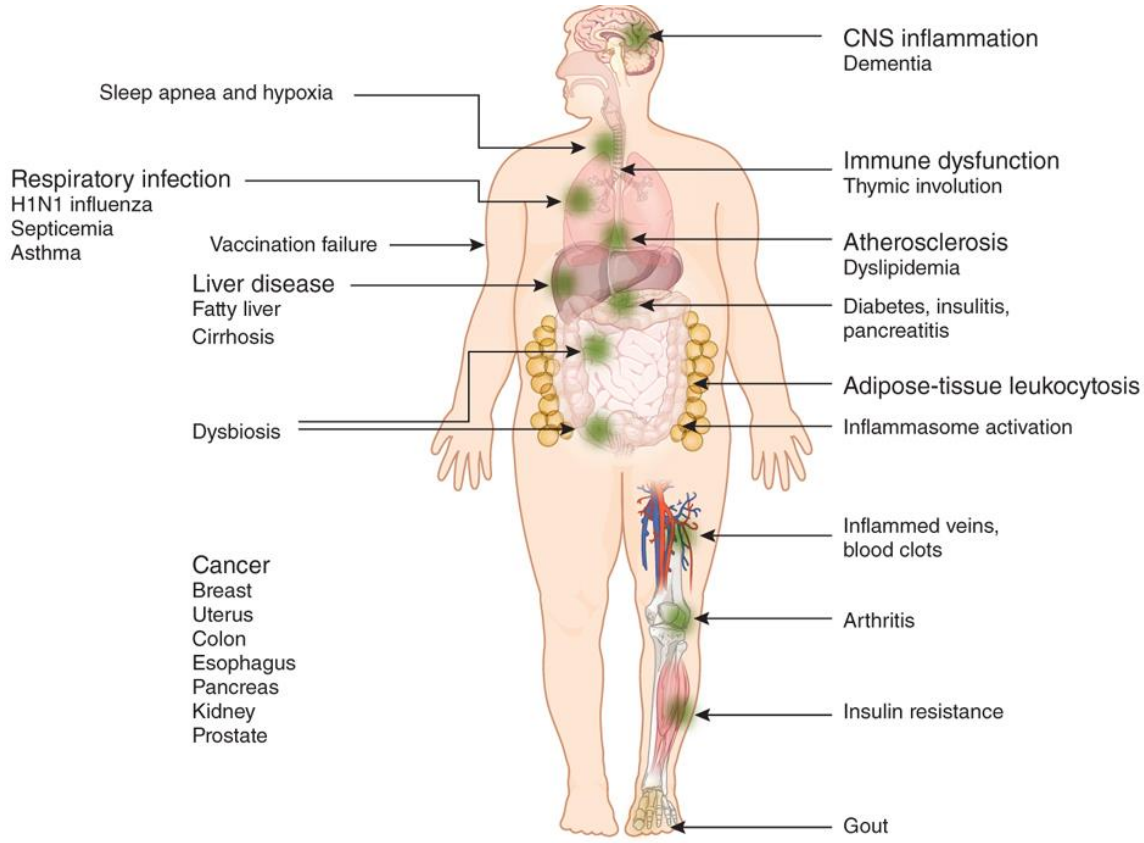

Figure 2. Obesity-related complications (from Kanneganti and Dixit 2012).

The elaboration of strategies to prevent and/or ameliorate both obesity and its comorbidities, have acquired extreme importance in the scientific community. These strategies are important not only to improve population health (life expectancy and

quality of life), but also to reduce the economic costs that obesity derives (Seidell and Halberstadt 2015). In this line, the current therapy for patients suffering from obesity is focused mainly on weight loss strategies, including life style intervention (Heymsfield and Wadden 2017). The therapies commonly used are dedicated to modify eating behaviours and to promote physical activity due to their low cost and feasibility (American College of Cardiology/American Heart Association Task Force on Practice Guidelines 2014). However, the compliance of obese patients to diet and to physical exercise is relatively low. These therapies usually fail either to lose weight or to maintain the normal weight achieve after the intervention (Heymsfield and Wadden 2017). Therefore, the finding of potential therapeutic agents is highly desirable in order to combat the global epidemic disease of the 21st century.

2. ADIPOSE TISSUE AND METABOLISM

Adipose tissue is a loose connective tissue composed predominantly of adipocytes (Rutkowski *et al.* 2015). Adipose tissue plays a key role in the pathogenesis of obesity and associated comorbidities (Kloting and Bluher 2014, Choe *et al.* 2016). In mammals, two types of adipose tissue are well distinguished: white adipose tissue (WAT) and brown adipose tissue (BAT) that have essentially antagonistic functions (Hassan *et al.* 2012, Kwok *et al.* 2016). Indeed, WAT functions as an energy storage organ, while BAT is a thermogenic tissue (Cinti 2012, Townsend and Tseng 2014). In addition, both depots have different precursor cells, phenotype, function and regulation (Berry *et al.* 2013). Interestingly, a third type of adipose tissue known as beige or “brite” (brown in white) adipose tissue has been identified (Petrovic *et al.* 2010, Bartelt and Heeren 2014, Wang and Seale 2016). These beige adipocytes are co-located within specific WAT depots, but display comparable functional and molecular features, as brown adipocytes. Interestingly, beige adipocytes seem to be induced by similar stimuli as brown adipocytes (Wu *et al.* 2012). The main morphological and functional characteristics of the three types of adipocytes are summarized in **Figure 3**.

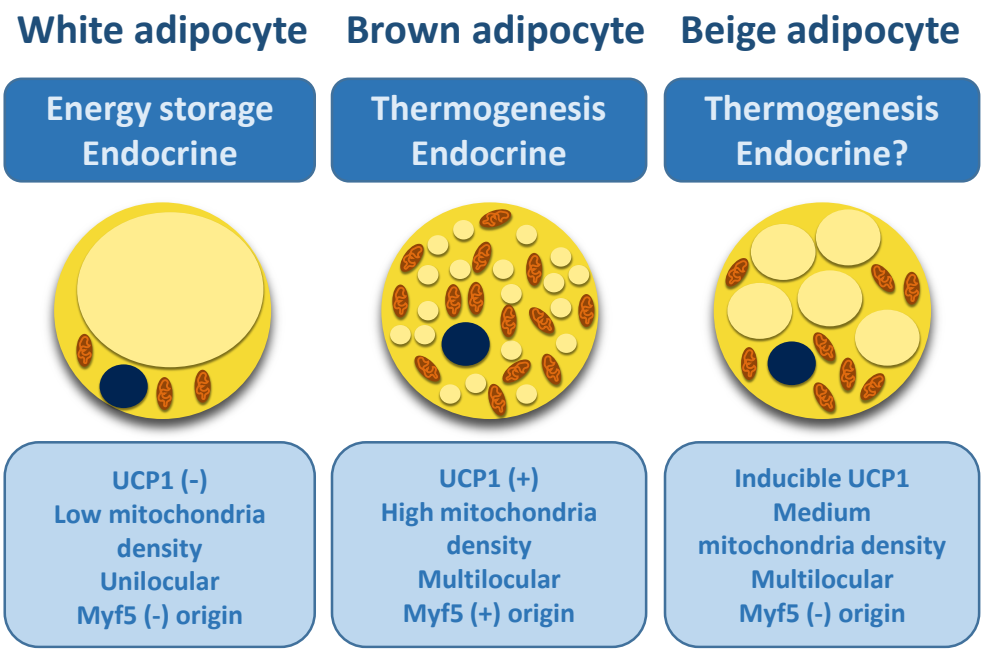

Figure 3. Principal characteristics and functions of white, brown and beige adipocytes.

2.1 White adipose tissue

White adipose tissue is the main energy storage organ, which also has a dynamic behaviour acting as an important endocrine organ able to secrete different molecules, widely known as adipokines. WAT is a very diverse tissue, composed by different types of cells, mainly mature adipocytes, specialized cells for fat storage (60-70 % of WAT) and the remaining cells that constitute the stromal vascular fraction, which gathers preadipocytes, fibroblasts, endothelial cells and immune cells, such as macrophages or lymphocytes (Scherberich *et al.* 2013).

White adipocytes have a typical morphology, presenting a prominent lipid droplet (LD) that occupies almost the entire cytoplasm (90%), therefore, the thin ring of cytoplasmic matrix contains other organelles in the cell periphery including the flattened, semilunar nucleus and few mitochondria (Le Lay *et al.* 2009). Moreover, white adipocytes mitochondria lack of uncoupling protein 1 (UCP1) (**Figure 3**). Furthermore, white adipocytes principally derivate from non-myogenic factor (*Myf5*) lineages (Shan *et al.* 2013).

WAT was traditionally considered as a passive storage organ, accumulating the excess of energy as triglycerides (TG), allowing a rapid mobilization under energy deprivation. Nowadays, it is known that WAT also acts as an extraordinary endocrine organ releasing a wide range of adipokines, which are involved in systemic energy homeostasis (Scherer 2006) by an overall cross-talk between key metabolic organs such as central nervous system (CNS), liver, skeletal muscle and pancreas (Trayhurn and Wood 2005). It is important to note that adipokines are also involved in the regulation of multiple physiological functions including appetite, satiety, energy expenditure, insulin sensitivity and secretion, glucose and lipid metabolism and fat distribution among others (Bluher 2013).

In adulthood, almost all the body adipose tissue is of white type. WAT can be one of the largest organs in the body and its weight is highly variable. The increase on body fat accumulation that occurs in obesity may be due to changes in size (hypertrophy) and number (hyperplasia) of adipocytes, mainly in subcutaneous fat (Virtue and Vidal-Puig 2010, Rutkowski *et al.* 2015). However, regional body fat distribution appears to be even more important than the total amount of fat accumulated. Indeed, central adiposity with visceral fat deposition correlates to more often elevated mortality, type 2 diabetes and cardiometabolic consequences of obesity (Farb and Gokce 2015). Nevertheless, these associations are less consistent in subcutaneous fat mass (Palou *et al.* 2010).

2.2 Brown adipose tissue

BAT is the main site for adaptive heat production through non-shivering thermogenesis. It is well known that the heat produced by BAT is essential for the survival of small mammals in cold environments and for arousal during hibernation (Harms and Seale 2013, Calderon-Dominguez *et al.* 2016). The principal cell type of BAT is the brown adipocyte, whose morphology is also characteristic, and entirely different from white adipocytes. It contains numerous lipid droplets (multilocular lipids), a round nucleus (usually situated centrally) and a large number of mitochondria (**Figure 3**). This high number of mitochondria gives a brown appearance to this adipose tissue and mitochondria are also responsible for the metabolic processes transforming energy

substrates and O_2 to produce heat. In this sense, BAT has more capillaries than WAT in order to get enough O_2 and nutrients to generate heat. Brown adipocytes derive from a *Myf5*-expressing cell lineage, differing from white adipocytes (Seale *et al.* 2008).

As previously stated, BAT mediated adaptive thermogenesis due to the existence of UCP1, a BAT-specific mitochondrial proton transport protein (Dempersmier and Sul 2015). UCP1 is located in the mitochondria inner membrane. When UCP1 is activated, uncouples the respiratory chain dissipating the proton gradient (proton leak) driving to heat production instead of adenosine triphosphate (ATP) (Bonet *et al.* 2013, Giralt and Villarroya 2013). It is well known that cold exposure or food intake can activate UCP1, inducing an increase of FA oxidation in order to maintain the levels required of uncoupled respiration, thus producing heat (Cannon and Nedergaard 2004, Kajimura *et al.* 2015). Since BAT consumes high levels of glucose, it has been proposed to act as a glucose clearance organ and therefore, improve hyperglycaemia. This strategy may be beneficial to reduce serum glucose levels in both diabetes and obesity (Chondronikola *et al.* 2014, Townsend and Tseng 2014).

BAT is found in almost every mammal. In humans, the amount of BAT is relatively abundant in neonates to maintain body heat, but gradually reduces during adulthood (Cypess *et al.* 2009, Cypess and Kahn 2010). Therefore, for a prolonged period of time, BAT was considered without physical relevance in adult humans. However, this view has radically changed since the identification of significant amounts of metabolically active BAT in healthy adults by ^{18}Fluoro-2-deoxy-D-glucose positron emission tomography (^{18}FDG-PET) scans (Cypess *et al.* 2009, Saito *et al.* 2009, van Marken Lichtenbelt *et al.* 2009, Virtanen *et al.* 2009, Zingaretti *et al.* 2009). In adults, BAT is mainly localized in the cervical and supraclavicular regions, but also in axillar, paravertebral, paraaortic and perirenal regions **(Figure 4)** (Nedergaard *et al.* 2007, Saito 2014). Moreover, it has been reported that BAT mass is inversely correlated with BMI and fat mass (Saito *et al.* 2009, Orava *et al.* 2013, Chondronikola *et al.* 2014). Interestingly, the sympathetic nervous system is involved in regulating not only the growth of BAT but also its thermogenic activity upon cold exposure (Cannon and Nedergaard 2004).

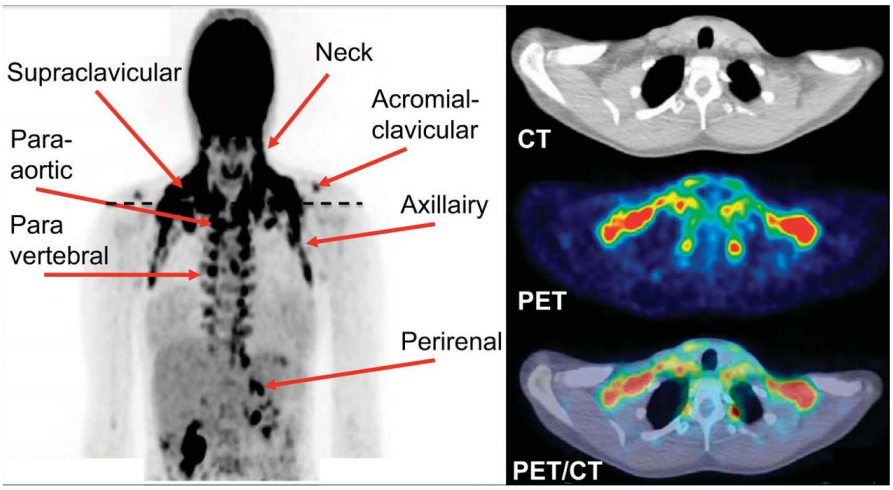

Figure 4. Depots of human BAT (from van der Lans *et al.* 2014).

2.3 Beige adipose tissue

In the last few years, the browning/beiging process, involving sporadic appearance of brown-like adipocytes, in common WAT depots have reached extraordinary interest in the scientific community (Townsend and Tseng 2012, Peschechera and Eckel 2013, Kim and Plutzky 2016). These brown-like adipocytes are now named as beige or brite adipocytes (Wu *et al.* 2012, Min *et al.* 2016). Beige adipocytes have mixed characteristics of both white and brown adipocytes. They are also multilocular and have thermogenic properties due to increased mitochondrial function and expression of inducible UCP1, similarly to brown adipocytes (Wu *et al.* 2012). However, beige adipocytes do not derive from the *Myf5* linage, they derive from *Myf5* negative progenitors as white adipocytes, and express beige-specific genes, such as *T-box 1 (TBX1), transmembrane protein 26 (TMEM26)* and *CD137* (Wu *et al.* 2012, Peirce *et al.* 2014, Wang and Seale 2016).

Concerning the origin of beige adipocytes, there are still some controversial hypotheses (**Figure 5**). One of the theories proposes that beige adipocytes may develop "*de novo*" from specific (Wu *et al.* 2012) or multiple (Wang *et al.* 2013) precursor cells within white depots that differentiate into beige adipocytes. Another theory supports that direct conversion from white to beige adipocytes takes place, a process recognised

as “transdifferentiation” (Cinti 2002, Barbatelli *et al.* 2010, Smorlesi *et al.* 2012). In fact, it is believed that paucilocular adipocytes (which shows UCP1 and have a lipid droplets distribution between white and brown adipocytes), are intermediate forms of adipocytes transitioning from white to inducible, beige/brite adipocytes (Himms-Hagen *et al.* 2000, Giralt and Villarroya 2013). Additionally, it is also suggested that both mechanisms may occur in WAT, varying from depot localization. Furthermore, some studies have demonstrated that the transdifferentiation process may be bidirectional depending of thermogenic and energy requirements (Rosenwald *et al.* 2013).

Recently, beige adipocytes have been identified in WAT depots of adult humans (Wu *et al.* 2012), making the inducible process of browning an attractive therapeutic target to combat obesity and its comorbidities (Bartelt and Heeren 2014). In this sense, the browning process and expression of the inducible UCP1, have been observed after different stimulus, such as chronic cold exposure, exercise, and several pharmacological and nutritional factors such as peroxisome proliferator-activated receptor γ (PPARγ) agonists, thiazolinediones (TZD), irisin, natriuretic peptides, fibroblast growth factor 21 (FGF21), cardiotrophin-1, curcumin, resveratrol and quercetin among others (Moreno-Aliaga *et al.* 2011, Bonet *et al.* 2013, Villarroya and Vidal-Puig 2013, Montanari *et al.* 2017). Importantly, it has been recently recognised the significance of the immune system inducing beige adipose tissue. Eosinophils and alternatively activated macrophages pathways have been proposed to promote the expression and production of factors required for browning in WAT (Qiu *et al.* 2014). However, there is some controversial recent findings about the direct role of alternatively activated macrophages in adaptive thermogenesis (Fischer *et al.* 2017).

On the other hand, there are also several key transcription factors that plays an important role in the regulation of the browning process, including Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), CCAAT-enhancer-binding proteins (C/EBP) and PR domain containing 16 (PRDM16) (Petrovic *et al.* 2010, Seale *et al.* 2011, Cohen *et al.* 2014). Indeed, PRDM16 is known to supress classic white adipocyte genes while induces the expression of some proteins involved in BAT thermogenesis such as PGC-1α (master regulator on mitochondrial biogenesis) and UCP1. In this context, the improvement of mitochondrial biogenesis and the browning

of WAT have been suggested as a novel therapeutic strategy to counteract obesity and metabolic-associated diseases (Liu *et al.* 2009b, Bartelt and Heeren 2014).

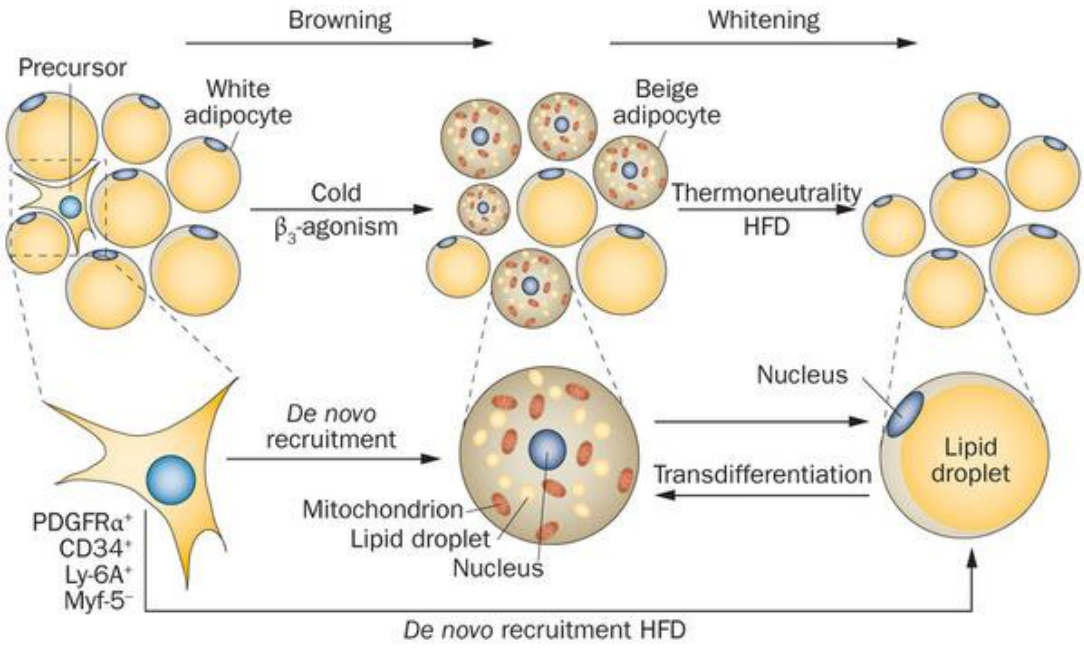

Figure 5. Browning/whitening of adipose tissue (from Bartelt and Heeren 2014).

2.4 Adipose tissue lipid metabolism

Several organs are involved in regulation of systemic lipid metabolism, however adipose tissue plays a key role in this complex process (Frayn *et al.* 2006). In this sense, adipose tissue as described above, it is the main organ for energy storage as TG. The amount of TG within the adipocyte is mainly regulated by the build-up of FA (lipogenesis) and their mobilization of through lipolysis and FA oxidation within the mitochondria (Rutkowski *et al.* 2015). Interestingly, it has also been recently suggested the involvement of lipophagy in this lipid breakdown (Singh *et al.* 2009a, Singh and Cuervo 2012). In obese adipose tissue, the physiological regulation of lipid metabolism may be impaired thus, promoting development of dyslipidaemia, which might lead to metabolic syndrome (MetS) (Borén 2013).

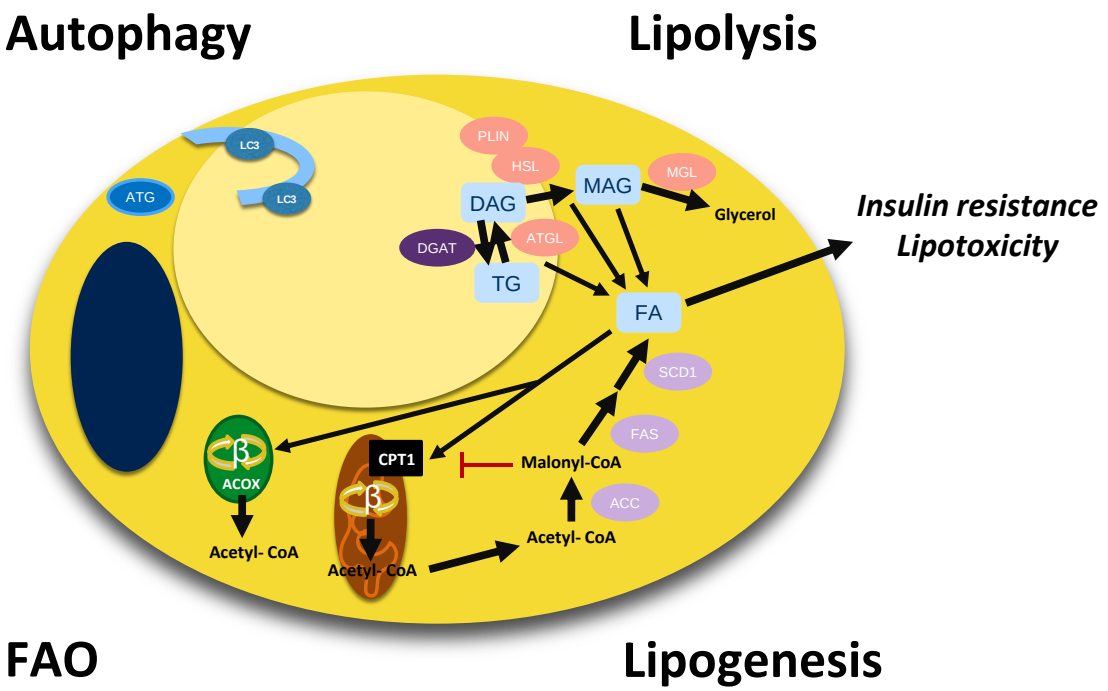

Figure 6. Scheme of the main biochemical pathways involved in adipocyte lipid metabolism. Acetyl CoA carboxylase (ACC); Acyl-coenzyme A oxidase (ACOX); Autophagy related protein (ATG); Adipose triglyceride lipase (ATGL); Carnitine palmitoyltransferase (CPT1); Diacylglycerol (DAG); Diacylglycerol acyltransferase (DGAT); Fatty acid (FA); Fatty acid oxidation (FAO); Fatty acid synthase (FAS); Hormone sensitive lipase (HSL); Microtubule-associated protein 1A/1B-light chain 3 (LC3); Monoacylglycerol (MAG); Monoacylglycerol lipase (MGL); Perilipin (PLIN); Stearoyl-CoA desaturase-1 (SCD1); Triglyceride (TG).

2.4.1 Lipogenesis

In adipocytes, triglycerides storage can be a consequence of either a reesterification of dietary fatty acids or *de novo* fatty acids synthesis from non-lipid substrates. Although the most prominent process is the first one, *de novo* lipogenesis has also an important role not only in liver but also notably in adipocytes (Strawford *et al.* 2004).

After dietary intake, chylomicrons and VLDL transport fatty acids in the blood stream to be stored as TG within the adipocyte lipid droplets. In order to enter into the adipocyte, it is necessary to hydrolyse TG into non-esterified fatty acids (NEFA). The lipoprotein lipase (LPL), located in the vascular endothelium of adipose tissue and stimulated by insulin, is in charge of this step. Circulating FA can be taken into adipocytes through specific facilitating transporters, including fatty acid binding protein

(FABP), fatty acid transport proteins (FATP), fatty acid translocase (FAP) among others (Febbraio *et al.* 2001, García-Arcos *et al.* 2013). Once FA are located inside the adipocytes, they are esterified into triglycerides and stored within the lipid droplet. In this sense, Diglyceride O-acyltransferase (DGAT), is a key enzyme involved in triglycerides esterification, by catalysing the final step in triglycerides synthesis. DGAT has two isoforms: DGAT1, mainly expressed in small intestine and WAT and DGAT2 principally expressed in liver (Choi *et al.* 2007, Yen *et al.* 2008).

On the other hand, *de novo* lipogenesis specifically refers to the formation of triglycerides from carbon skeletons, mostly glucose. It is believed to have a slight contribution in the whole body lipid stores (McDevitt *et al.* 2001). However, *de novo* lipogenesis seems to play an essential role in the generation of lipid storage also in adipocytes. Interestingly, it has been reported that inhibiting adipose tissue lipogenesis is able to reprogram thermogenesis in diet-induced obese mice (Lodhi *et al.* 2012).

Furthermore, triglycerides, cholesterol and diacylglycerol are synthesized by the action of Acetyl-CoA Carboxylase (ACC), a rate-limiting enzyme that converses acetyl-coA to manolyl-CoA. Interestingly, AMPK activation inactivates ACC by phosphorylation (Zhou *et al.* 2001), which leads to decreased levels of manolyl-CoA, which could also promote mitochondrial fatty acid oxidation (Ceddia 2013). Then, fatty acid synthase (FAS) is required for the conversion to saturated fatty acids, following by stearoyl-CoA desaturase (SCD1), an endoplasmic reticulum enzyme, which catalyses the synthesis of monounsaturated fatty acids from saturated fatty acids (Ntambi *et al.* 2002) (See **Figures 6 and 7**).

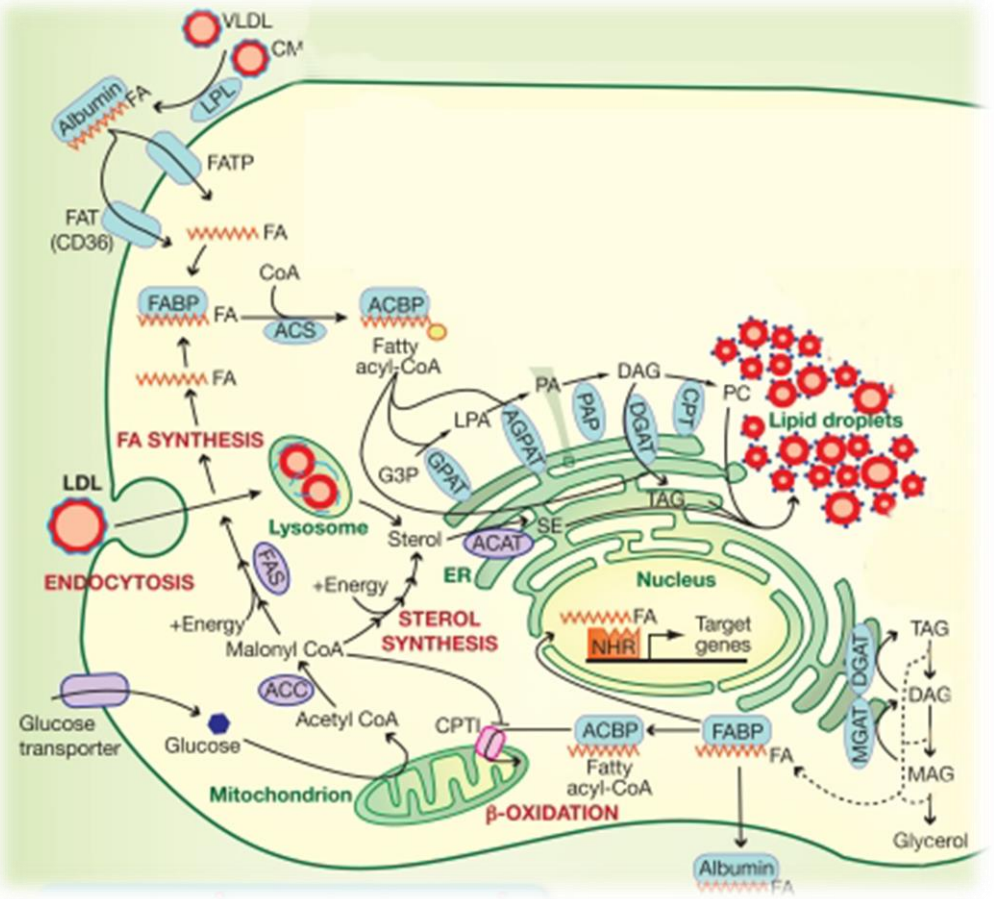

Figure 7. Scheme of adipocyte lipogenesis modified (from Guo *et al.* 2009).

2.4.2 Lipolysis

In fasting periods or during physical exercise, energy demands are highly increased. Thus, triglycerides are hydrolysed to glycerol and FA, which can be used as energetic substrates, a process known as lipolysis. During lipolysis, an intracellular TG breakdown occurs by the consecutive actions of three major lipases: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MAGL) (Zechner *et al.* 2012) (**Figures 6 and 8**). ATGL is the first lipase involved in lipolysis that cleaves the first FA from triglycerides and then HSL acts on diacylglycerol, and MAGL on monoacylglycerol respectively, releasing the two additional fatty acids and one glycerol molecule (Gaidhu *et al.* 2010). Both, ATGL and HSL are considered responsible for 95% of triglycerides lipase activity due to complementary actions between them (Zechner *et*

al. 2009, Thompson *et al.* 2010). Perilipin is a protein located on the lipid droplets surface, that significantly block lipases to reach the lipid droplet surface, thus preventing TG breakdown (Tansey *et al.* 2004). Moreover, Perilipin, in basal conditions, is associated to comparative gene identification-58 (CGI-58), an ATGL coactivator, thus inhibiting ATGL activity. ATGL function can also be regulated by G0/G1 switch gene 2 (G0S2) that is the main selective inhibitor of ATGL activity (Yang *et al.* 2010). On the other hand, HSL activity is regulated by several mechanisms including reversible phosphorylation at different serine residues. In this sense, HSL activation occurs by phosphorylation at Ser-600, Ser-660 and Ser-563, but HSL function is reduced after phosphorylation at Ser-565 since an allosteric inhibition to Ser-563 takes place (Watt *et al.* 2006).

Lipolysis in adipocytes is tightly modulated by several hormones and biochemical signals (**Figure 8**). These molecules regulate the activity of main lipases and proteins involved in this catabolic process (Duncan *et al.* 2007, Rutkowski *et al.* 2015). In this sense, cathecolamines are major activators in fasting lipolysis through β adrenergic receptor stimulation. Furthermore, glucagon also promotes adipocyte lipolysis in starvation (Duncan *et al.* 2007). The binding of catecholamines or glucagon to their receptor induces adenylate cyclase (AC) activity, thus increasing intracellular cyclic adenosine monophosphate (cAMP) and subsequently protein kinase A (PKA) activation. PKA promotes poli-phosphorylation of perilipin and HSL activity through the phosphorylation at the activating sites of HSL, Ser-563 and Ser-660, which are necessary for activated lipolysis and the transit of HSL to the surface of lipid droplet (Langin and Arner 2006, Watt *et al.* 2006, Jocken and Blaak 2008). Additionally, other important regulators of lipolysis are natriuretic peptides (NPs). These hormones stimulate the activation of the protein kinase G (PKG), which activates lipolysis by phosphorylation of HSL and perilipin, similarly to PKA (Sengenes *et al.* 2003). Moreover, the pro-inflammatory cytokine TNF-α is also known to promote lipolysis by modulating key lipases and reducing perilipin protein expression (Ryden *et al.* 2004). On the other hand, insulin is a key antilipolytic hormone. Insulin binding causes phosphorylation of its receptor activating phosphatidylinositol 3-kinase (PI3K) and subsequently triggering protein kinase B/Akt (PKB/Akt). These activations lead to cAMP degradation, releasing PKA from activation. Thus, inhibiting lipolysis during feeding state. The main target of

the antilipolytic action of insulin was believed to be phosphodiesterase 3B (PDE3B), whose phosphorylation by Akt leads to accelerated degradation of the prolipolytic second messenger cAMP, but some recent data suggest that phosphorylation of PDE3B by Akt seems not to be required for insulin to suppress adipocyte lipolysis (DiPilato *et al.* 2015). Moreover, another lipolysis inhibitor is adenosine (Johansson *et al.* 2008) whose binding to its adipocyte receptor leads to adenylate cyclase inhibition (Duncan *et al.* 2007).

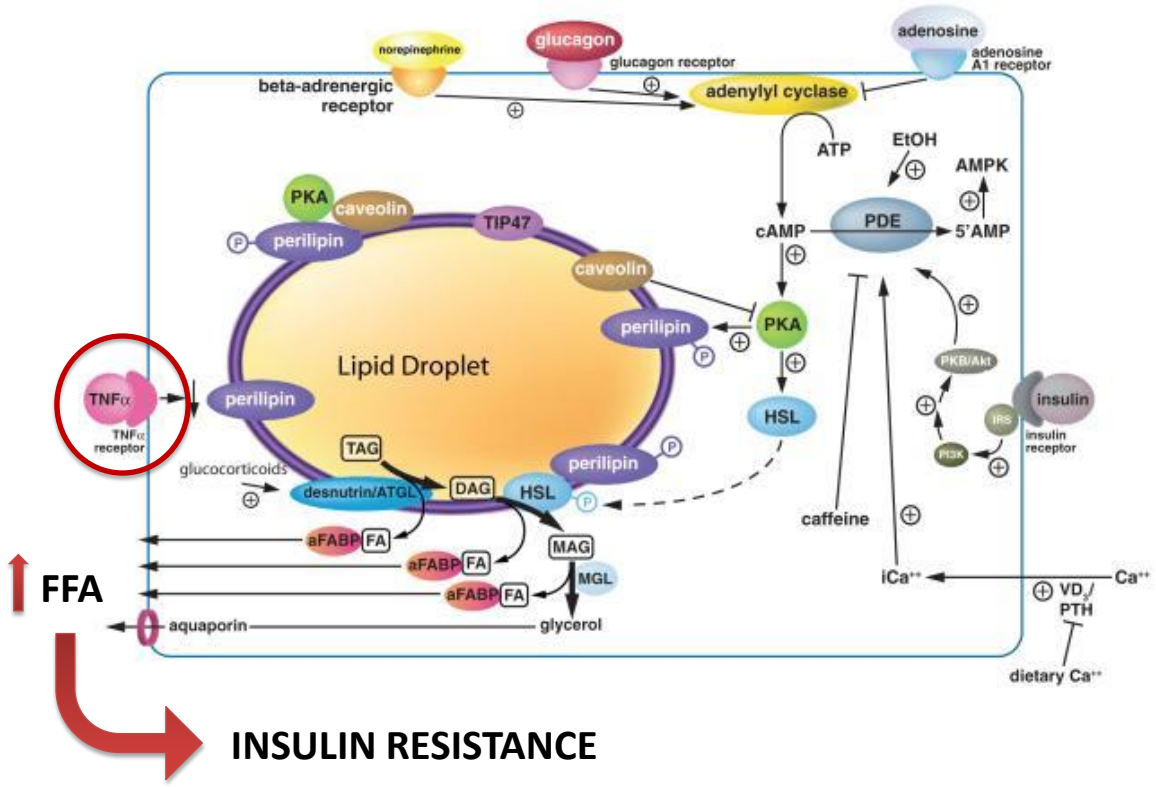

Figure 8. Scheme of adipocyte lipolysis modified (from Duncan *et al.* 2007).

Increased lipolysis has been highly linked to obesity, because it induces detrimental metabolic consequences including insulin resistance and MetS (Morigny *et al.* 2016). In fact, excess of circulating FA derived from excessive adipose tissue lipolysis are stored in insulin-sensitive tissues including liver and muscle. This ectopic fat accumulation, known as lipotoxicity is highly associated with insulin resistance (Virtue and Vidal-Puig 2010, Samuel and Shulman 2012). Additionally, increased lipolysis rate can also modulate both adipocyte and macrophages secretory pattern of cytokines that significantly influence insulin sensitivity (Suganami *et al.* 2005, Ertunc *et al.* 2015). In this sense, TNF-α, a pro-

inflammatory cytokine whose production is elevated in obesity (Hotamisligil *et al.* 1995) strongly promotes lipolysis, which may decrease insulin anti-lipolytic effects and lead to insulin resistance (Guilherme *et al.* 2008, Morigny *et al.* 2016) aggravating ectopic lipid accumulation (Langin and Arner 2006). In this context, it has been suggested that anti-lipolytic agents that decrease circulating FA, may be an attractive therapeutic strategy to counteract insulin resistance (Morigny *et al.* 2016).

2.4.3 Fatty acid oxidation

In mammals, β-oxidation is a key catabolic pathway to breakdown FA. This FA catalysis provide energy to cells, and can take place in both mitochondria and peroxisomes (**Figures 6 and 9**). Interestingly, both organelles have a cooperative function for FA oxidation (Demarquoy and Le Borgne 2015).

Mitochondrial FA β-oxidation pathway plays a key role in energy homoeostasis. Indeed, it is the main pathway involved in the degradation of FA (Bartlett and Eaton 2004, Houten and Wanders 2010). The mitochondrial membrane is impermeable to acyl-CoAs. Thus, they need to be catalysed to be imported into mitochondria by the carnitine shuttle carnitine palmitoyltransferase I (CPT1). In mammals there are three different isoforms of CPT1: CPT1A, CPT1B and CPT1C (Serra *et al.* 2013). CPT1 is in charge of acyl-CoA conversion into an acylcarnitine, which can enter into the mitochondria. Interestingly, CPT1 is presented at the outer mitochondrial membrane and inhibited by malonyl-CoA, which is cleaved by ACC as explained above (Ramsay *et al.* 2001, Bonnefont *et al.* 2004). Once inside the mitochondria, the four enzyme reactions series recognised as β-oxidation, catalyse acyl-CoAs into acetyl-CoA. Thereafter, acetyl-CoA go to the citric acid cycle and the electron carriers derived from β-oxidation reactions, transport electrons to the electron transport chain to generate energy (ATP).

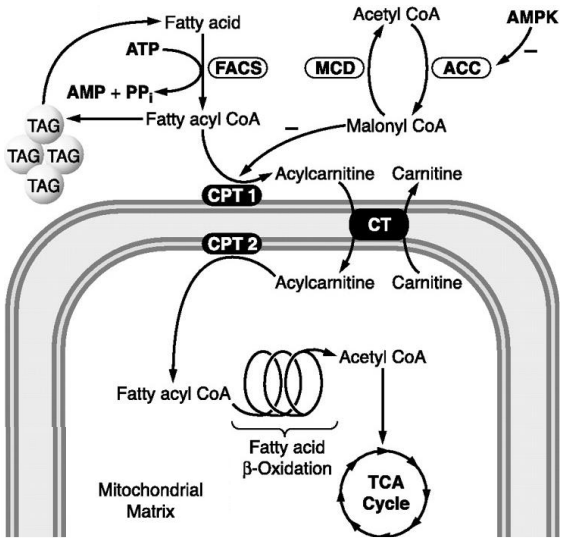

Figure 9. Scheme of adipocyte fatty acid oxidation (modified from Lopaschuk *et al.* 2010).

On the other hand, peroxisomes are also responsible of β-oxidation in animal cells. Indeed, mainly long-chain dicarboxylic and very long-chain monocarboxylic fatty acids are oxidised within the peroxisome. Although there are similarities to β-oxidation in mitochondria, the first reaction in the peroxisomal β-oxidation is catalysed by acyl-CoA oxidase (ACOX), which controls the pathway flux (Poirier *et al.* 2006). Remarkably, peroxisomal β-oxidation is not regulated by energy requirement, though it is essentially involved in biosynthesis pathways instead of energy production, as occur in mitochondrial β-oxidation (Demarquoy and Le Borgne 2015).

2.4.4 Autophagy

Autophagy ("self-devouring") is a self-degradative highly conserved evolutionary process that involves degradation of cellular constituents by lysosomes. Autophagy is important for balancing sources of energy as required (during development or nutrient deprivation periods), because it is in charge of recycling intracellular components to maintain energy homeostasis. Additionally, autophagy also ensure quality control removing misfolded proteins and eliminating damaged organelles (Glick *et al.* 2010). Depending on the molecular components involved in the degradation, three types of

autophagy have been recognised: chaperone-mediated autophagy, microautophagy and macroautophagy. This last one usually refers as autophagy unless otherwise specified (Schneider and Cuervo 2014).

Chaperone-mediated autophagy is referred to degradation of the substrate proteins identified by a cytosolic chaperone, which delivers them directly to the lysosome. Interestingly, this form of autophagy has been recently related to facilitate lipolysis and may take part in lipid degradation (Kaushik and Cuervo 2015, Schweiger and Zechner 2015). However, in microautophagy cytosolic components are internalized for degradation in single-membrane vesicles formed directly from invaginations of the lysosomes surface and it is generally a non-selective process (Schneider and Cuervo 2014).

On the other hand, in macroautophagy (hereafter referred to as autophagy), cytosolic components including lipids, proteins and mitochondria among others, are sequestered inside double-membrane vesicles, called autophagosomes, which deliver the cargo to lysosomes through vesicular fusion. Autophagy is modulated by a complex network of proteins, which regulate the autophagosome formation and fusion with the lysosome. Autophagy machinery involves a complex integration of genes and their protein products, where the main representative proteins are known as autophagy-related proteins (ATGs). These ATGs contribute to the activation, nucleation of the autophagosome membrane, elongation and sealing of the autophagosome, delivery and fusion to the lysosome (Singh and Cuervo 2012). Additionally, microtubule-associated proteins 1A/1B light chain 3 (LC3) are also involved in autophagosome formation. Upon induction of autophagy LC3 is activated to LC3I (cytoplasmic) that is converted to its lipidated form LC3II by an ATG-dependent mechanism. LC3II is found in both internal and external membranes of the autophagosome where it plays a key role selecting cargo for degradation and might promote autophagosome biogenesis (Glick *et al.* 2010). Moreover, LC3II may function as a receptor for p62/*SQSTM1* (a multifunctional protein that directly interacts with cargo), whose degradation is preferentially through autophagy and it is accumulated in autophagy-deficient cells (Bjorkoy *et al.* 2009). Thus, LC3II and p62/ *SQSTM1* are commonly used for autophagy monitorization (Mizushima 2007).

Autophagy has recently been suggested as a master regulator on metabolic homeostasis. In fact, during nutrient deprivation, autophagy can cleavage lipid droplet triglycerides into FA (lipophagy) (Singh *et al.* 2009a) and glycogen stores into oligosaccharides and glucose (Kotoulas *et al.* 2006). Interestingly, it has been described that lipophagy could play a similar role as lipolysis although they involve different proteins and regulation (**Figure 6 and 10**), but both are active in fasting periods (Singh and Cuervo 2012).

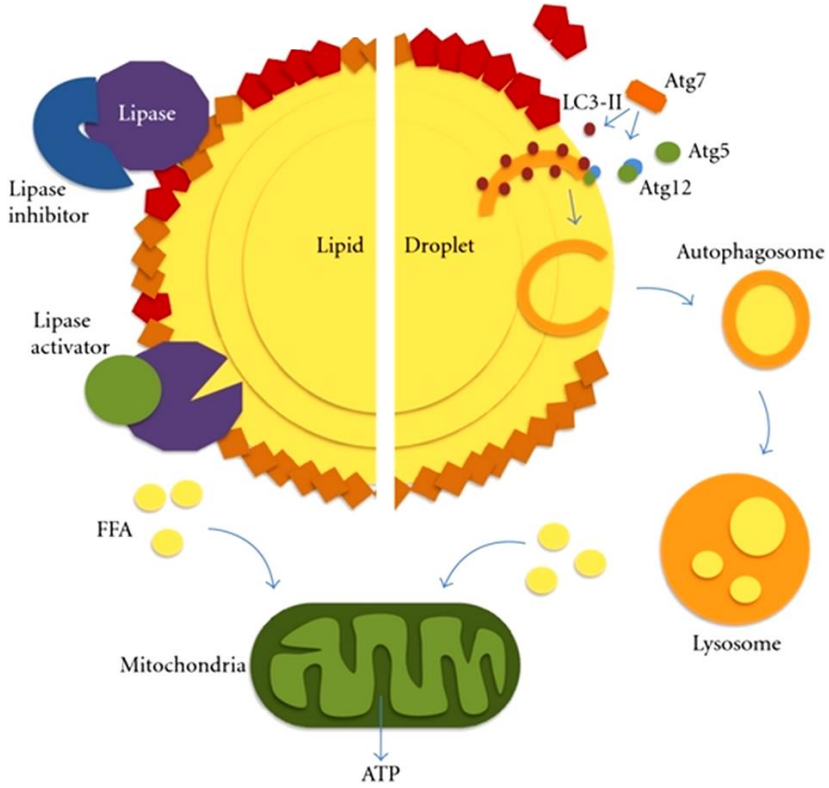

Figure 10. TG breakdown by key lipases and lipophagy machinery (modified from Singh and Cuervo 2012).

It has been shown that lipophagy may serve as a protective mechanism to reduce lipotoxicity in obesity. In fact, the up-regulation of the transcription factor EB, which regulate lysosomal biogenesis and activates autophagy, prevents of diet-induced obesity and MetS (O'Rourke and Ruvkun 2013, Settembre *et al.* 2013). In adipose tissue, it has been demonstrated that autophagy inhibition blocks adipogenic differentiation (Singh *et al.* 2009b, Zhang *et al.* 2009, Zhang *et al.* 2013), and reduces adipose tissue mass

(Goldman *et al.* 2010). Thus, it could be a target to counteract obesity and type 2 diabetes (Zhang *et al.* 2013). Additionally, the decrease on adiponectin secretion that occur in obesity, has been suggested as a link between increased autophagy and adipocyte endocrine dysfunction (Slutsky *et al.* 2016). Although, the effects of autophagy in obesity seem to be tissue-specific (different outcomes in diverse metabolic organs, such as liver and adipose tissue) and the role of autophagy in obesity and its comorbidities is still controversial (Nuñez *et al.* 2013, Kosacka *et al.* 2015, Soussi *et al.* 2016, Mizunoe *et al.* 2017) it has emerged as a potential target in metabolic-related diseases.

2.4.5 Mitochondrial function

Mitochondria are multifunctional cytoplasmic organelles found in human and animal cells (Wang *et al.* 2010). The mitochondrion is a very active, pleomorphic organelle that is responsible for several functions such as cell growth and energy production (Nuñez *et al.* 2006), as well as apoptosis (Green and Reed 1998). Among these functions, mitochondria play a key role in lipid metabolism pathways such as lipogenesis and more importantly FA oxidation (De Pauw *et al.* 2009, Wrighton 2015).

Different cellular pathways and stimuli modulate mitochondrial content, which requires the expression of several transcription activators such as the master regulator PGC-1α (Gleyzer and Scarpulla 2011). The effects of PGC-1α on mitochondrial biogenesis are driven through the activation of both nuclear respiratory factors 1 and 2 (NRF1 and NRF2), which are also potent stimulators of the mitochondrial transcription factor A (TFAM) that induces mitochondrial DNA replication causing mitochondrial duplication (Scarpulla 2006, Jornayvaz and Shulman 2010). Additionally, PGC-1α activity is mainly regulated by AMPK and Sirtuin 1 (SIRT1) actions. This last one can activate PGC-1α by deacetylation and by induction of AMPK-mediated phosphorylation, which drives to mitochondrial biogenesis stimulation too (Canto and Auwerx 2009, Scarpulla 2011, Tang 2016).

In the mitochondria take place the aerobic respiration or mitochondrial oxidative phosphorylation, which requires O_2 in order to synthetize ATP, the master cellular energy molecule. Most of the ATP produced (up to 90%) of the cellular energy is created

through oxidative phosphorylation (OXPHOS) (Chen *et al.* 2009). Interestingly, OXPHOS is normally used as a marker of proper mitochondrial function (Brand and Nicholls 2011). In this process, electrons are transferred from donors to acceptors, including O_2 in redox reactions that release energy. These redox reactions are carried out by a series of protein complexes, which are known as the electron transport chain that couples this electron transfer with the transport of protons through the inner membrane of the mitochondria. Thus, the electrochemical proton gradient that is created drives to the synthesis of ATP through the enzyme ATP synthase (Dimroth *et al.* 2000, Schultz and Chan 2001, Rich 2003). Interestingly, in brown and beige adipocytes this respiratory process can be uncoupled by UCP1, which allows the proton gradient across the membrane to be dissipated to generate heat instead of ATP, when it is stimulated by the sympathetic nervous system (Nicholls *et al.* 1978, Klingenberg 2017).

Mitochondrial dysfunction can derive from either a decrease in mitochondrial biogenesis and content or by a decrease in mitochondrial activity (Vamecq *et al.* 2012, Montgomery and Turner 2015). Several studies have proposed that mitochondria play a major role affecting individual susceptibility to develop obesity. Thus, adipocyte mitochondrial dysfunction is related to both obesity and type 2 diabetes development (Rong *et al.* 2007). Moreover, several gene transcripts encoding mitochondrial proteins are decreased in obesity, along with reduction on mitochondrial content in adipocytes from obese individuals (Mustelin *et al.* 2008). Furthermore, fatty acid β-oxidation is decreased due to mitochondrial dysfunction leading to accumulation of lipids within the adipocytes. Eventually, this lipid accumulation may lead to insulin resistance and contribute to obesity development (Monsenego *et al.* 2012). In this context, therapeutic interventions to reduce these causes may improve mitochondrial function and could be a useful approach to prevent/counteract obesity (Scarpulla *et al.* 2012, Peschechera and Eckel 2013).

3. NON-ALCOHOLIC FATTY LIVER DISEASE

NAFLD is characterized by an excess of lipid accumulation in liver (TG infiltration in >5% of hepatocytes) due to several non-alcohol related causes (Brunt *et al.* 2015, Neuschwander-Tetri 2017). NAFLD is a chronic inflammatory liver disease that covers a spectrum of pathologies ranging from simple accumulation of fat within the hepatocytes or liver steatosis, to late stages of the disease such as cirrhosis, going through non-alcoholic steatohepatitis and fibrosis, as summarized in **Figure 11** (Mark *et al.* 2010, Vanni *et al.* 2010, Hardy *et al.* 2016). NAFLD is considered one of the most common causes of liver disease worldwide (Mavrogiannaki and Migdalis 2013) and its incidence increase in tandem with the growing obesity rates. Indeed, its global prevalence is 20-30%, with the highest frequency in the Middle East and South America and the lowest in Africa (Younossi *et al.* 2016). Despite the NAFLD public health implications, most of the individual affected persist undiagnosed (Younossi *et al.* 2016).

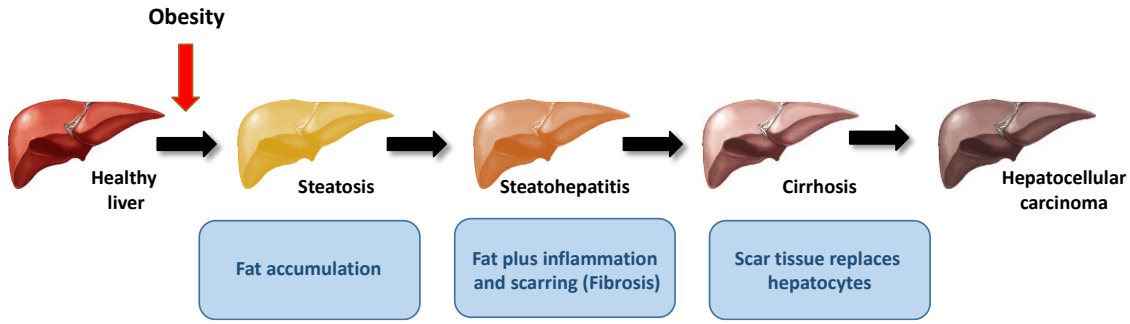

Figure 11. Scheme of NAFLD progression.

During the first stages of the disease, NAFLD is reversible and in some cases, might be even asymptomatic, which is one of the main difficulties for the diagnosis. However, the development towards fibrosis and cirrhosis can lead to multisystemic failure and death (Day 2006, Qureshi and Abrams 2007). Importantly, NAFLD is tightly associated with MetS features such as obesity and other metabolic-related diseases including type 2 diabetes, hyperlipidaemia and hypertension (Vanni *et al.* 2010, Anstee *et al.* 2013). Hence, NAFLD is considered as the hepatic manifestation of this syndrome (de Alwis and

Day 2008). In this context, the specific mechanisms of how NAFLD develops are still widely unknown, due to the fact that it is a complex disease modulated by multifaceted interaction of several factors including metabolic, genetic, microbiota and environmental, among others (Rinella 2015).

As described before, obesity may lead to metabolic complications. It is known that excessive adipose tissue can induce alterations on both lipid and glucose metabolism systemically, which lead to ectopic fat accumulation in liver and other peripheral organs (Virtue and Vidal-Puig 2010). Thus, it progressively induces liver injury due to lipotoxicity, inflammation and oxidative stress (Rinella 2015). In fact, it is believed that the initiation of NAFLD depends of severity of obesity and insulin resistance development in both adipose tissue and liver (Anstee *et al.* 2013).

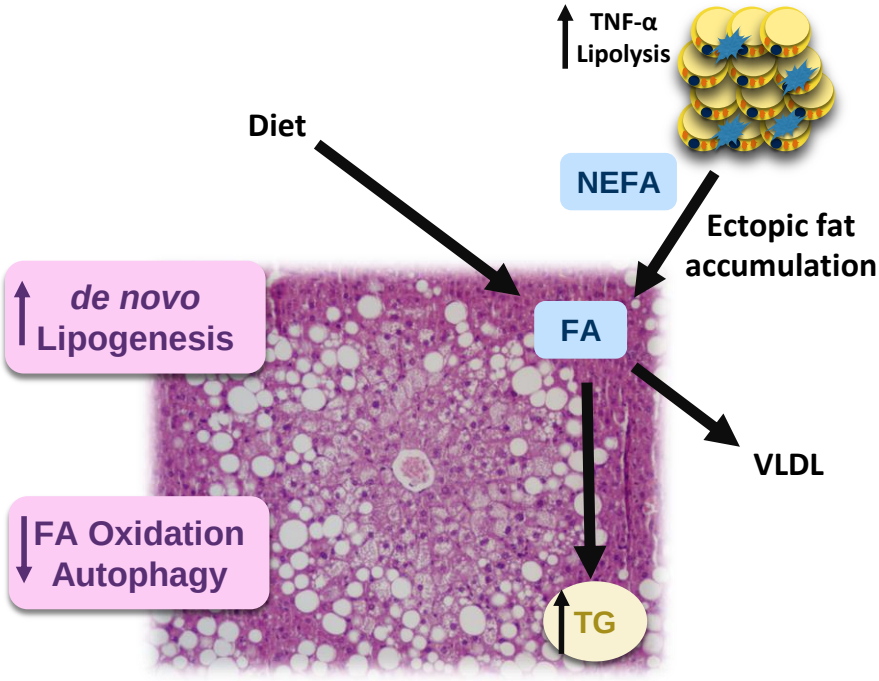

Figure 12. Summary of impaired lipid metabolism in fatty liver disease.

Several cellular biochemical pathways are involved in hepatic lipid accumulation, briefly summarized in **Figure 12**. Indeed, liver plays a key role in lipid metabolism. The homeostasis of liver lipid metabolism is maintained by a balance among lipid synthesis and fatty acids uptake into the liver, and lipid breakdown. On the one hand, in *de novo*

hepatic lipogenesis ACC and FAS, are the main predominant enzymes that catalyze hepatic fatty acid synthesis (Sanders and Griffin 2016). *De novo* hepatic lipogenesis is also controlled on a transcriptional level by glucose and insulin via carbohydrate-responsive element–binding protein (ChREBP) and sterol regulatory element-binding protein 1c (SREBP-1c), respectively (Sanders and Griffin 2016). These transcription factor actions connect both hepatic glucose and lipid metabolism. Additionally, hepatic fat can also be uptaken from circulating NEFA derived from adipose tissue (Rui 2014). On the other hand, the liver may oxidize FA by β-oxidation pathway to produce energy for its metabolic activities and fasting periods. FA oxidation occurs predominantly in mitochondria and alternatively in peroxisomes to shorten very-long-chain FA. Indeed, FA have to travel from the cytoplasm to the mitochondria using CPT1, a protein that shuttle fatty acid across mitochondria membranes in order to be catalysed, as described above (McGarry and Brown 1997). Also, ACOX is responsible for FA dehydrogenation promoting β-oxidation within peroxisomes (Poirier *et al.* 2006).

Recently, autophagy has raised significance in liver. It has been described that autophagy not only maintain liver homeostasis, but also may have an important role in liver fat accumulation (Singh *et al.* 2009a, Singh and Cuervo 2012). In fact, hepatocyte lipids are also degraded by the selective autophagy pathway, lipophagy. During nutrient deprivation, hepatocytes specifically target lipids for degradation through autophagy, which may have a key role in the ability of hepatocytes to rapidly mobilize great amount of lipids (Czaja 2011). Autophagy seems to play an important role in the development of NAFLD (Cursio *et al.* 2015), since it has been shown that liver steatosis is accompanied by defective hepatic autophagy (Singh *et al.* 2009a, Singh and Cuervo 2012). It has also been described that autophagy inhibition in cultured hepatocytes and mouse liver enlarged TG storage within lipid droplets (Singh *et al.* 2009a). Furthermore, autophagy markers are decreased in liver of obese mice (Liu *et al.* 2009a, Yang *et al.* 2010), which further suggest a link between obesity-related liver steatosis and autophagy. Consequently, the finding of new agents able to modulate hepatic lipid metabolism biochemical pathways, in order to treat and prevent NAFLD development is highly desired.

4. OBESITY AND INFLAMMATION

There is broad evidence pointing out the relevance of the immune system as a connection between increased adiposity and the development of obesity comorbidities (Kloting and Bluher 2014). Obesity-associated inflammation is a type of non-infectious, systemic and low-grade inflammation, which is the result of body response to metabolic disruption (Sun *et al.* 2012).

In this sense, in obesity there is an activation of certain inflammatory pathways, usually a physiological response used as host defence (Monteiro and Azevedo 2010). Moreover, the resolution of inflammation is also impaired in obesity (Spite *et al.* 2014). It is believed that dysfunctional WAT plasticity and immune cells mobilization, can promote the activation of inflammatory cascades that may disturb insulin signalling leading to insulin resistance. Although, inflammation plays a role in the development of insulin resistance, up to the present time the exact mechanisms involved in this chronic low-grade inflammation are not completely understood yet. However, it is known that cytokines play an important role in this chronic inflammation (Monteiro and Azevedo 2010, Gregor and Hotamisligil 2011).

Recent evidence suggests that both brown and white adipose tissue are able to secrete different cytokines, which may act in autocrine, paracrine and endocrine signalling as immunomodulatory agents (Hauner 2005, Villarroya *et al.* 2017). In this context, increased levels of pro-inflammatory cytokines, such as TNF-α, interleukin 6 (IL-6) and monocyte chemoattractant protein-1 (MCP1), are found within the obese adipose tissue. Contrary, the levels of several anti-inflammatory adipocytokines such as interleukin 10 (IL-10) or adiponectin are decreased in this tissue (Hotamisligil *et al.* 1995, Maury and Brichard 2010). The misbalance of anti-inflammatory/pro-inflammatory cytokines that is established in obesity occurs in parallel to macrophage infiltration. Actually, a switch on macrophages phenotype, from M2 macrophages (anti-inflammatory) to M1 macrophages (proinflammatory) arises in obese adipose tissue (Castoldi *et al.* 2015).These macrophages have an important role in obesity-inflammation development since they are the primary source for cytokine production (Castoldi *et al.* 2015). This inflammatory state could lead to adipose tissue dysfunction

and later on to systemic metabolic disorders such as dyslipidaemia and insulin resistance (Moller 2000).

Interestingly, the observation of elevated TNF-α production by adipose tissue in obesity by Hotamisligil group back in 1993, accelerate the study of the role of inflammation in this disease. Nowadays, it is well known that the proinflammatory cytokine TNF-α induces insulin resistance, one of the main components of type 2 diabetes (Hotamisligil *et al.* 1993). It also takes an important role in adipose tissue insulin resistance. Indeed, it is believed that elevated levels of TNF-α, could alter adipocyte lipid metabolism, promoting high lipolysis rate (Laurencikiene *et al.* 2007). Although, this might seem favourable to eliminate excess of fat accumulated, it has been proved that increased lipolysis levels lead to high levels of circulating free FA. FA might be accumulated ectopically when they are not consumed immediately, and can also interfere with the insulin signalling cascade, inducing systemic insulin resistance (Arner 2002, Guilherme *et al.* 2008, Morigny *et al.* 2016).

Accumulating evidence supports that inflammation, along with increased levels of proinflammatory cytokines, are strongly related with not only insulin resistance but also with NAFLD. It is known that immune activation is a master factor generating hepatic inflammation in NAFLD (Asrih and Jornayvaz 2013, Arrese *et al.* 2016). Moreover, insulin resistance has been characterized as the crucial pathophysiological factor in NAFLD development (Mavrogiannaki and Migdalis 2013). It is believed that the antilipolytic effect of insulin is compromised under insulin-resistant conditions. Thus, inducing triglyceride synthesis also in liver, which is recognized as a typical outcome of MetS (Williams 2015). Moreover, the consequence of adipocyte insulin resistance and the chronic release of free FA to blood stream by adipose tissue, lead to storage of lipids, ectopically in other distant organs such as muscle, heart or liver (Cusi 2010, Gaggini *et al.* 2013). Consequently, the storage of lipids in liver may produce lipotoxicity and inflammation promoting the development of NAFLD. However, NAFLD is a multifactorial complex disease, in which several physiopathological factors participate on its development including impaired glucose and lipid metabolism (Hardy *et al.* 2016). Therefore, the study of NAFLD treatment and development need to be further considered (Karlas *et al.* 2013).

Taking all these evidences together, it could be hypothesized that the resolution of chronic inflammation is a critical component in the strategies for tackling obesity and the clustering of obesity-associated conditions.

5. OMEGA-3 POLYUNSATURATED FATTY ACIDS

The organism can receive two different types of fat through diet: unsaturated and saturated fat. This last one can be found naturally in food, mainly from animal sources, such as meat and dairy products, and have been related with higher risk of heart disease (de Souza *et al.* 2015). On the other hand, unsaturated fats are predominantly found in food derived from fish or plants, such as olive oil, nuts and seeds (Lee *et al.* 2009). These unsaturated fats can be differentiated in monounsaturated and polyunsaturated fats.

In this sense, the long chain omega-3 polyunsaturated fatty acids (n-3 PUFAs) are derived from marine or vegetal sources (**Figure 13**). Among marine n-3 PUFAs, the most significant ones are Eicosapentaenoic acid (EPA, n-3, 20:5) and Docosahexaenoic acid (DHA, n-3, 22:6). EPA and DHA can be found mainly in oily fish including salmon, tuna, mackerel and anchovy among others (Lorente-Cebrián *et al.* 2013 and 2015) On the other hand, the most relevant n-3 PUFA derived from plants including walnut, linseeds and flaxseeds, is the α-linolenic acid (ALA, 18:3). ALA is considered as an essential fatty acid, since mammals are not able to synthesize it. Nevertheless, mammals can obtain ALA through diet and metabolized it through desaturation and elongation to EPA and DHA into the organism. However, the rate of conversion from ALA to EPA and DHA is limited, so external supply through diet is necessary to reach optimal endogenous levels (Burdge *et al.* 2002, Burdge and Wootton 2002).

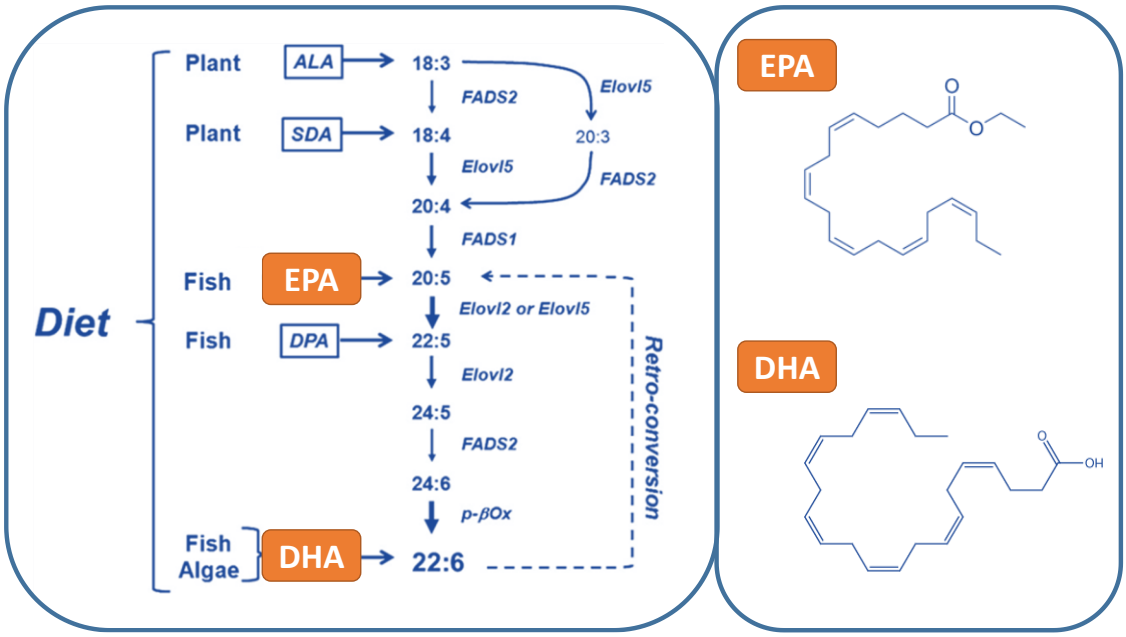

Figure 13. N-3 PUFAs biosynthesis and chemical structure (modified from Jump *et al.* 2012).

Marine n-3 PUFAs have been widely recognised to exert favourable anti-inflammatory actions on inflammatory-related pathologies such as cardiovascular diseases, atherosclerosis, Alzheimer disease, asthma, arthritis, colitis, obesity and MetS (Calder 2015). It has been suggested that increased intake of n−3 PUFAs might reduce cardiovascular disease risk, improve some MetS features and have potential anti-obesity properties (Abete *et al.* 2011, Lorente-Cebrián *et al.* 2013). Based on their beneficial effects, some health organizations recommend a daily intake of 250 to 500 mg of EPA and DHA for healthy adults for primary prevention of disease (EFSA Panel on Dietetic Products 2009, FAO/WHO 2010). On the other hand, different international organizations have also recognised the beneficial actions of the marine n-3 PUFAs reducing hypertriglyceridemia and cardiovascular risk (Kris-Etherton *et al.* 2003, Lavie *et al.* 2009, Mozaffarian and Wu 2011). The Food and Drug Administration (FDA) stated that an intake up to 3 g/day of marine n-3 PUFAs can be safe and the American Heart Association (AHA) recommends the use of EPA/DHA supplements in a dose of 2-4 g/day for hypertriglyceridemia treatment (Kris-Etherton *et al.* 2003, Bradberry and Hilleman 2013).

On the other hand, several investigations have suggested favourable effects of n-3 PUFAs in obesity. Thus, plasma n-3 PUFAs levels have been negatively associated with obesity (Micallef *et al.* 2009). Moreover, it has been suggested that an increase in the n-6/n-3 PUFAs ratio favours the risk for obesity (Simopoulos 2016). However, it remains unclear whether dietary supplementation with n-3 PUFAs may be helpful to promote weight loss in obese subjects (Huerta *et al*. 2016). As previously explained, obesity is considered as a chronic low-grade inflammatory disease, which increases the risk of both cardiovascular diseases and insulin resistance (Gregor and Hotamisligil 2011). In this context, murine models of obesity and clinical trials in humans strongly proposed n-3 PUFAs supplementation as a potential therapeutic strategy to protect from diet-induced inflammatory state in obesity (Martínez-Fernández *et al.* 2015). Moreover, the ability of n-3 PUFAs to ameliorate obesity-linked metabolic disorders, have been associated to their ability to modulate adipocyte metabolism and reduce inflammation in adipose tissue (Martínez-Fernández *et al.* 2015).

5.1 N-3 PUFAs and lipid metabolism

Considering the beneficial properties of marine n-3 PUFAs to improve impaired lipid metabolism in obesity, research interest has been focused in getting a better knowledge of the mechanisms involved. These studies have revealed that n-3 PUFAs modulate lipid metabolic pathways in main insulin-sensitizing organs including liver, skeletal muscle and adipose tissue (Flachs *et al.* 2009, Kopecky *et al.* 2009, Pérez-Echarri *et al.* 2009, Sun *et al.* 2011, Martínez-Fernández *et al.* 2015).

In this sense, n-3 PUFAs are able to regulate key lipogenic enzymes such as SCD1 and FAS (Sebokova *et al.* 1996, Raclot *et al.* 1997, Manickam *et al.* 2010, Barber *et al.* 2013), which may reduce fat accumulation in both WAT and BAT.

On the other hand, it has been reported that n-3 PUFAs can also modulate catabolic pathways in adipose tissue and thus, improve fatty acid balance. Several studies have shown the ability of n-3 PUFAs to modulate lipolysis through the action on key lipases and lipid droplet proteins, although, some controversial findings have been observed. Some studies have suggested that both EPA and DHA up-regulate lipolysis in murine

adipocytes (Kim *et al.* 2006, Manickam *et al.* 2010, Barber *et al.* 2013), while other research found that EPA decreases basal lipolysis and it is also able to prevent the deleterious lipolytic effect of the pro-inflammatory cytokine, TNF-α in rat primary cultured adipocytes (Lorente-Cebrián *et al.* 2012).

In addition, it has also been described the capability of n-3 PUFAs to induce FA oxidation in both mitochondria and peroxisome by upregulating *Cpt1a* and *Acox1,* two key genes involved in these processes in mice (Flachs *et al.* 2005, Guo *et al.* 2005). Interestingly, it has also been proposed that n-3 PUFAs could modulate mitochondrial biogenesis and induce browning of WAT in murine adipocytes and in mice (Flachs *et al.* 2005, Zhao and Chen 2014). Flachs *et al.* (2011) also found that n-3 PUFAs improve mitochondrial function and biogenesis in WAT through UCP1 independent mechanisms in mice. Supporting this observation, it has also been shown that the anti-obesity effects of n-3 PUFAs occur independently of cold-induced thermogenesis (Janovska *et al.* 2013).

Taking all these data together, it can be concluded that n-3 PUFAs orchestrate a complex regulation of different enzymes involved in key metabolic pathways responsible for lipid balance (lipid storage, lipid breakdown and oxidation), promoting in overall a reduction of triglycerides accumulation in adipose tissue. Further studies are needed to better characterize the potential britening properties of n-3 PUFAs and the ability of these fatty acids to modulate lipid metabolism in human adipocytes and especially in human adipose tissue from obese subjects.

5.2 N-3 PUFAs and adipose tissue inflammation

The impact of n-3 PUFAs on the functional responses of cell to acute and chronic inflammation has been broadly studied. There is strong evidence indicating the important anti-inflammatory properties of marine n-3 PUFAs, and thus they might be useful in the management of inflammatory diseases such as obesity and MetS (Calder 2013, Lorente-Cebrián *et al.* 2013 and 2015). Several mechanisms have been linked to n-3 PUFAs anti-inflammatory actions including: 1) The increase of anti-inflammatory cytokines and the reduced production of the pro-inflammatory ones; 2) the decrease of macrophage infiltration; 3) the reduced formation of n-6 derived pro-inflammatory lipid

mediators; and 4) being substrates for the formation of proresolutive lipid mediators (**Figure 14**).

1) It is widely known that n-3 PUFAs can alleviate adipocyte dysregulation and the consequent inflammation by modulating cytokines release. In this line, it has been demonstrated that n-3 PUFAs down-regulate the production of several pro-inflammatory adipocytokines including MCP-1, IL-6, TNF-α or resistin, and induce the release of anti-inflammatory adipocytokines such as adiponectin or IL-10 (Kang and Weylandt 2008, Pérez-Echarri *et al.* 2008, Kalupahana *et al.* 2010, Sato *et al.* 2010, Figueras *et al.* 2011, Rossmeisl *et al.* 2012, Ding *et al.* 2014).

Interestingly, studies in *fat-1* transgenic mice (expressing the *Caenorhabditis elegans fat-1* gene encoding an n-3 fatty acid desaturase that converts n-6 to n-3 fatty acids) have also demonstrated the ability of n-3 PUFAs to modulate adipocytokines, and thus reducing inflammation. These animals exhibited decreased mRNA levels of some pro-inflammatory cytokines including *Mcp-1, Rantes, Il1β, Il2* and *Il6* in epididymal adipose tissue. These effects occur in parallel with a reduction in the percentage of $F4/80^{+}$ macrophages and the characteristic crown-like structures (macrophages surrounding dying or dead adipocytes), and with increased expression of genes related with anti-inflammatory actions in adipose tissue (White *et al.* 2010, López-Vicario *et al.* 2015). All these data strongly prove that the anti-inflammatory effects of n-3 PUFAs are in part mediated by their ability to modulate adipocytokines production.

2) It has also been proposed that these fatty acids have the capability to reduce leukocyte chemotaxis, and thus decrease macrophage infiltration. In this context, it has been found down-regulated expression of markers linked with macrophages infiltration (*Mrs1, Il1rn, CD14*) in diabetic mice after being fed with n-3 PUFAs-enriched diet (Todoric *et al.* 2006). Additionally, n-3 PUFAs are able to promote a macrophage switch from M1 (pro-inflammatory) phenotype to M2 (anti-inflammatory). Thus, DHA administration to high fat diet fed mice promoted a phenotype switch in macrophage polarization towards an M2-like phenotype (Titos *et al.* 2011). Furthermore, studies in transgenic *fat-1* mice revealed a fall in macrophage migration markers within the adipose tissue of these mice, together with an induction to M2 macrophage phenotype, further supporting the previous information (Li *et al.* 2015, López-Vicario *et al.* 2015).

3) Subsequently, numerous studies have shown that n-3 PUFAs anti-inflammatory actions may be mediated through a reduction of n-6 PUFAs-derived pro-inflammatory lipid mediators, including prostaglandins and leukotrienes derived from arachidonic acid (n6, 20:4). In this line, it has been demonstrated that both EPA and DHA compete with arachidonic acid as substrates for several enzymes, thus preventing the formation of classical proinflammatory mediators derived from arachidonic acid (Needleman *et al.* 1979, Corey *et al.* 1983, González-Périz and Clària 2010). Remarkably, it has also been found that n-3 PUFAs-enriched diet can reduce the production of n-6 PUFAs derivatives including PGE2, PGF2α, TXB2, 5-HETE, 12-HETE and 15-HETE in adipose tissue of *ob/ob* mice (González-Périz *et al.* 2009).

4) Few years ago, Serhan and collaborators discovered that n-3 PUFAs can be enzymatically converted to the generically known as specialized proresolving lipid mediators (SPMs) within the organism (Serhan 2007, Poulsen *et al.* 2008, Serhan 2009). These novel bioactive lipid mediators play an active role in the resolution of acute and chronic inflammation, thus restoring tissue homeostasis, and exerting potent anti-inflammatory actions (Serhan 2014, Chiang and Serhan 2017, Serhan 2017a). Therefore, the anti-inflammatory actions of n-3 PUFAs could also be mediated in part by their metabolic conversion to these SPMs, including resolvins, protectins and maresins.

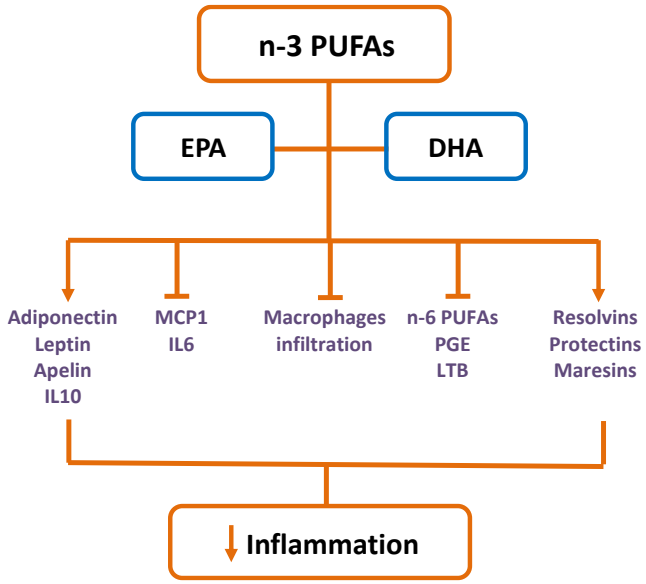

Figure 14. Summary of the anti-inflammatory actions of EPA and DHA on obese adipose tissue.

In summary, these mechanisms could be underlying the n-3 PUFAs ability to reduce inflammation in obese adipose tissue, and to ameliorate the low-grade inflammation that is established in obesity and metabolic complications. Therefore, it may be a potential therapy to combat these deleterious disorders.

6. SPECIALIZED PRO-RESOLVING LIPID MEDIATORS (SPMs)

As previously described, n-3 PUFAs serve as precursors of several SPMs that have beneficial effects on inflammatory-related diseases. These novel bioactive lipid mediators are classified reliant on their precursor (**Figure 15**). Thus, EPA-derived SPMs are known as E-series Resolvins (RvE1-3) and DHA-derived lipid mediators are named as D-series Resolvins (RvD1-6), Protectins (NPD1 and PDX), and Maresins (MaR1-2) (Serhan 2014). These SPMs derived from n-3 PUFAs by the action of different enzymes, including the three major oxygenase enzymes in humans (5-, 12- and 15-LOX) and also through epoxidation and peroxidation reactions. Additionally, the cyclooxygenase-2 (COX2), a key enzyme in the biosynthesis of prostaglandins, and the cytochrome P450 monooxygenases also play a role in some SPMs synthesis (Serhan 2007). Remarkably, there are also certain isomers of Rvs that are termed as aspirin-triggered resolvins (AT-Rvs) since their synthesis is initiated by the drug-modified COX2 enzyme, enhancing 18R and 17R epimers (Serhan 2017b). In this sense, it has been very recently described a T series Rvs that are derived from another n-3 PUFA, Docosapentaenoic Acid (DPA) after aspirin or atorvastatin treatment (Dalli *et al.* 2013a and 2015).

Interestingly, the n-6 PUFA arachidonic acid, which is known to have pro-inflammatory actions, also serves as a substrate of SPMs. The arachidonic acid-derived SPMs are called lipoxins. These lipoxins promote resolution and exert similar properties as n-3 PUFAs derivates, instead of having pro-inflammatory actions as their precursors (Borgeson *et al.* 2015, Chiang and Serhan 2017, Serhan 2017a).

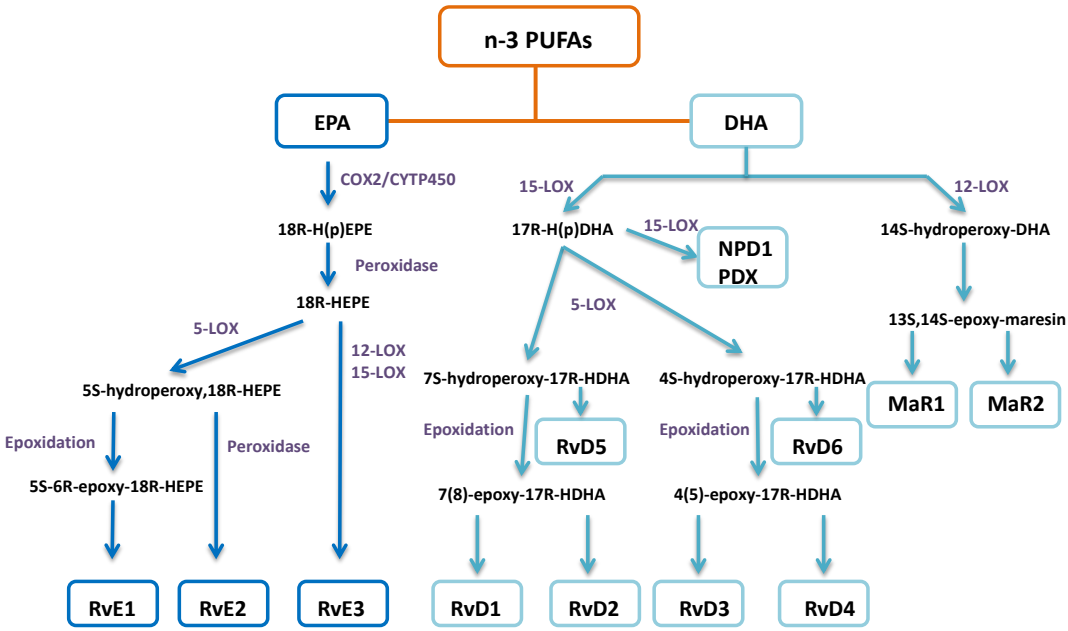

Figure 15. Human SPM biosynthetic routes (modified from Martínez-Fernández *et al.* 2015).

6.1 Profiling of SPMs

Historically, the resolution of inflammation was thought to be a passive process, while is now known to involve several active molecules such as SPMs, which play a major role facilitating inflamed tissues to recover homeostasis (Serhan *et al.* 2008). Thus, SPMs activate tissue protection and healing through resolving physiological inflammatory responses and acting as more potent anti-inflammatory agents than their precursors. Indeed, it has been described that some of these SPMs also have anti-inflammatory effects on inflamed obese adipose tissue and other metabolic organs such as liver (Spite *et al.* 2014, Clària *et al.* 2017).

Interestingly, a variety of these SPMs have recently been detected in different organs, as well as in human subcutaneous adipose tissue (**Figure 16**). In inflamed adipose tissue several SPMs have been distinguished including RvD1-2, PD1, Lipoxin A4, and the monohydroxy biosynthetic pathway markers of RvD1, PD1 (17-HDHA), RvE1 (18-HEPE), and MaR1 (14-HDHA) (Clària *et al.* 2013). Regarding obesity, it has been proved that the endogenous levels of these lipid mediators are decreased because an impaired

local production in adipose tissue (Clària *et al.* 2012, Neuhofer *et al.* 2013). In addition to adipose tissue, these deficiencies have been also noted in other metabolic organs including liver and skeletal muscle (White *et al.* 2010). In this context, there is evidence that supports that a dietary marine origin n-3 PUFAs supplementation can promote an increment of the synthesis of these SPMS in adipose tissue of obese mice (González-Périz *et al.* 2009, Neuhofer *et al.* 2013). In fact, it is believed that SPMs levels correlate with n-3 PUFAs intake in humans (Mas *et al.* 2012). Supporting this concept, highly purified n-3 PUFAs treatment to obese-nondiabetic patients significantly raised the production of some SPMs in visceral adipose tissue, along with a reduction of adipose tissue and systemic inflammation (Itariu *et al.* 2012).

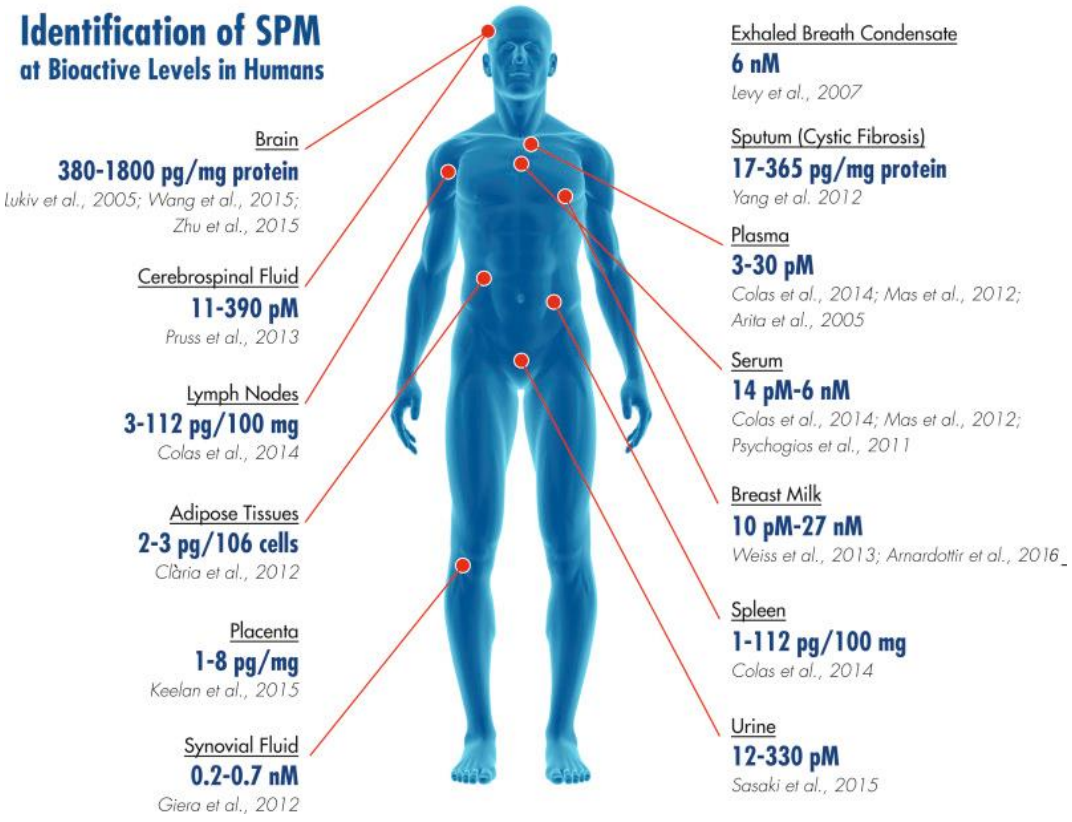

Figure 16. Bioactive levels of SPMs in humans (from Serhan 2017a).

6.2 SPMs actions in obesity

In the last years, several research groups have focused their studies in analysing the ability of some SPMs to ameliorate obesity and their associated metabolic complications including insulin resistance and NAFLD. **Table 1** briefly summarizes the currently available studies describing the effect of SPMs in animal models of obesity and associated disorders. Interestingly, it is noteworthy to mention that unlike their precursors, these SPMs exert potent actions at pico to nanomolar range, suggesting a potential powerful therapeutic approach to improve obesity and metabolic-related diseases.

Briefly, it has been reported that treatment with RvE1 and PD1 to *ob/ob* mice have similar beneficial actions as n-3 PUFAs supplementation (González-Périz *et al.* 2009). Hence, RvE1 attenuated hepatic steatosis and up-regulated gene expression levels of adiponectin, *Glut4, Irs1, Ppar-γ* in parallel with an induction of AMPK phosphorylation in adipose tissue. PD1 also increases adiponectin expression in adipose tissue explants of *ob/ob* mice (González-Périz *et al.* 2009). Additionally, RvD1 has been proposed to have beneficial effects not only on inflammatory processes and on the modulation of cytokine secretion, but also improving insulin sensitivity in both, genetic and diet-induced obese mice. In this line, RvD1 treatment improved glucose tolerance by increasing adiponectin serum levels and promoting Akt and AMPK activation in WAT. These effects are also accompanied by decreased pro-inflammatory adipokines expression and crown-like structures, as well as by a rise in the ratio M2:M1 macrophages in RvD1-treated *db/db* mice (Hellmann *et al.* 2011).

In accordance with these results, other study revealed that RvD1 treatment down-regulated pro-inflammatory cytokines along with an induction of macrophage polarization toward M2-like phenotype in adipose tissue (Titos *et al.* 2011). Moreover, RvD1 and RvD2 *ex vivo* treatment of inflamed obese adipose tissue explants showed decreased pro-inflammatory cytokines levels including leptin, TNF-α, IL-6, and IL-1β. Both resolvins reduced MCP1 and LTB_4-stimulated monocyte adhesion to adipocytes conjointly with monocyte transadipose migration (Clària *et al.* 2012). Interestingly, RvD1 also promoted the resolution process initiated by calorie restriction in obesity-induced steatohepatitis thus, improving liver steatosis (Rius *et al.* 2014).

Table 1. Effects of SPMs in animal models of obesity (modified from Martínez-Fernández *et al.* 2015)

SPM	Animal	Treatment	Results	References
RvE1	*ob/ob* mice	1.2 ng/g BW every 24 h during 4 days	↑ PPARγ, GLUT4 and IRS-1 gene expression ↑ Adiponectin	(Gonzalez-Periz *et al*. 2009)
RvD1	*db/db* mice	2 µg/kg BW for 8 to 16 days	↑ Glucose tolerance ↑ Adiponectin ↑ AMPK and Akt phosphorylation in WAT ↓ IL-6 expression in WAT ↓ Crown-like structures rich in inflammatory $F4/80^+$ $CD11c^+$ macrophages ↑ $F4/80^+$ cells expressing MGL-1.	(Hellmann *et al*. 2011)
RvD1	C57BL/6J mice HFD	300 ng every 24 h during 3 weeks + Calorie restriction	↑ Adiponectin, IL-4, IL-10 ↓ Macrophage innate immune response in liver	(Rius *et al.* 2014)
17- HDHA	*db/db* mice	50 ng/g BW every 12 h during 8 days, or continuous application for 15 days	↓ Expression of MCP1, TNF-α and IL-6 in WAT ↑ Adiponectin ↑ Glucose tolerance and insulin sensitivity	(Neuhofer *et al*. 2013)
Protectin DX	*db/db* mice	1 µg intravenously immediately before and 2.5 h into the 6 h lipid infusion	↑ IL-6 expression in skeletal muscle ↑ AMPK phosphorylation in skeletal muscle ↑ Insulin sensitivity in skeletal muscle	(White *et al.* 2014)
Lipoxin A4	DIO mice Adiponetin KO	5 ng/g three times per week between weeks 5 and 12 of the 3 months on HFD	↓ Liver weight ↓ ALT, hepatic TG ↓ Adipose tissue Inflammation Modulate autophagy	(Borgeson *et al.* 2015)

Protein kinase B (Akt); Alanine aminotransferase (ALT); AMP-activated protein kinase (AMPK); Body weight (BW); Diet induced obesity (DIO); Glucose transporter type 4 (GLUT4); High fat diet (HFD); Interleukin (IL); Insulin receptor substrate 1 (IRS-1); Knock out (KO); Monocyte chemoattractant protein-1 (MCP1); Peroxisome proliferator-activated receptor gamma (PPARγ); Triglycerides (TG); Tumor necrosis factor alpha (TNF-α).

Further evidence supporting SPMs positive effects on metabolic disturbances, may be that 17-HDHA reduces adipose tissue expression of inflammatory cytokines (MCP-1, TNF-α and IL-6) and increased adiponectin levels in *db/db* mice. These actions are accompanied by reduced insulin resistance and improved glucose tolerance in obese-diabetic mice (Neuhofer *et al.* 2013).

Moreover, it has been demonstrated that Protectin DX (PDX), also derived from DHA, exerts important insulin-sensitizing and glucoregulatory actions by stimulating the release of the myokine IL-6 from skeletal muscle in *db/db* mice (White *et al.* 2014).

Furthermore, Lipoxin A4 attenuates obesity-induced fatty liver, reducing TG content and weight in parallel with decreased serum transaminases. Additionally, lipoxin A4 also reduces adipose inflammation by promoting a switch on macrophages towards M2 phenotype. Interestingly, in adiponectin knockout mice, Lipoxin A4 restores adipose expression of autophagy markers such as LC3-II and p62 (Borgeson *et al.* 2015).

On the basis of the evidence currently available, it can be concluded that SPMs seem to have an important role in tissue homeostasis, and in ameliorating inflammation-related disorders such as obesity and its metabolic complications, including insulin resistance or liver steatosis. Nevertheless, the cellular and molecular mechanism of actions of all these series of SPMs on metabolic regulation still remains to be better elucidated. Indeed, to date, only five receptors have been characterized for these molecules: the LX4 receptor ALX; the RvE1 receptors, ChemR23 and BLT1; the RvD1 receptor Gpr120; and the recently described RvD2 receptor Gpr18 (Serhan *et al.* 2011, Chiang *et al.* 2017).

7. MARESIN 1

The "**Ma**crophage mediator in **res**olving **in**flammation" (Maresins) are a family of lipid mediators biosynthesized from DHA via 12-LOX through macrophages action. Maresin 1 (7R,14S-dihydroxy-docosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid or MaR1), was the first member of this family identified. Indeed, Serhan and collaborators identified MaR1 during the resolution of mouse peritonitis exudates (Serhan *et al.* 2009). Maresins have been predominantly detected as products synthetized by

monocytes/macrophages upon acute inflammatory response. In this context, MaR1 has already been detected in several locations including the joints synovial fluid from rheumatoid arthritis patients (Serhan and Chiang 2013), and in a murine model of acute respiratory distress syndrome (Abdulnour *et al.* 2014). MaR1 has been suggested to exert strong anti-inflammatory effects and potent proresolutive actions (Serhan *et al.* 2009). Additionally, it has been related to have pro-healing properties at similar extent to other members of the n-3 PUFAs-derived SPMs (Serhan *et al.* 2012, Tang *et al.* 2013).

7.1 MaR1 biosynthesis

The biosynthesis of MaR1 takes place during inflammation resolution (**Figure 17)**. In fact, one of the intermediate in MaR1 biosynthesis, 14S-hydroperoxydocosa-4Z,7Z,10Z,12E,16Z,19Z-hexaenoic acid (14-HpDHA) derived from endogenous DHA has been reported to be accumulated in the exudates of mouse peritonitis during resolution. It is known that DHA is enzymatically converted by an initial oxygenation with molecular oxygen by 12-LOX to 14-HpDHA (Serhan *et al.* 2009). Then, 14-HpDHA is converted to 13S,14S-epoxy-maresin through epoxidation (Dalli *et al.* 2016). Finally, an enzymatic hydrolysis through hydrolases converts 13S,14S-epoxy-maresin into MaR1. Recently, supporting the idea that MaR1 is made by macrophages (Deng *et al.* 2014), it has been isolated 13S,14S-epoxy-maresin from human macrophages (Serhan *et al.* 2009) and from recombinant human 12-LOX after incubation using alcohol trapping (Dalli *et al.* 2013b).

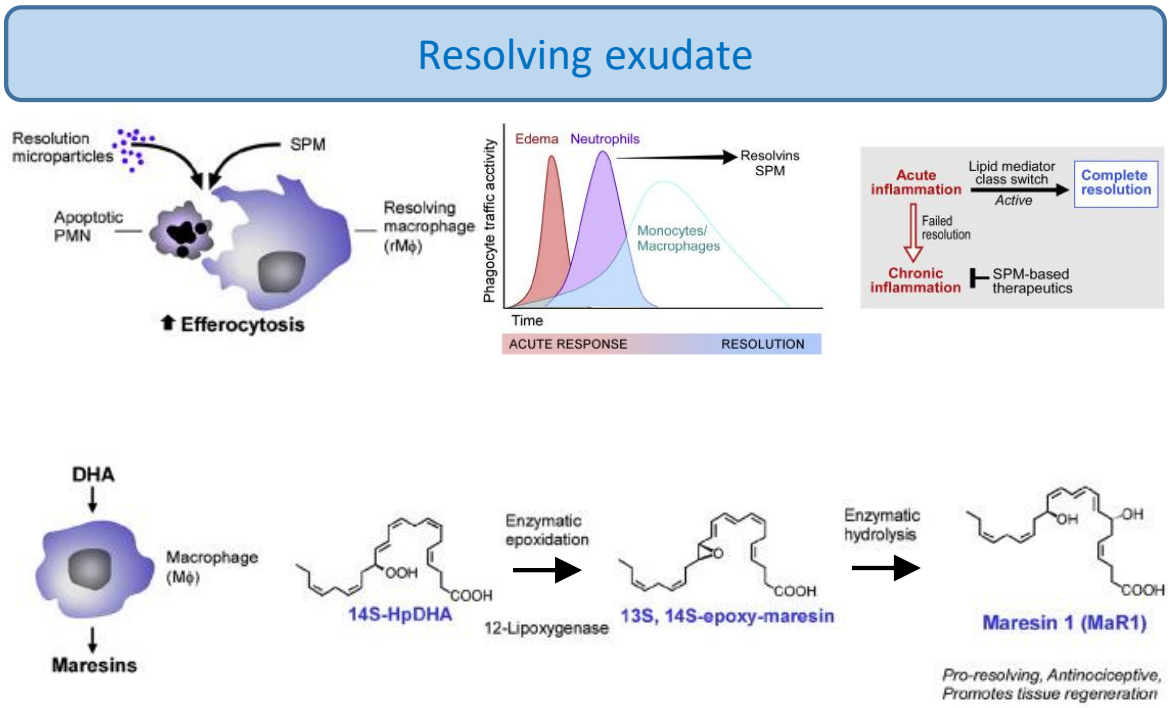

Figure 17. Maresin 1 biosynthesis pathway (modified from Chiang and Serhan (2017) and Serhan (2017a)).

7.2 Biological actions of MaR1

Despite MaR1 was identified few years ago, important effects on several inflammatory-related diseases, such as colitis and atheroprogression have been already discovered. It has been described that MaR1 has a protective key role in the body by reducing inflammation. Several effects have been shown for MaR1 effects in vascular endothelial cells, lung, brain, gut, kidney and adipose tissue (Serhan *et al.* 2012, Abdulnour *et al.* 2014, Chatterjee *et al.* 2014, Gong *et al.* 2014, Gong *et al.* 2015, Nordgren *et al.* 2015, Wang *et al.* 2015, Li *et al.* 2016, Viola *et al.* 2016, Xian *et al.* 2016, Martínez-Fernández *et al.* 2017, Tang *et al.* 2017). The mechanisms of action of MaR1 in these tissues included: decreasing neutrophil infiltration, increasing macrophage phagocytosis, inhibiting NFκB activation as well as limiting pro-inflammatory cytokines production among others (Chatterjee *et al.* 2014, Dalli *et al.* 2016).

In this context, studies analysing MaR1 effects on systemic and local inflammation have shown important anti-inflammatory and proresolutive actions of MaR1 in the

inflammation process. Thus, MaR1 is able to decrease inflammation and promote its resolution in a model of zymosan-induced peritonitis at similar levels of other SPMs such as RvE1 and PD1 (Serhan *et al.* 2009, Serhan 2014). Moreover, MaR1 persistently reduced inflammatory cell infiltration together with a decrease on macrophage activation in endothelial cells, which may ameliorate vascular inflammation. These effects are found in parallel with a decrease in pro-inflammatory cytokines production in vascular smooth cells as well as NFκB inhibition (Chatterjee *et al.* 2014).

Also in the circulatory system, MaR1 has also been described to have an important role in the nonresolved inflammation that accompanied atheroprogression. In fact, during atheroprogression an imbalance between inflammatory and proresolving lipid mediators occurs, and treatment with MaR1 successfully prevented this misbalance and leads to resolve arterial inflammation (Viola *et al.* 2016). In addition, MaR1 attenuated intimal hyperplasia in mice, influencing vessel remodelling. Thus, the administration of MaR1 may modulate resolution pathways in vascular injury response to promote vascular homeostasis (Akagi *et al.* 2015).

Interestingly, MaR1 seems to also have a role in haemostasis since a recent study has proved that MaR1 enhanced haemostatic function of platelets by inducing spreading and aggregation as well as dampening inflammatory mediators release. These effects may suggest that MaR1 promotes a favourable scenario for wound healing (Lannan *et al.* 2017). In fact, it has been demonstrated that the production of MaR1 by macrophages has indeed tissue regenerative actions, and MaR1 treatment reduced regeneration times and pain in the flatworm *Dugesia tigrina* (Serhan *et al.* 2012). Given the importance of tissue regeneration in different diseases for example healing of wounds encountered in diabetes, it may be hypothesized that MaR1 may have an important function in wound healing as other SPMs such as RvD1-2 (Tang *et al.* 2013, Spite *et al.* 2014).

Additionally, the potential actions of MaR1 in acute ischemic brain stroke, in which inflammation has an important role, are remarkable. MaR1 treatment by intracerebroventricular injection reduces the infarct volume and neurological defects associated, alleviating pro-inflammatory cascades and NFκB activation in a mouse brain ischemia reperfusion model (Xian *et al.* 2016).

Furthermore, resolution of inflammation also occurs after MaR1 treatment in other organs, including lungs (Krishnamoorthy *et al.* 2015). In fact, MaR1 mitigated lung injury induced by lipopolysaccharide (LPS), a strong elicitor of immune responses. MaR1 blocked the interaction between neutrophils and platelets and reduced pro-inflammatory cytokines release (Gong *et al.* 2014 and 2015). Remarkably, MaR1 also seems to have a noteworthy role not only as an anti-inflammatory agent but also diminishing the progression of pulmonary fibrosis (Wang *et al.* 2015).

Regarding gut, there is also crucial inflammatory component in ulcerative colitis and it has been proved that the precursor of MaR1, DHA, has important beneficial effects in this disease. In this line, MaR1 treatment also attenuated both DSS and 2,4,6-trinitrobenzene sulfonic acid-induced colonic inflammation. This was observed by histological analysis and also determined by reduced levels of pro-inflammatory cytokines, as well as the NFκB pathway inhibition (Marcon *et al.* 2013).

Moreover, in metabolic organs such as liver, the effects of MaR1 have also been studied under inflammatory stress. In this context, MaR1 ameliorated hepatic injury induced by carbon tetrachloride through reduced oxidative stress and lipid peroxidation. Similar to previous results in other diseases, MaR1 also attenuated inflammatory cytokines including, TNF-α, IL-6, IL-1β and MCP1 among others. Interestingly, MaR1 inhibited NFκB activation in the liver of carbon tetrachloride injured mice, further supporting the evidence of MaR1 as an anti-inflammatory molecule (Li *et al.* 2016).

Similar results have been recently found in diabetic nephropathy mouse model characterized with microvascular complications and inflammation. It has been demonstrated that MaR1 mitigated high glucose-induced glomerular cell injury by reducing pro-inflammatory cytokines production and reactive oxygen species generation, which not only mitigated inflammation but also reduced early fibrosis (Tang *et al.* 2017).

A recent study from our group has shown that MaR1 improved insulin sensitivity and attenuate WAT inflammation in obese mice (Martínez-Fernández *et al.* 2017), suggesting that MaR1 might be an attractive therapeutic approach to counteract the dysfunctional inflamed WAT and the subsequent insulin resistance associated to obesity.

To conclude, several research studies have been focused on clarifying the function of MaR1 and other SPMs regulating immune response to infection, wound healing and other inflammatory diseases. In fact, the current evidence suggests that MaR1 may play a key role modulating resolution programs of both acute and chronic inflammation. However, little evidence exist about the role of MaR1 in metabolic-related diseases including obesity and its comorbidities, such as insulin resistance and NAFLD, and the potential mechanisms involved.

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II. HYPOTHESIS AND AIMS

1. Justification for the study

Obesity is a chronic low-grade inflammatory pathology, and this inflammatory state has been proposed as a key link with the metabolic disorders associated to obesity, such as insulin resistance, type 2 diabetes and MetS (Esser *et al.* 2014).

Several studies have suggested the importance of both WAT and BAT metabolism and their secreted bioactive molecules in the development of obesity and its related comorbidities (Christodoulides *et al.* 2009, Villarroya *et al.* 2017). Inflammation originating from the adipose tissue is considered an important contributor for the development of insulin resistance and type 2 diabetes in obesity. In fact, obese adipose tissue is characterized by increased infiltration of proinflammatory M1 macrophages, and reduced content of anti-inflammatory M2 macrophages (Martínez-Fernández *et al.* 2015). Moreover, hypertrophied dysfunctional adipocytes secrete higher pro-inflammatory adipocytokines, which can affect adipose storage function, promoting lipolysis and causing an increase in the release of free fatty acids, which leads to insulin resistance (Lorente-Cebrián *et al.* 2012, Morigny *et al.* 2016). There is also evidence that this inflammatory state may also contribute to the alteration of autophagy, which may play a role in body fat accumulation (Chen *et al.* 2010, Harris 2011, Maixner *et al.* 2012).

During obesity development, liver accumulates ectopic fat within hepatocytes, which leads to disturbances on lipid metabolism and autophagy in this key metabolic organ (Singh *et al.* 2009, Hardy *et al.* 2016). Fatty liver plays also an important role in the development of metabolic complications associated to obesity. Indeed, it is clearly associated with insulin resistance, which is a risk factor to progress to type 2 diabetes (Gaggini *et al.* 2013).

Interestingly, BAT activation has recently been brought to attention, since it provides a defensive mechanism against excessive body weight accumulation (Yoneshiro *et al.* 2013) due to its thermogenic properties. Moreover, recent investigations have suggested emerging roles of BAT activation in the regulation of metabolism, including control of triglyceride clearance, glucose homeostasis and insulin sensitivity (Bartelt *et al.* 2011, Chondronikola *et al.* 2014, Townsend and Tseng 2014). Beige adipocytes can also dissipate energy as heat, and white-to-beige

conversion has also been pointed out as a potential therapy targeting obesity (Jeremic *et al.* 2017). BAT activation and adipose tissue browning occurs in response to some stimuli such as chronic cold exposure and β_3-adrenergic signalling (Villarroya and Vidal-Puig 2013). However, their uncomfortableness and important side effects clearly limit their utilization. Therefore, the identification of new pharmacological/nutritional agents able to counteract the disturbed adipocyte lipid metabolism induced by chronic inflammation, to reduce liver steatosis, as well as to activate BAT and WAT browning can be a therapeutic strategy to ameliorate obesity-associated disorders, such as type 2 diabetes.

In recent years, several studies and clinical investigations have suggested beneficial effects of marine n-3 PUFAs DHA and EPA on obesity and metabolic-related disorders, including hyperlipidaemia and cardiovascular disease (Martínez-Fernández *et al.* 2015). Some studies have pointed out towards the capability of n-3 PUFAs to induce mitochondrial biogenesis and adipose tissue browning in mouse models (Zhao and Chen 2014). However, there was no evidence about their potential ability to promote mitochondrial biogenesis and activate the beiging process in human subcutaneous adipocytes. The beneficial metabolic actions of these n-3 PUFAs seem to be also related to their anti-inflammatory properties. Thus, n-3 PUFAs have been shown to promote a decline in the production of proinflammatory cytokines. In addition, n-3 PUFAs are converted *in vivo* to a novel series of bioactive lipid mediators known as resolvins (Rv), protectins (PD) and maresins (MaR) (Serhan 2017a). These lipid mediators exert not only potent anti-inflammatory effects, but also actively participate in the resolution of tissue inflammation. In this context, these specializing proresolving lipid mediators (SPMs) may help to reduce inflammation in adipose tissue, and thus contribute to the beneficial actions of n-3 PUFAs in obesity and associated metabolic complications (Spite *et al.* 2014). Recent studies have revealed that the administration of some of these SPMs such as RvD1, RvD2 and PDX could be beneficial to counteract obesity-related inflammation and insulin resistance in animal models of obesity (Hellmann *et al.* 2011, Clària *et al.* 2012, White *et al.* 2014). MaR1 is a DHA-derived SPM that was first identified in macrophages and more recently in several tissues (Serhan *et al.* 2009). MaR1 exerts potent anti-inflammatory and proresolving activity (Serhan, Yang et al. 2009), and MaR1 administration has shown

beneficial effects in several chronic inflammatory diseases, such as colitis and atheroprogression (Marcon *et al.* 2013, Viola *et al.* 2016). However, the metabolic actions of MaR1 and its potential therapeutic properties in obesity and associated comorbidities have been scarcely studied.

2. Hypothesis

Based on all the previously mentioned observations, the hypothesis of the present Doctoral Thesis was that EPA beneficial effects on obesity might be also related to its ability to promote browning of human adipocytes, and that the DHA-derived bioactive lipid mediator MaR1 may constitute a good candidate to reverse inflammation-induced alterations on adipocyte function, and to promote BAT activation and WAT browning, as well as to ameliorate obesity-related fatty liver disease.

3. Objectives

The **general objective** of the present study was to determine the ability of EPA to remodel metabolism and phenotype of human subcutaneous adipocytes, as well as to characterize the ability of MaR1 to counteract inflammation-induced adipocyte disorders and to elucidate MaR1 actions on brown/beige adipose tissue and liver function in animal models of obesity and in culture cells.

The specific aims were:

1. To study the actions of EPA on mitochondrial biogenesis and its potential ability to promote beiging markers in cultured human subcutaneous adipocytes from obese/overweight subjects, as well as to evaluate the cellular mechanisms that could be responsible for these effects *(Chapter 1).*

2. To determine the effects of MaR1 on TNF-α-induced lipolysis and autophagy by characterizing its actions on main lipases and lipid droplet proteins involved in the control of the lipolytic pathway, as well as on key autophagy markers in murine adipocytes *(Chapter 2).*

3. To characterize the potential ability of MaR1 to ameliorate obesity-induced liver steatosis in genetic and diet-induced obese mice models, as well as to identify the possible mechanisms underlying MaR1 actions in fatty liver and in primary cultured hepatocytes *(Chapter 3).*

4. To analyse whether MaR1 could regulate brown adipose tissue activity and induce browning of white adipose tissue, and to characterize the major genes/proteins implicated, both in DIO mice and in cultured murine brown adipocytes and in human mesenchymal stem cells (hMSC)-adipocytes from subcutaneous fat *(Chapter 4).*

1. Justificación del estudio

La obesidad es una patología metabólica asociada a un estado de inflamación crónico de bajo grado a nivel sistémico. Este estado inflamatorio se ha establecido como un nexo clave entre la obesidad y sus trastornos metabólicos asociados tales como la resistencia a la insulina, la diabetes tipo 2 y el síndrome metabólico (Esser *et al*. 2014).

Varios estudios han sugerido la importancia tanto del metabolismo del tejido adiposo blanco (TAB), como del tejido adiposo pardo (TAP), y de las moléculas bioactivas secretadas por los mismos, en el desarrollo de la obesidad y sus comorbilidades asociadas (Christodoulides *et al*. 2009, Villarroya *et al*. 2017). La inflamación que se origina en el tejido adiposo se considera un contribuyente importante para el desarrollo de la resistencia a la insulina y la diabetes tipo 2 en la obesidad. De hecho, el tejido adiposo obeso se caracteriza por un aumento de la infiltración de macrófagos proinflamatorios M1, y una reducción en el contenido de macrófagos anti-inflamatorios M2 (Martínez-Fernández *et al.* 2015). Por otra parte, los adipocitos disfuncionales hipertrofiados secretan más adipocitoquinas pro-inflamatorias, que pueden afectar a la función de almacenamiento del tejido adiposo, además de promover lipólisis causando un aumento en la liberación de ácidos grasos libres, que favorecen al desarrollo de resistencia a la insulina (Lorente-Cebrián *et al,* 2012, Morigny *et al.* 2016). Además, hay también evidencias de que esta inflamación puede contribuir a la alteración de la autofagia, un proceso que podría desempeñar un papel clave en la acumulación de grasa corporal (Chen *et al.* 2010, Harris 2011, Maixner *et al.* 2012).

Durante el desarrollo de obesidad, el hígado acumula grasa ectópica dentro de los hepatocitos, lo que conlleva la aparición de lipotoxicidad, acompañada del desarrollo de alteraciones del metabolismo lipídico y de la autofagia en este órgano metabólico clave (Singh *et al*. 2009, Hardy *et al.* 2016). El hígado graso tiene un papel importante en el desarrollo de complicaciones metabólicas asociadas a la obesidad. Así se ha descrito que el desarrollo de hígado graso está claramente asociado con la resistencia a la insulina, factor de riesgo para el progreso de la diabetes tipo 2 (Gaggini *et al*. 2013).

Recientemente, se ha prestado mucha atención a la investigación de factores que modulan la activación del TAP, ya que gracias a sus propiedades termogénicas,

proporciona un mecanismo de defensa contra la acumulación excesiva de peso corporal (Yoneshiro *et al.* 2013). Por otra parte, existen evidencias que indican que la activación del TAP desempeñaría también un papel fisiológico relevante en el control del aclaramiento y la homeostasis de los triglicéridos y de glucosa, así como en la regulación de la sensibilidad a la insulina (Bartelt *et al.* 2011, Chondronikola *et al.* 2014, Townsend y Tseng. 2014). Los adipocitos beige también pueden disipar energía en forma de calor, y el incremento de la formación de adipocitos beige en el TAB (pardeamiento), también se ha señalado como una potencial diana terapéutica contra la obesidad (Jeremic *et al*. 2017).

La activación del TAP y el pardeamiento del TAB se producen en respuesta a algunos estímulos tales como la exposición crónica al frío y señales β_3-adrenérgicas (Villarroya *et al.* 2013). Sin embargo, la incomodidad que la terapia con frío supondría, y los efectos secundarios no deseados de los agonistas β-adrenérgicos, limitan su utilización terapéutica. Por lo tanto, la identificación de nuevos agentes farmacológicos/nutricionales capaces de contrarrestar las alteraciones en el metabolismo lipídico de los adipocitos inducidas por la inflamación crónica, además de reducir la esteatosis hepática podría utilizarse como posibles terapias para reducir las complicaciones derivadas de la obesidad. Igualmente, la activación del TAP y el pardeamiento del TAB, podrían también ser una adecuada estrategia terapéutica para mejorar los trastornos asociados con la obesidad como la diabetes tipo 2.

En los últimos años, varios estudios e investigaciones clínicas han sugerido efectos beneficiosos de los ácidos grasos omega 3 (n-3 PUFAs) de origen marino (DHA y EPA) sobre la obesidad y los trastornos relacionados con el metabolismo, incluyendo la hiperlipidemia y enfermedades cardiovasculares (Martínez-Fernández *et al.* 2015). Algunos estudios han apuntado hacia la capacidad de los n-3 PUFAs para inducir la biogénesis mitocondrial y el pardeamiento del tejido adiposo blanco en modelos animales (Zhao *et al.* 2014). Sin embargo, no se había demostrado su potencial capacidad para promover la biogénesis mitocondrial y activar el proceso de *beiging* en adipocitos subcutáneos humanos. Las acciones metabólicas beneficiosas de estos n-3 PUFAs parecen estar también relacionadas con sus propiedades anti-inflamatorias. Así pues, se ha demostrado que los n-3 PUFAs promueven la disminución de la producción de citoquinas proinflamatorias. Además, se ha demostrado que los n-3 PUFAs son

capaces de ser metabolizados *in vivo* a una nueva serie de mediadores lipídicos bioactivos conocidos como resolvinas (Rv), protectinas (PD) y maresinas (MaR) (Serhan 2017). Estos mediadores lipídicos no sólo poseen potentes efectos anti-inflamatorios, sino que además participan activamente en la resolución de la inflamación de los tejidos. En este contexto, estos mediadores lipídicos proresolutivos especializados (SPMs) podrían ayudar a reducir la inflamación del tejido adiposo, y contribuir así a las acciones beneficiosas de n-3 PUFAs en obesidad y sus complicaciones metabólicas asociadas (Spite *et al.* 2014).

Estudios recientes han revelado que la administración de algunos de estos SPMs como RvD1, RvD2 y PDX podrían ser beneficiosos para contrarrestar la inflamación relacionada con la obesidad y la resistencia a la insulina en modelos animales de obesidad (Hellmann *et al.* 2011, Clària *et al.* 2012, White *et al.* 2014). MaR1 es un SPM derivado del DHA que se identificó inicialmente en los macrófagos, y más recientemente se ha detectado su presencia también en varios tejidos (Serhan et al. 2009). Esta molécula tiene una potente actividad antiinflamatoria y proresolutiva (Serhan *et al.* 2009), y su administración ha mostrado efectos beneficiosos en varias enfermedades inflamatorias crónicas, como la colitis y la aterosclerosis (Marcon *et al.* 2013; Viola *et al.* 2016). Sin embargo, las acciones metabólicas de MaR1 y sus posibles acciones terapéuticas en obesidad y sus comorbilidades asociadas han sido poco estudiadas.

2. Hipótesis

En base a todas las observaciones mencionadas anteriormente, la **hipótesis** de la presente Tesis Doctoral es que los efectos beneficiosos del EPA sobre la obesidad estarían también relacionados con su capacidad de promover el pardeamiento de los adipocitos humanos, y que el mediador lipídico bioactivo derivado del DHA, MaR1 podría constituir un buen candidato para revertir las alteraciones funcionales de los adipocitos inducidas por la inflamación, y para promover la activación del TAP y el pardeamiento del TAB, así como para mejorar el hígado graso asociado a obesidad.

3. Objetivos

El **objetivo general** del presente estudio fue determinar la capacidad del EPA para remodelar el metabolismo y el fenotipo de los adipocitos subcutáneos humanos, así como caracterizar la capacidad de MaR1 para contrarrestar las alteraciones funcionales de los adipocitos inducidas por la inflamación y dilucidar las acciones de MaR1 sobre el tejido adiposo marrón/blanco y sobre la función del hígado en modelos animales de obesidad y en cultivos celulares.

Los **objetivos específicos** fueron:

1. Estudiar las acciones del EPA sobre la biogénesis mitocondrial y su potencial capacidad para promover pardeamiento en cultivos de adipocitos subcutáneos humanos de sujetos con sobrepeso/obesidad, así como determinar los mecanismos celulares que podrían estar implicados (*Capítulo 1*).

2. Determinar los efectos de MaR1 sobre la lipólisis y la autofagia inducida por TNF-α mediante la caracterización de sus acciones sobre las principales lipasas y proteínas de la gota lipídica implicadas en el control de la lipólisis, y sobre genes/proteínas claves en la regulación de la autofagia en adipocitos murinos (*Capítulo 2*).

3. Caracterizar la potencial eficacia de MaR1 para mejorar la esteatosis hepática en modelos de obesidad genética o inducida por la dieta, así como identificar los posibles mecanismos subyacentes a las acciones MaR1 en hígado graso y en hepatocitos primarios (*Capítulo 3*).

4. Analizar si MaR1 podría regular la actividad del tejido adiposo marrón e inducir pardeamiento de tejido adiposo blanco, y caracterizar los principales genes/proteínas implicados, tanto en ratones con obesidad inducida por la dieta, como en cultivos de adipocitos marrones murinos y en adipocitos derivados de células mesenquimales obtenidas de grasa subcutánea humana (*Capítulo 4*).

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III. EXPERIMENTAL DESIGN AND METHODS

CHAPTER 1. "Eicosapentaenoic acid promotes mitochondrial biogenesis and beige-like features in subcutaneous adipocytes from overweight subjects"

- *Cell model and experimental design*

Human subcutaneous pre-adipocytes cryopreserved from overweight females (BMI: 28.1-29.8 kg/m^2) were purchased from Zen-Bio Inc. and differentiated as previously described (Fernández-Galilea *et al.* 2015). When cells displayed typical mature adipocyte phenotype, EPA (100-200 µM) was added to cell media and incubated for additional 24 h. After incubation period, cells were harvested and frozen at -80 °C for further analysis.

Figure 18 summarizes the experimental design and assays performed in chapter 1 whose aim was to analyse the potential beiging effects of EPA in human subcutaneous adipocytes (more detailed information about the protocols is described in the methods section of the corresponding manuscript).

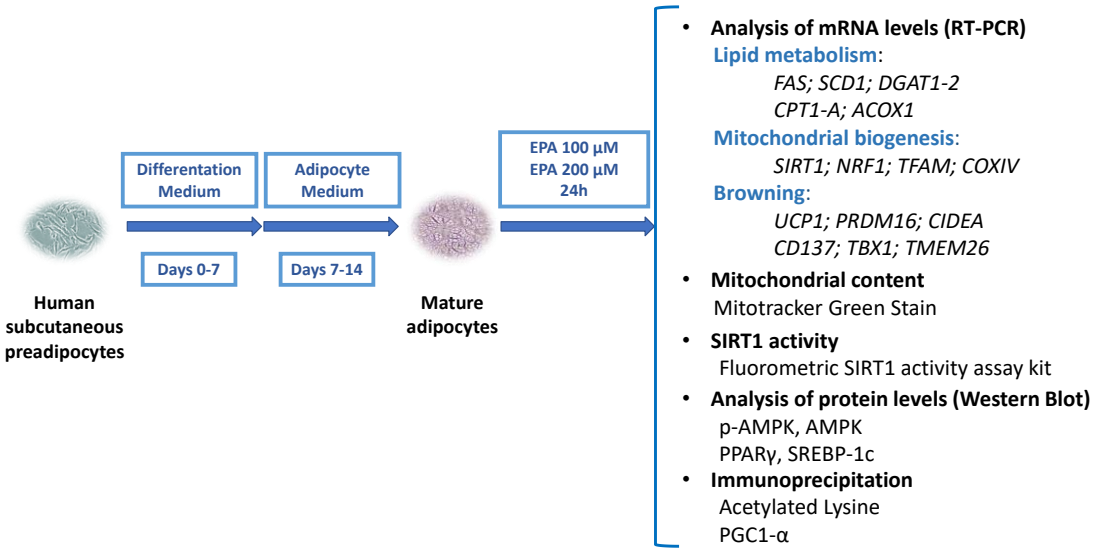

Figure 18. Experimental design for chapter 1 in human subcutaneous adipocytes.

- *Analyses of mRNA levels*

mRNA expression levels of *FAS, SCD-1, DGAT1-2, CPT-1, ACOX1, SIRT1, NRF-1, TFAM, COXIV, UCP1, PRDM16, CIDEA, CD137, TBX1, TMEM26* and *18S* (as housekeeping gene) were measured by real-time-PCR using ABI PRISM 7900HT Fast Sequence Detection System and Taqman Universal Master Mix (see **Table 2** on page 95).

- *Mitochondrial content*

The mitochondrial content of mature subcutaneous adipocytes was evaluated using MitoTracker Green FM stain and quantified by Polarstar Galaxy fluorimeter.

- *SIRT1 activity*

The activity of SIRT1 was determined in nuclear protein fractions from human subcutaneous adipocytes using a fluorometric SIRT1 activity assay kit (Abcam, Cambridge, UK) and measured in Polarstar Galaxy fluorimeter.

- *Western Blots*

Whole cell lysates from mature human subcutaneous adipocytes were prepared as previously described (Fernández-Galilea *et al.* 2014). The protein expression levels were determined by Western blot. The following antibodies were used: phospho-AMPK, total AMPK and β-Actin in cell lysates. Moreover, PPARγ and SREBP-1c were determined in nuclear extracts (see **Table 3** on page 96).

- *Immunoprecipitation*

Mature human subcutaneous adipocytes whole lysates were collected to determine PGC1-α acetylation. Immunoprecipitation was carried out using protein A/G PLUS-Agarose-beads and the specific antibody PGC1-α. Thereafter, Western Blot was used to determine acetylated Lysine and total PGC1-α as previously described (Fernández-Galilea *et al.* 2015) (see **Table 3** on page 96).

CHAPTER 2. "Maresin 1 inhibits TNF-alpha-induced lipolysis and autophagy in 3T3-L1 adipocytes"

- *Cell model and experimental design*

3T3-L1 mouse embryo fibroblast (American Type Culture Collection, Rockville, MD) were cultured and differentiated to adipocytes as previously described (López-Yoldi *et al.* 2014). Once they reached typical features of mature adipocytes, cells were serum starved for 4 h. Then, mature 3T3-L1 adipocytes were treated with or without MaR1 at several concentrations (1, 10, 100 nM) in the presence or absence of TNF-α (10 ng/mL) for 6, 24 and 48 h. After treatment period, cell media was collected and stored to determine the total amount of glycerol release (as an index of lipolysis) and cells were lysed and kept at -80 °C for future determinations.

Figure 19 summarizes the experimental design and assays performed in chapter 2, whose aim was to analyse the actions of MaR1 on TNF-α-induced lipolysis and autophagy in murine 3T3-L1 adipocytes. In this study, we also evaluated its potential actions on the regulation of the main lipases, on lipid droplet proteins involved in the hydrolysis of TG and on modulation of major autophagy markers (more detailed information about the protocols is described in the section of Methods of the corresponding manuscript).

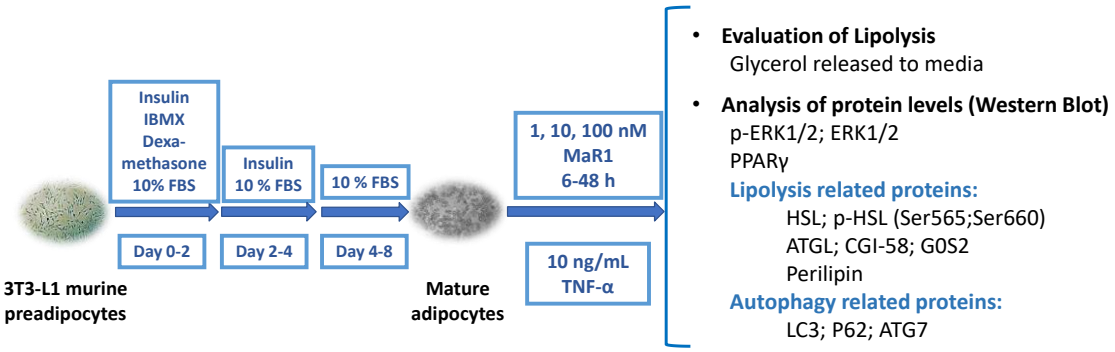

Figure 19. *In vitro* experimental design for Chapter 2 in 3T3-L1 adipocytes.

- *Evaluation of lipolysis in 3T3-L1 adipocytes*

Glycerol measurements in cell culture media were performed using the Pentra C200 autoanalyser.

- *Western Blots*

The extractions of proteins were carried out as described above. Analysis of key adipocyte lipases, regulatory proteins involved in lipolysis and autophagy markers were carried out by **Western blot**. The following antibodies were used: phospho-HSL (Ser565), phospho-HSL (Ser660), HSL, ATGL, CGI-58, G0S2, perilipin, LC3, p62, ATG7, p-ERK1/2, ERK1/2, PPARγ and β-Actin (see **Table 3** on page 96).

CHAPTER 3. "Maresin 1 mitigates liver steatosis in *ob/ob* and diet-induced obese mice"

***In vivo* studies**

- *Animal models and experimental design*

Genetically obese mice

Eight-week-old *ob/ob* mice were obtained from Janvier Laboratories (Le Genest St. Isle, France). The animals were established at the animal facilities of the University of Navarra (UN). They were kept in plastic cages under controlled conditions (22 ± 2 °C, with a 12 h light–dark cycle, relative humidity, 55 ± 10%). All experiments were performed according to national and institutional animal care guidelines, and with the approval of the Ethics Committee for Animal Experimentation (CEEA) of the UN (Protocol 029-12).

After an acclimation period, mice were separated into three experimental groups **(Figure 20)**:

- ***ob/ob* + vehicle** (n=6): Treated with vehicle (100 µl of sterile saline-0.1% ethanol) by daily intraperitoneal (i.p.) injection for 20 days.
- ***ob/ob* + MaR1 (2 µg/kg)** (n=7): Treated with MaR1 (2 µg/kg of BW) by daily i.p. injection for 20 days.
- ***ob/ob* + MaR1 (10 µg/kg)** (n=7): Treated with MaR1 (10 µg/kg of BW) by daily i.p. injection for 20 days.

Animals were sacrificed after 20 days of treatment, and blood samples were collected and processed. Moreover, livers were isolated and quickly snap frozen in liquid nitrogen and stored at -80 °C for further determinations.

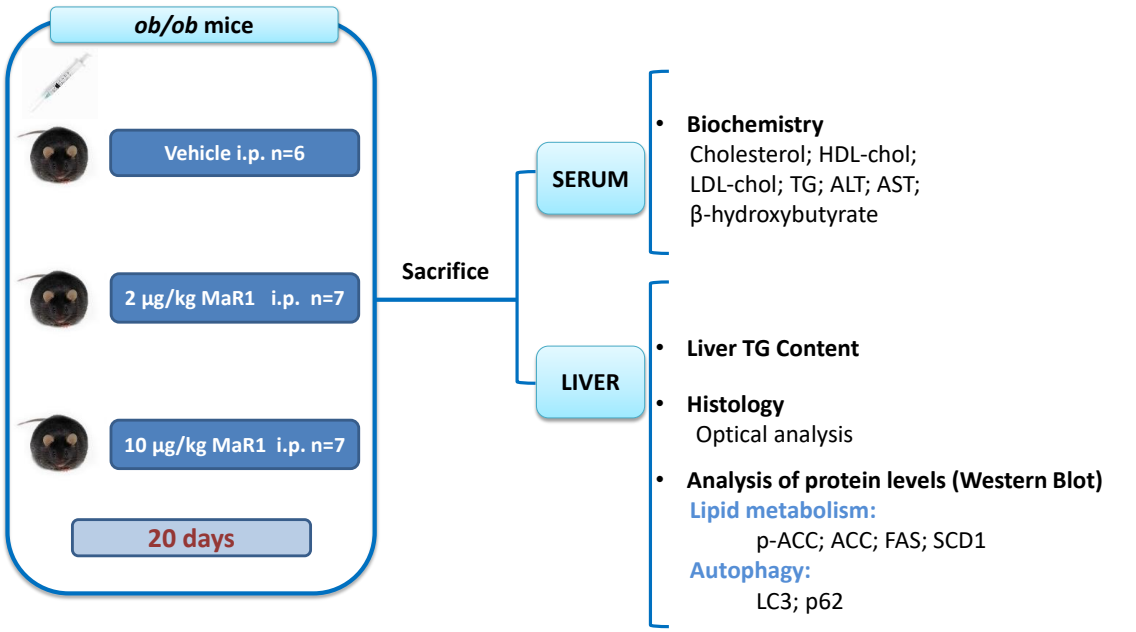

Figure 20*:* Experimental design for chapter 3 in *ob/ob* mice treated with MaR1 (intraperitoneal injection for 20 days).

Diet-induced obesity (DIO) mice

Seven-week old C57BL/6J mice were purchased from Harlan Laboratories (Barcelona, Spain). Animals were established at the animal facilities of UN, where they were kept in cages under the same controlled conditions of light, temperature and humidity as previously described. All experiments were performed according to national and institutional animal care guidelines, and with the approval of the Ethics Committee for Animal Experimentation (CEEA) of the UN (Protocols 029-12 and 047-15). After 7 days acclimation period, mice were fed *ad libitum* with a high-fat diet (HFD, 60% of kcal from fat, 20% from carbohydrates and 20% from protein, ResearchDiets, New Brunswick, NJ, USA) for 3 months in order to induce obesity. After 3 months on HFD, two different trials were performed in these diet induced obese (DIO) mice:

In trial 1, DIO mice were separated into two sub-groups (**Figure 21**):

- **DIO + vehicle** (n=11): Treated with vehicle (100 µl of sterile saline-0.1% ethanol) by daily i.p. injection for 10 days.
- **DIO + MaR1 (2 µg/kg)** (n=11): Treated with MaR1 (2 µg/kg of BW) by daily i.p. injection for 10 days.

In trial 2, DIO mice were assigned into other two sub-groups **(Figure 21)**:

- **DIO + vehicle** (n=8): Treated with vehicle (100 µl of sterile saline-0.1% ethanol) by daily oral gavage for 10 days.
- **DIO + MaR1 (50 µg/kg)** (n=8): Treated with MaR1 (50 µg/kg of BW) by daily oral gavage for 10 days.

After treatments, mice were sacrificed and blood and tissues were collected, processed and kept at -80 °C for further analysis.

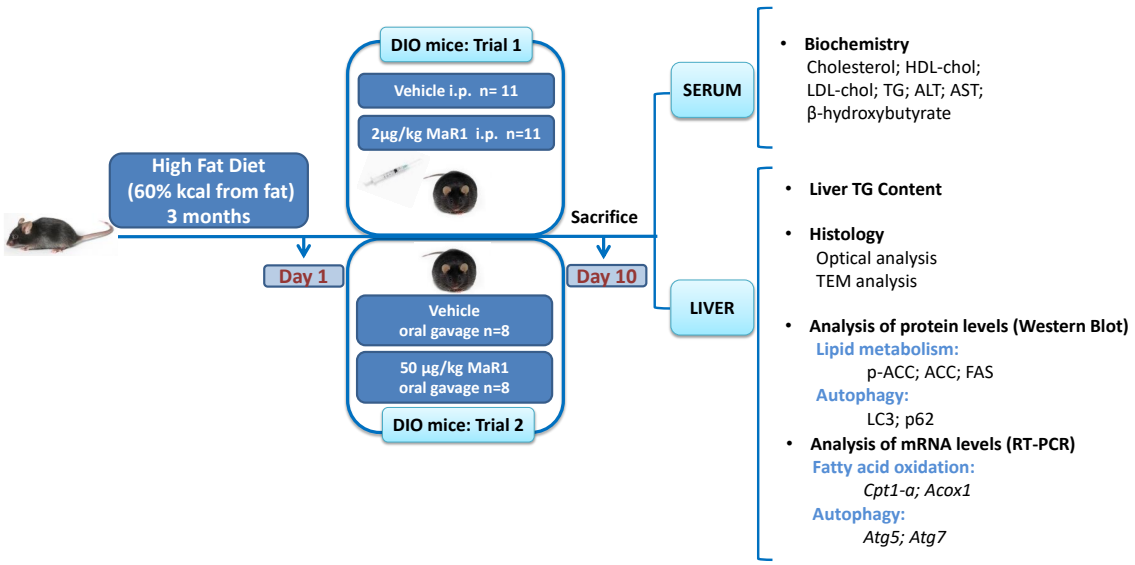

Figure 21: Experimental design for Chapter 3 in DIO mice treated with MaR1 (intraperitoneal injection or oral gavage for 10 days).

- *Serum biochemistry*

Biochemical analysis were carried out after an over-night (12 h) fasting period. Serum levels of cholesterol, high-density lipoprotein cholesterol (HDL-chol), TG, alanine amino transferase (ALT), aspartate amino transferase (AST) and β-

hydroxybutyrate were determined using Pentra C200 autoanalyser (Roche Diagnostic, Basel, Switzerland). Values of low-density lipoprotein cholesterol (LDL-chol) were calculated using the Friedewald equation (Friedewald *et al.* 1972).

- *Liver TG content*

Folch method was used to extract lipids and triglycerides from liver (Folch *et al.* 1957). Liver TG content was quantified by Infinity Triglycerides Liquid Stable Reagent (Thermo Electron Corporation, CO, USA), using Multiskan Spectrum microplate spectrophotometer (Thermo-Scientific, DE, USA).

- *Light microscopy analysis*

Liver pieces were fixed in 10% neutral formalin (pH=7.4) for 24 h, dehydrated and embedded in paraffin. 5 µm thick sections were deparaffinised and stained with hematoxylin-eosin and visualized with Nikon Eclipse E800 microscope (Nikon Instruments Europe, Amsterdam, Netherlands). Images were taken with the camera Nikon Eclipse E800 (Nikon Instruments Europe). Additionally, fat-red stain was performed in frozen samples and images were taken with Digital Pathology Slide Scanner Leica Aperio CS2 (Leica Biosystems, Wetzlar, Germany). Relative areas of lipid accumulation were quantified by ImageJ software (National Institute of Health, MD, USA).

- *Transmission electron microscopy analysis*

Ultra-thin sections of liver were examined with a transmission electron microscope (TEM) Libra 120 (Zeiss GMgH, Oberkochen, Germany). Images were taken with iTEM 5.1 (Olympius Soft Imaging Solutions GmbH, Münster, Germany). Autophagic vacuoles (AV) were examined and the number of AV observed was referred to each hepatocyte analysed.

- *Western Blot*

The extractions of proteins were carried out as previously described (López-Yoldi *et al.* 2014). Analysis of main proteins involved in autophagy and lipid metabolism were determined by Western blot**.** The following antibodies were used: FAS, phospho-ACC, ACC, LC3, p62, SCD1 and β-Actin (see **Table 3** on page 96).

- *Analyses of mRNA levels*

Gene expression levels of *Cpt1a, Acox1, Atg5, Atg7* and *Cyclophilin A* (as housekeeping gene), were measured by real-time PCR using the ABI PRISM 7900HT Fast Sequence Detection System and Taqman Universal Master Mix (see **Table 2** on page 95).

***In vitro* studies**

Primary hepatocytes and experimental design

Liver collagenase perfusion via portal vein was used to isolate hepatocytes from 6 months old C57BL/6J mice after being fed with a standard chow diet or with a HFD (45% of Kcal from fat, 35% from carbohydrates and 20% from protein, ResearchDiets) for 3 months. Primary hepatocytes were isolated and cultured until complete adhesion to the well (Rodriguez-Ortigosa *et al.* 2014). For the study of the potential intracellular mechanisms involved in the actions of MaR1 on fatty acid oxidation and autophagy, pretreatment for 1 h with specific inhibitor of AMPK (Compound C) was performed. Then MaR1 was added in concomitant treatment to the media for different time periods (6-24h). **Figure 22** illustrates the experimental design and the techniques carried out to evaluate the effects of MaR1 in primary cultured hepatocytes.

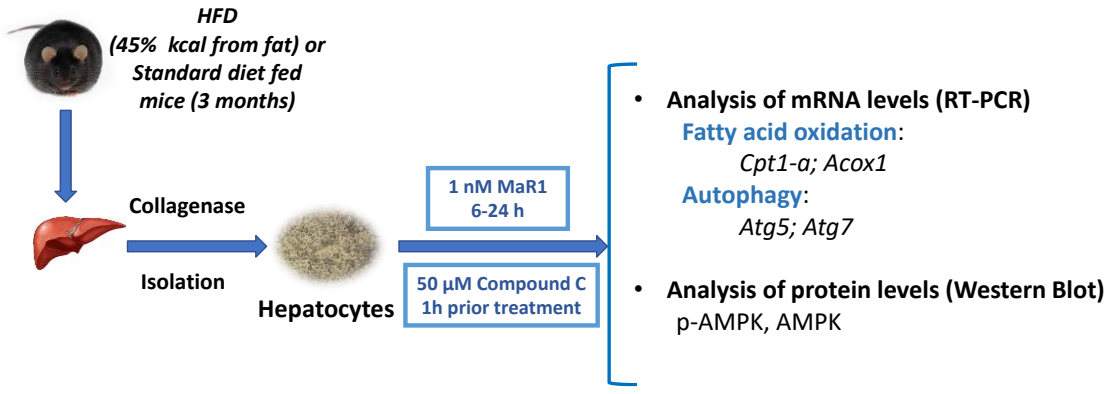

Figure 22. Experimental design in cultured primary hepatocytes for Chapter 3.

- *Western Blots*

The extractions of proteins were carried out as described above. MaR1 effects on AMPK phosphorylation levels were determined by Western Blot (see **Table 3** on page 96).

- *Analyses of mRNA levels*

mRNA expression levels of *Cpt1a, Acox1, Atg5, Atg7* and *Cyclophilin A* (as housekeeping gene) were measured by real-time PCR using the *ABI PRISM 7900HT Fast Sequence Detection System and Taqman Universal Master Mix* (see **Table 2** on page 95).

CHAPTER 4. "Maresin 1 induces brown adipose tissue activation and promotes browning of white adipose tissue in diet-induced obese mice"

***In vivo* studies**

- *Animal models and experimental design*

Seven-week-old male C57BL/6J mice were obtained from Harlan Laboratories (Barcelona, Spain). Animals were fed with a standard mouse pelleted chow diet (13% of kcal from fat, 67% from carbohydrates and 20% from protein, Harlan Teklad Global Diets, Harlan Laboratories, IN, USA) for an adaptation period of 7 days. Then, one group (control, n=7) was fed *ad libitum* with standard mouse chow diet, and the second group (DIO, n=16) with a high-fat diet (60% of kcal from fat, 20% from carbohydrates and 20% from protein, ResearchDiets, New Brunswick, USA) for three months. Animals were established at the animal facilities of UN, where they were kept in cages under the same controlled conditions of light, temperature and humidity as previously described. All experiments were performed according to national and institutional animal care guidelines, and with the approval of the Ethics Committee for Animal Experimentation (CEEA) of the UN (Protocol 047-15).

After 3 months on a HFD, DIO mice were also randomly divided into two experimental sub groups **(Figure 23)**:

- **Control + vehicle** (n=7): Mice fed on standard diet and treated with vehicle (100 µl of sterile saline-0.1% ethanol) by daily oral gavage for 10 days.
- **DIO + vehicle** (n=8): High fat fed mice treated with vehicle (100 µl of sterile saline-0.1% ethanol) by daily oral gavage for 10 days.
- **DIO + MaR1 (50 µg/kg)** (n=8): High fat fed mice treated with MaR1 (50 µg/kg of BW) by daily oral gavage for 10 days.

After treatments, mice were sacrificed, blood samples were processed and organs were snap frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

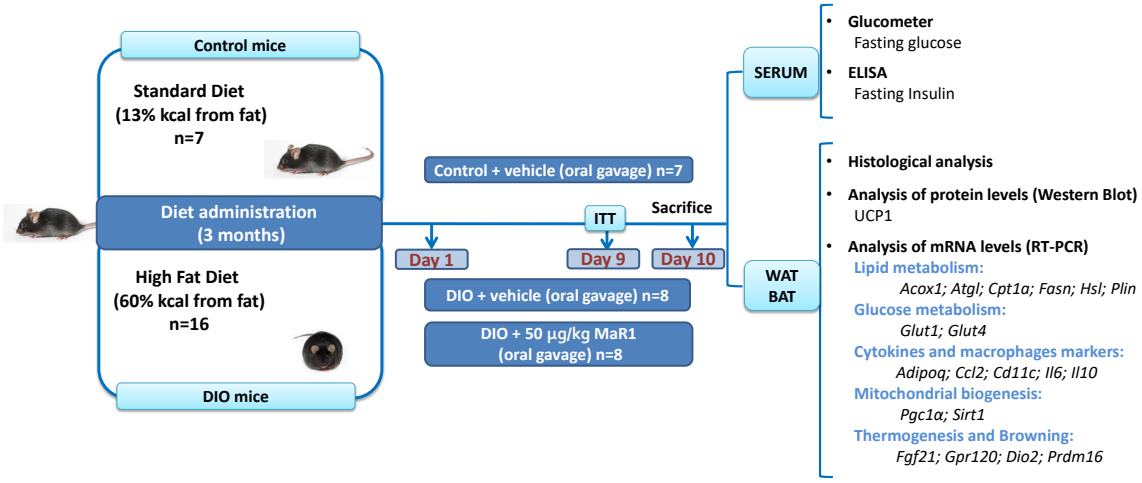

Figure 23. Experimental design for chapter 4 in DIO-mice treated with MaR1 (50 µg/kg for 10 days).

- *Biochemistry and insulin tolerance test (ITT)*

All serum measurements were done on mice fasted for 12 h. Fasting glucose was determined by a standard glucometer and fasting insulin levels were analysed using a commercial mouse ELISA kit (see **Table 4** on page 97). ITT was performed on day 9 of treatment. After 8 h of fasting, baseline glucose levels were measured with a glucometer, and then mice were intraperitoneally injected with 0.375 mU/g BW human insulin (NovoRapid®, Novo Nordisk A/S, Bagsvaerd, Denmark). Blood glucose levels were measured at 30, 60, 120 and 180 min after the injection.

- *Histological analysis*

BAT pieces were fixed in 10% neutral formalin (pH=7.4) for 24 h, dehydrated and embedded in paraffin. 5 µm thick sections were deparaffinized and stained with hematoxylin-eosin and visualized with Nikon Eclipse E800 microscope (Nikon Instruments Europe B.V. Amsterdam, Netherlands) and images were taken with the camera Nikon Eclipse E800 (Nikon Instruments Europe B.V.).

- *Western blot*

UCP1 protein expression levels were determined by Western blot as described above. (See **Table 3** on page 96 for antibody information).

- *mRNA analysis*

mRNA expression levels of *Acox1, Adipoq, Atgl, Ccl2, Cd11c, Cpt1α, Cidea, Fasn, Fgf21, Gpr120, Hsl, Il6, Il10, Dio2, Tfam, Nrf-1, Plin, Pgc1α, Prdm16, Sirt1, Glut1, Glut4, Tbx1, Tmem26, Ucp1* and *Cyclophilin A* (as housekeeping gene) mRNA levels were determined using predesigned Taqman® Assays-on-Demand and Taqman Universal Master Mix (Applied Biosystems) (see **Table 2** on page 95).

- *In vivo* study of BAT activation by positron emission tomography (PET)

BAT activation after MaR1 acute treatment was studied *in vivo* by PET with the radiotracer ^{18}F-fluorodeoxyglucose (^{18}F-FDG). Mice were randomly divided into two experimental sub groups **(Figure 24)**:

- **Control + vehicle** (n=5): Mice fed on standard diet and treated with vehicle (100 µl of sterile saline-0.1% ethanol) by i.p. injection.
- **DIO + MaR1 (50 µg/kg)** (n=5): Mice fed on standard diet and treated with MaR1 (50 µg/kg of BW) by i.p. injection.

Thirty minutes after MaR1 or vehicle administration, BAT was stimulated by cold exposure during 1 h at 4 °C (Wu *et al.* 2011). After cold exposure, ^{18}F-FDG (10.1±0.9 MBq) was injected through the tail vein. PET static images were acquired 1, 2 and 4 h post ^{18}F-FDG injection in a small animal PET scanner (Mosaic, Philips). For each study, animals were anesthetized with 2% isoflurane in 100% O_2 gas.

All studies were exported and analysed using PMOD software (PMOD Technologies Ltd., Adliswil, Switzerland). For semiquantitative analysis, 18F-FDG uptake by BAT was evaluated drawing volume-of-interest (VOIs) on coronal PET images including the interscapular BAT. From each VOI, maximum standardized uptake value (SUVmax) was calculated using the formula SUV = [tissue activity concentration (Bq/cm^3)/injected dose (Bq)] × body weight (g).

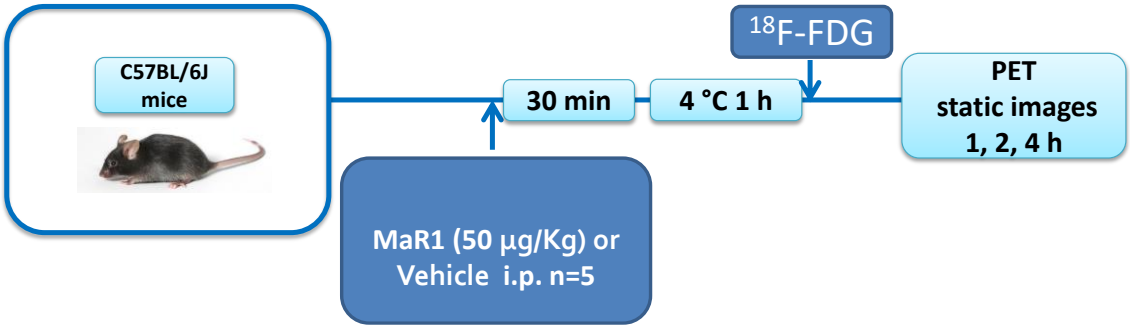

Figure 24. Experimental design for PET studies (chapter 4) in control mice acutely treated with MaR1 (intraperitoneal injection).

***In vitro* studies**

- Brown adipocytes

Immortalized brown pre-adipocytes from interscapular BAT of neonatal mice were kindly provided by Dr. Valverde. Pre-adipocytes were differentiated as previously described (Ortega-Molina *et al.* 2012). For treatments, MaR1 (0.1 and 1 nM) was added to the media for 24 h **(Figure 25)**.

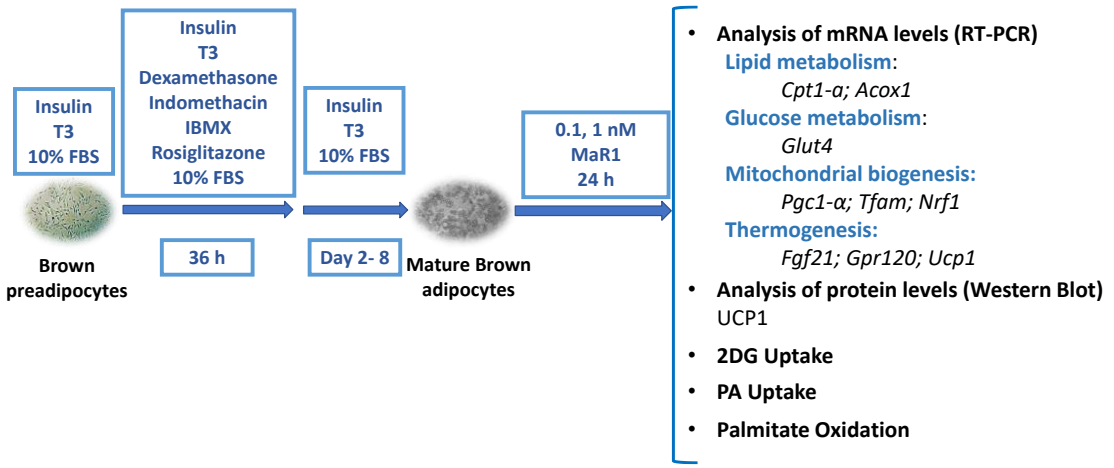

Figure 25. Experimental design and assays carried out to evaluate the effects of MaR1 in cultured brown adipocytes (Chapter 4).

Adipose tissue-derived human mesenchymal stem cells (hMSC)

Isolated hMSC from subcutaneous adipose tissue human subjects obtained from elective laparoscopic surgery were kindly provided by Dr. Arbones-Mainar from the Instituto Aragonés de Ciencias de la Salud. All donors signed the written consent and the study was approved by the local Institutional Review Board (CEIC-A) and the Ethics Committee of the University of Navarra. The hMSC were differentiated as previously described (Martínez-Fernández *et al.* 2017). Cells were treated either across the differentiation process (MaR1 1-100 nM) or were treated acutely (1-10 nM, 24 h), when already differentiated **(Figure 26)**.

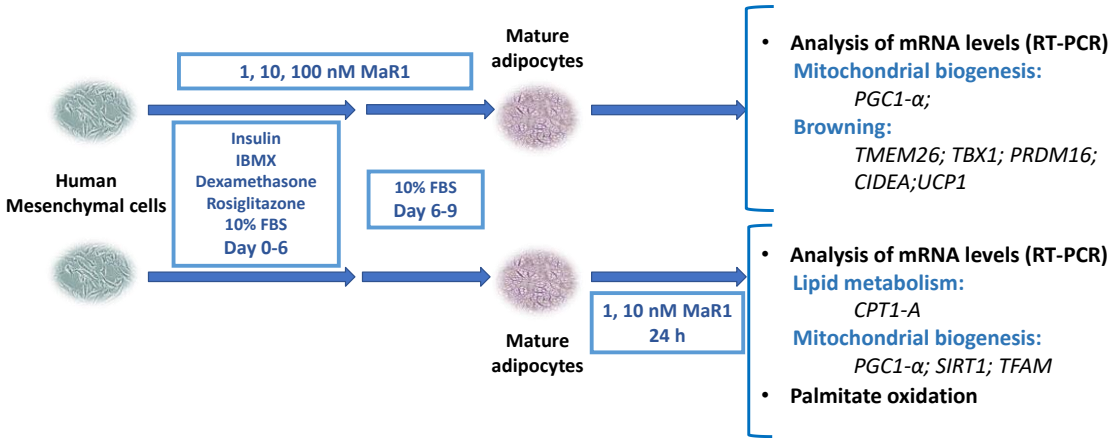

Figure 26. Experimental design and assays carried out to determine the effects of MaR1 in hMSC-derived adipocytes (Chapter 4).

- *Western Blots*

  As described before.

- *mRNA analysis*

  As described before (see **Table 2** on page 95).

- *Glucose uptake*

Mature brown adipocytes treated with vehicle or MaR1 (0.1-1 nM) for 24 h, were incubated for 2 h with DMEM without serum or glucose at 37 °C prior the experiments. Some wells were treated with 0.5 nM insulin during the last 15 minutes of deprivation. After, plates were incubated for 15 min with 0.1 mM of 2-Deoxy-D-glucose (Sigma) and

traces of ^{14}C-2-Deoxy-D-glucose (Moraveck Biochemicals). After, cell lysates were taken to measure ^{14}C-2-Deoxy-D-glucose radioactivity by liquid scintillation counting, HIDEX 300 SL scintillation counter (Hidex Oy, Turku, Finland).

- *FFA uptake*

Mature brown adipocytes were incubated in Krebs-Ringer buffer during 50 min. Then, palmitic acid, L-carnitine and the non-metabolizable analogue 20 µM ^{14}C-2-Bromopalmitic acid (Moraveck Biochemicals, Brea, CA) were added to the media and incubated for 10 min. After that, cells were lysed and intracellular ^{14}C-2-Bromopalmitic acid radioactivity was measured as above.

- *FFA oxidation*

Triglyceride-derived fatty acid oxidation was determined by the sum of $^{14}CO_2$ liberated to the media and ^{14}C-acid soluble metabolites as previously described (Fernández-Galilea *et al.* 2015). Mature white and brown adipocytes were incubated for 4 h in Krebs-Ringer buffer with ^{14}C-palmitic acid (Perkin Elmer; Waltham, MA). $^{14}CO_2$ from medium was liberated by acidification and collected after 2 h. $^{14}CO_2$ was measured by scintillation counting HIDEX 300 SL (Hidex Oy). ^{14}C-acid soluble metabolites from cell lysates were separated. Specific activity for ^{14}C-acid soluble metabolites were measured as above, and used to calculate total oxidation as equivalent of oxidized palmitic acid.

- *Analysis of adipocyte oxygen consumption*

Oxygen consumption in mature brown adipocytes (treated with vehicle or MaR1 0.1 nM for 24 h) was measured using Seahorse Extracellular Flux (XF) 24 Analyser (Agilent Technologies, CA, USA) with the aim to measure the oxygen consumption rate (OCR), an indicator of mitochondrial respiration. Briefly, adipocytes were incubated in XF24 microplates. 1 h prior the assay, the cells were washed thoroughly with assay medium (unbuffered DMEM supplemented with 50 mM glucose and 1 mM sodium pyruvate) and incubated in a CO_2-free incubator at 37 °C. After incubation, microplates were loaded into the XF24 Analyser. During the assay, oligomycin (1 µg/mL), carbonylcyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; 0.6 µM), and a mixture of rotenone and antimycin A (2 µM of each one), were sequentially injected for OCR measurements. The results were normalized to total protein content.

Table 2: List of TaqMan probes used for RT-PCR for human and mouse models.

Human			
Name	**Ref**	**Gene Symbol**	**Specie**
FAS	Hs01005622_m1	*FASN*	Homo sapiens
SCD1	Hs01682761_m1	*SCD1*	Homo sapiens
DGAT1	Hs00201385_m1	*DGAT1*	Homo sapiens
DGAT2	Hs01045913_m1	*DGAT2*	Homo sapiens
CPT1-A	Hs00912671_m1	*CPT1*	Homo sapiens
ACOX1	Hs01074241_m1	*ACOX1*	Homo sapiens
SIRT1	Hs01009005_m1	*SIRT1*	Homo sapiens
NRF1	Hs00192316_m1	*NRF1*	Homo sapiens
TFAM	Hs01082775_m1	*TFAM*	Homo sapiens
COX IV	Hs00971639_m1	*COX4I1*	Homo sapiens
UCP1	Hs00222453_m1	*UCP1*	Homo sapiens
PRDM16	Hs00223161_m1	*PRDM16*	Homo sapiens
CIDEA	Hs00154455_m1	*CIDEA*	Homo sapiens
CD137	Hs00155512_m1	*TNFRSF9*	Homo sapiens
TBX1	Hs00962556_m1	*TBX1*	Homo sapiens
TMEM26	Hs00415619_m1	*TMEM26*	Homo sapiens
PPARγ	Hs01115513_m1	*PPARG*	Homo sapiens
PGC1α	Hs01016719_m1	*PPARGC1A*	Homo sapiens
18S	Hs03003631_m1	*18S*	Homo sapiens
Mouse			
Name	**Ref**	**Gene Symbol**	**Specie**
Cpt1-a	Mm01231183_m1	*Cpt1a*	Mus musculus
Acox1	Mm01246834_m1	*Acox1*	Mus musculus
Atg5	Mm00504340_m1	*Atg5*	Mus musculus
Atg7	Mm00512209_m1	*Atg7*	Mus musculus
Adipoq	Mm00456425_m1	*Adipoq*	Mus musculus
Atgl	Mm00503040_m1	*Atgl*	Mus musculus
Ccl2	Mm00441242_m1	*Ccl2*	Mus musculus
Ctf1	Mm00432772_m1	*Ctf1*	Mus musculus
Cidea	Mm00432554_m1	*Cidea*	Mus musculus
Fasn	Mm00662319_m1	*Fasn*	Mus musculus
Fgf21	Mm00840165_m1	*Fgf21*	Mus musculus
Gpr120	Mm00725193_m1	*Ffar4*	Mus musculus
Hsl	Mm00495359_m1	*Hsl*	Mus musculus
Il6	Mm00446190_m1	*Il6*	Mus musculus
Il10	Mm01288386_m1	*Il10*	Mus musculus
Dio2	Mm00515664_m1	*Dio2*	Mus musculus
Tfam	Mm00447485_m1	*Tfam*	Mus musculus
Nrf1	Mm01135606_m1	*Nrf1*	Mus musculus
Plin	Mm00558672_m1	*Plin1*	Mus musculus
Pparγ	Mm00440940_m1	*Pparγ*	Mus musculus
Pgc1α	Mm01208835_m1	*Ppargc1α*	Mus musculus
Prdm16	Mm00712556_m1	*Prdm16*	Mus musculus
Sirt1	Mm01168521_m1	*Sirt1*	Mus musculus
Glut1	Mm00441480_m1	*Slc2a1*	Mus musculus
GLu4	Mm00436615_m1	*Slc2a4*	Mus musculus
Tbx1	Mm00448949_m1	*Tbx1*	Mus musculus
Tmem26	Mm01173641_m1	*Tmem26*	Mus musculus
Ucp1	Mm01244861_m1	*Ucp1*	Mus musculus
Cyclophilin A	Mn02342430_m1	*Cyclophilin A*	Mus musculus

Table 3: List of primary antibodies used for Western Blot studies.

Antibody	Manufacturer	Ref	Source
Phospho AMPK (Thr172)	Cell signaling technology	2535	Rabbit
AMPK	Cell signaling technology	2532	Rabbit
PPARy	Cusabio	PA018424LA01HU	Rabbit
SREBP-1C	Santa Cruz Biotechnologies	sc-366	Rabbit
Acetylated-lysine	Cell signaling technology	9814	Rabbit
PGC-1a	Santa Cruz Biotechnologies	sc-67286	Rabbit
Phospho HSL (Ser565)	Cell signaling technology	4137	Rabbit
Phospho HSL (Ser660)	Cell signaling technology	4126	Rabbit
HSL	Cell signaling technology	4107	Rabbit
ATGL/Desnutrin	Cell signaling technology	2138	Rabbit
G0S2	Santa Cruz Biotechnologies	sc-133423	Rabbit
CGI58	Santa Cruz Biotechnologies	sc-130934	Rabbit
Perilipin	Cell signaling technology	9349	Rabbit
LC3A/B	Cell signaling technology	4108	Rabbit
p62	Sigma-Aldrich	P0067	Rabbit
ATG7	Cell signaling technology	8558	Rabbit
Phospho ERK 1/2 (Thr202/Tyr204)	Cell signaling technology	4370	Rabbit
MAPK (ERK 1/2)	Cell signaling technology	9102	Rabbit
FAS	Cell signaling technology	3180	Rabbit
Phospho-ACC (Ser79)	Cell signaling technology	3661	Rabbit
ACC	Cell signaling technology	3662	Rabbit
SCD1	Abcam	Ab23331	Rabbit
UCP1	Abcam	Ab10983	Rabbit
Actin	Sigma-Aldrich	A1978	Mouse

Table 4: ELISA kit used in chapter 4.

Antibody	Manufacturer	Ref	Sample type
Insulin	Mercodia Mouse Insulin ELISA	10-1247-01	Mouse

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IV. RESULTS

CHAPTER 1

Eicosapentaenoic acid promotes mitochondrial biogenesis and beige-like features in subcutaneous adipocytes from overweight subjects

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ABSTRACT

Eicosapentaenoic (EPA), a n-3 long-chain polyunsaturated fatty acid, has been reported to have beneficial effects in obesity-associated metabolic disorders. The objective of the present study was to determine the effects of EPA on the regulation of genes involved in lipid metabolism, and the ability of EPA to induce mitochondrial biogenesis and beiging in subcutaneous adipocytes from overweight subjects.

Fully differentiated human subcutaneous adipocytes from overweight females (BMI: 28.1-29.8 kg/m^2) were treated with EPA (100-200 µM) for 24 h. Changes in mRNA expression levels of genes involved in lipogenesis, fatty acid oxidation and mitochondrial biogenesis were determined by qRT-PCR. Mitochondrial content was evaluated using MitoTracker® Green stain. The effects on peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (PGC-1α) and AMP-activated protein kinase (AMPK) were also characterized.

EPA down-regulated lipogenic genes expression while up-regulated genes involved in fatty acid oxidation. Moreover, EPA-treated adipocytes showed increased mitochondrial content, accompanied by an up-regulation of nuclear respiratory factor-1, mitochondrial transcription factor A and cytochrome c oxidase IV mRNA expression. EPA also promoted the activation of master regulators of mitochondrial biogenesis such as sirtuin 1, PGC1-α and AMPK. In parallel, EPA induced the expression of genes that typify beige adipocytes such as fat determination factor PR domain containing 16, uncoupling protein 1 and cell death-inducing DFFA-like effector A, T-Box protein 1 and *CD137*. Our results suggest that EPA induces a remodelling of adipocyte metabolism preventing fat storage and promoting fatty acid oxidation, mitochondrial biogenesis and beige-like markers in human subcutaneous adipocytes from overweight subjects.

Key words: n-3 PUFAs, EPA, lipogenesis, fatty acid oxidation, mitochondrial biogenesis, browning.

INTRODUCTION

Obesity is getting importance as a worldwide epidemic disease, which leads to the development of several co-morbidities such as insulin resistance, dyslipidaemia and MetS (WHO 2011).

Obesity is characterized by altered lipid metabolism and mitochondrial activity, promoting accumulation of triglycerides in white adipose tissue, liver and muscle (Rong *et al.* 2007, Christodoulides *et al.* 2009). White adipocytes are poor in mitochondria and have a low oxidative capacity (Tourniaire *et al.* 2015). Moreover, a recent study has revealed defective mitochondrial biogenesis and oxidative metabolic pathways in subcutaneous adipose tissue during acquired obesity, preceding the metabolic disturbances in obesity (Heinonen *et al.* 2015). Therefore, stimulation of mitochondrial biogenesis and oxidative capacity of white adipocytes by pharmacological and nutritional agents has been proposed as a potential target for obesity and related metabolic disorders (Bonet *et al.* 2013).

During the last years a new type of adipose tissue has been described and named as beige adipose tissue (Wu *et al.* 2012). Beige or "brite" (brown-in-white) adipocytes are multilocular and have thermogenic properties due to increased mitochondrial function and expression of inducible uncoupling protein 1 (UCP1). Interestingly, beige adipocytes have been identified in adult humans (Wu *et al.* 2012), making this cell type an attractive therapeutic target for the management of obesity and metabolic-related diseases (Bartelt and Heeren 2014). In addition to the canonical activators of browning (cold exposure and β_3 adrenergic agonist), recent studies have described several bioactive molecules (cardiac natriuretic peptides, irisin, fibroblast growth factor 21, cardiotrophin-1) and food components (curcumin, resveratrol, quercetin) able to induce adipose tissue beiging (Moreno-Aliaga *et al.* 2011, Bonet *et al.* 2013, Wang *et al.* 2015, Lone *et al.* 2016).

The marine long-chain polyunsaturated omega-3 fatty acids (n-3 PUFAs), docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA), have been associated to beneficial effects on obesity and related-metabolic disorders. Several studies have evidenced that adipose tissue is one of the main target organs regulated by n-3 PUFAs and involved in the metabolic responses (Moreno-Aliaga *et al.* 2010, Lorente-Cebrián

et al. 2013, Martínez-Fernández *et al.* 2015). Thus, EPA and DHA have been shown to reduce inflammation (Todoric *et al.* 2006, Pérez-Matute *et al.* 2007, Pérez-Echarri *et al.* 2008), modulate adipokine secretion (Pérez-Matute *et al.* 2005, Lorente-Cebrián *et al.* 2009 and 2010, Pérez-Echarri *et al.* 2009) and promote mitochondrial biogenesis and improve oxidative capacity of white adipocytes in animal models (Flachs *et al.* 2005 and 2011). Nevertheless, the ability of n-3 PUFAs to induce UCP1 in adipose tissue and their thermogenic properties are still controversial (Flachs *et al.* 2011, Janovska *et al.* 2013). Interestingly, it has been suggested that EPA is able to enhance energy dissipation in subcutaneous adipose tissue by promoting britening and inducing oxidative metabolism in mice (Zhao and Chen 2014). However, there is not evidence about EPA ability to promote mitochondrial biogenesis and activate the beiging process in human subcutaneous adipocytes. For these reasons, this study aimed to characterize the actions of EPA on the modulation of genes/proteins involved in lipogenesis, fatty acid oxidation, mitochondrial biogenesis and adipocytes beiging in cultured subcutaneous primary adipocytes from overweight subjects.

MATERIAL AND METHODS

Chemicals and cell culture reagents

EPA was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and dissolved in ethanol (Sigma-Aldrich, St. Louis, MO, USA). All other reagents/chemicals were of analytical grade. Human subcutaneous pre-adipocytes and cell culture reagents including PM-1, DM-2 and AM-1 were obtained from Zen-Bio Inc. (Research Triangle Park, NC, USA). Moreover, MitoTracker Green FM fluorescent mitochondrial stain was obtained from Molecular Probes (Life Technologies Ltd, Paisley, UK).

Cell culture of human subcutaneous pre-adipocytes and treatments

Human subcutaneous pre-adipocytes cryopreserved from overweight females (BMI: 28.1-29.8 kg/m^2) were obtained from Zen-Bio Inc. and differentiated according to the manufacturer's procedures. As previously described (Fernández-Galilea *et al.* 2015), pre-adipocytes were cultured in 12 wells plates at 40.000 cells/cm^2 and incubated at 37 °C in an humidity atmosphere of 5% CO_2 in presence of pre-adipocytes

medium PM-1 ZenBio (DMEM/Ham's F-12 medium, HEPES pH=7.4, fetal bovine serum, penicillin, streptomycin, amphotericin B). Pre-adipocytes were feed every two days with 1 mL of PM-1 until confluence. After that, PM-1 medium was replaced with 1 mL of DM2 (ZenBio, PM-1 with biotin, pantothenate, human insulin, dexamethasone, isobutylmethylxanthine, PPARγ agonist) in order to induce differentiation to adipocytes (days 0-7). After 7 days of differentiation, 600 µL of DM2 medium were removed and 800 µL of AM1 medium (Zen-Bio) was added, which includes PM-1, biotin, pantothenate, human insulin and dexamethasone. Cells were additionally incubated in AM1 medium, which was replaced every two days. At day 14 post-differentiation, cells displayed typical mature adipocyte phenotypes characterized by the presence of multilocular small lipid droplets and were ready for EPA treatment. Cell media was replaced with 1mL of new AM1 medium, and EPA was added to a final concentration of 100-200 µM and incubated for 24 h. Control adipocytes were treated with the same amount of vehicle (ethanol). After incubation period, cells were harvested and frozen at -80 °C for further analysis. Cell viability was measured by lactate dehydrogenase (LDH) activity in the culture medium as an indicator of cell membrane integrity, and, consequently, as a measurement of cell necrosis/apoptosis (Fernández-Galilea *et al.* 2015). No statistical differences between control and EPA-treated adipocytes were found, indicating that treatment did not alter cell viability (data not shown).

Analysis of mRNA levels

Total RNA was isolated from mature human subcutaneous adipocytes using TRIzol® reagent (Invitrogen, CA, USA) according to manufacturer's procedures. RNA-concentrations and quality were measured using Nanodrop Spectrophotometer ND1000 (Thermo Scientific, DE, USA). RNA was then incubated with a RNAse-free kit DNAse (Ambion, Austin, TX) for 30 min at 37 °C. RNA was reverse transcribed to cDNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen). Fatty acid synthase (*FAS*), stearoyl-CoA desaturase-1 (*SCD-1*), diacylglycerol O-acyltransferase (*DGAT1-2*), carnitine palmitoyltransferase (*CPT-1A*), acyl-coenzyme A oxidase (*ACOX1*), sirtuin 1 (*SIRT1*), nuclear respiratory factor-1 (*NRF-1*), mitochondrial transcription factor A (*TFAM*), cytochrome c oxidase IV *(COX IV), UCP1,* PR domain

containing 16 (*PRDM16*), cell death-inducing DFFA-like effector a *(CIDEA), CD137,* T-box 1 transcription factor *(TBX1)* and Transmembrane Protein 26 *(TMEM26)* mRNA levels were determined using predesigned Taqman® Assays-on-Demand and Taqman Universal Master Mix (Applied Biosystems, CA, USA). Amplification and detection of specific products were done using the ABI PRISM 7900HT Fast Sequence Detection System (Applied Biosystems) (Fernández-Galilea *et al.* 2014 and 2015).

The levels of mRNA were normalized to *18S* as housekeeping obtained from Applied Biosystems. Samples were analysed in duplicate. Ct values were generated by the ABI PRISM 7900HT (Applied Biosystems). Finally, the relative expression of the genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Mitochondrial content

The mitochondrial content of the mature subcutaneous adipocytes was assessed using MitoTracker Green FM fluorescent mitochondrial stain (Molecular Probes, Life Technologies). In order to determine mitochondrial content, 48000 cells *per* well were seeded in 12-wells plates and 2 - 3 wells *per* condition were used in three independent experiments. Mitochondria were labelled with a final concentration of dye of 100 nM for 30 min before visualization. In order to determine the fluorescence intensity, a Polarstar Galaxy fluorimeter (BMG labtech, Ortenberg, Germany) set up to 490 nm excitation and 516 nm emission wavelengths was used (Moreno-Aliaga *et al.* 2011).

SIRT1 activity

The activity of SIRT1 was determined in nuclear protein fractions isolated from mature adipocytes with or without EPA treatment, using a fluorometric SIRT1 activity assay kit as described by the manufacturer (Abcam, Cambridge, UK). Nuclei isolation and extraction was carried out following the supplier's instructions. The activity of SIRT1 was measured by mixing simultaneously fluorescence-labeled acetylated peptide (substrate), trichostatin A, NAD, lysylendopeptidase and the samples or the recombinant SIRT1 (as positive control) at room temperature. Fluorescence intensity (Excitation: 340 nm/Emission: 460 nm) was read for 48 min, at 2 min intervals, to determine the enzyme activity. The fluorescence signal generated was in proportion to the amount of deacetylation of the lysine by SIRT1.

Western blot analysis

Human subcutaneous adipocytes lysates were obtained by the addition of a buffer containing 2 mM Tris HCl (pH 8), 137 mM NaCl, 2 mM EDTA, 1% protease inhibitor cocktail 1 (Sigma-Aldrich), 2 mM orthovanadate, and 1 mM PMSF. Samples were centrifuged and protein concentrations were determined by the BCA method according to the supplier's instructions (Pierce-Thermo Scientific, Rockford, IL). Briefly, equivalent amounts of total protein (30-40 μg) were electro- phoretically separated by 12% SDS-PAGE in the presence of a reducing agent (2-mercaptoethanol). Proteins were electroblotted from the gel to polyvinylidene difluoride membranes (GE Healthcare Europe GmbH, Barcelona, Spain). Following the transfer of proteins, the membranes were stained with Ponceau S as control for total protein loaded, and then blocked and probed with specific primary antibodies diluted at 1:1000 against phospho-AMPK, total AMPK (rabbit, Cell Signaling Technology, Danvers, MA) and β-Actin (mouse, Sigma-Aldrich) with overnight stirring at 4 °C. Moreover, PPARγ and SREBP-1c were measured in nuclear extracts and probed with specific antibodies against PPARγ (1:1000, rabbit, Cusabio, College Park, MD) and SREBP-1c (1:200, mouse, Santa Cruz Biotechnology, Santa Cruz, CA). To determine total AMPK or β-Actin protein expression levels, stripping (ReBlot Plus Mild Solution, Millipore, Temecula, CA) of the membranes was carried out for 15 min. Then the membranes were blocked and probed overnight with specific total AMPK or β-Actin antibody at 4 °C. After that, infrared fluorescent secondary anti-rabbit (Cell Signaling Technology) or anti-mouse (LI-COR Biosciences, Lincoln, USA) antibodies were used (diluted 1:15000) and incubated for one hour at room temperature. Fluorescence signal was quantitated using an Odyssey® Sa infrared imaging system (LI-COR).

Immunoprecipitation

In order to determine the acetylation of PGC-1α, 2 μg of anti-PGC-1α (Santa Cruz Biotechnology) antibody was added to 200 μg of protein extracts (1 μg/μl) and incubated stirring for 2 h at 4 °C. After that, 20 μl of protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added and incubated at 4 °C with shaking overnight. The A/G PLUS-Agarose-beads were pelleted by centrifugation at 12,000*g* for 1 min at 4 °C and washed four times with PBS at 4 °C. Finally, protein was released from the beads by

treatment at 95 °C for 7 min in 2x sample buffer (Invitrogen, Carlsbad, CA). Equivalent amounts of total protein were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked and probed with specific antibodies against anti-acetylated Lysine diluted at 1:100 (rabbit, Cell Signaling Technology) and anti-PGC-1α diluted at 1:100 (rabbit, Santa Cruz Biotechnology) and detected as defined before (Fernández-Galilea *et al.* 2015).

Statistical analysis

Statistical analyses were performed using the program GraphPad Prism 5.0 (GraphPad Software Inc, CA, USA). Data were expressed as mean ± standard errors (SEM). The differences between two groups were assessed using t-Student. Comparisons between more than two groups were carried out with one-way ANOVA followed by *post-hoc* of Tukey test for normal distribution variables or Kruskal–Wallis test when variables did not follow normal distribution. Differences between groups were set up as statistically significant at $P < 0.05$.

RESULTS

EPA down-regulated mRNA expression levels of lipogenic genes

In human subcutaneous mature adipocytes, treatment with the highest concentration of EPA tested (200 µM) caused a noteworthy reduction ($P < 0.01$) of *FAS* gene expression, a key lipogenic enzyme involved in the first step of de novo lipogenesis (Fig. 1). Moreover, EPA (200 µM) strongly decreased ($P < 0.01$) the expression of *SCD1*, which catalyses the unsaturation of fatty acids in lipogenesis forming a double bond in Stearoyl-CoA. On the other hand, mRNA expression levels of *DGAT1* and *DGAT2*, two isoforms of diglyceride acyltransferase, were significantly downregulated ($P < 0.05$) upon treatment with EPA (200 µM). These results suggest a consistent inhibition of the lipogenic pathways by EPA, which seems to be concentration-dependent, as it was not observed in adipocytes treated with a lower concentration of EPA (100 µM). However, nuclear concentrations of SREBP-1c were apparently not modified in EPA-treated adipocytes (Supplemental Fig. 1).

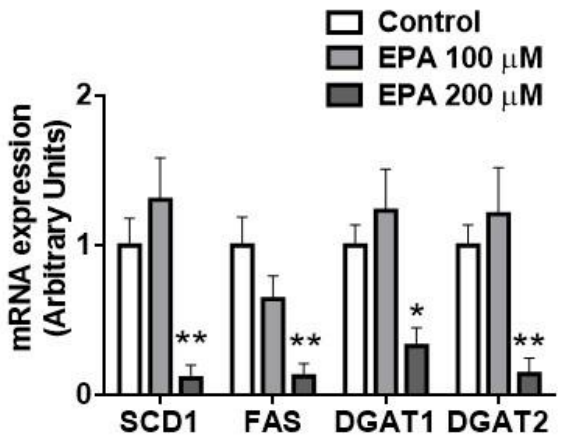


Fig 1. EPA down-regulates the expression of lipogenesis-related genes in subcutaneous abdominal adipocytes from overweight subjects. EPA effect was evaluated at two different concentrations (100 and 200 μM). Control cells were treated with the same amount of vehicle (ethanol). Data (mean ± SEM) are expressed as fold change ($2^{-\Delta\Delta CT}$) relative to control group considered as 1. (n = 4-6 independent experiments). **P* <0.05, ***P* <0.01 *vs*. control group.

EPA up-regulated fatty acid oxidation genes

Next, we determine whether EPA treatment might affect the expression of genes involved in fatty acid oxidation. Interestingly, EPA upregulated mRNA expression of both *CPT-1A* (*P* <0.001 and *P* <0.05 for 100 and 200 μM respectively) and *ACOX* (*P* <0.01 and *P* <0.05 for 100 and 200 μM), two enzymes that participate in fatty acid oxidation in mitochondria and peroxisomes respectively (Fig. 2). On the other hand, EPA treatment did not significantly change the nuclear protein concentrations of PPARγ (Supplemental Fig. 1).

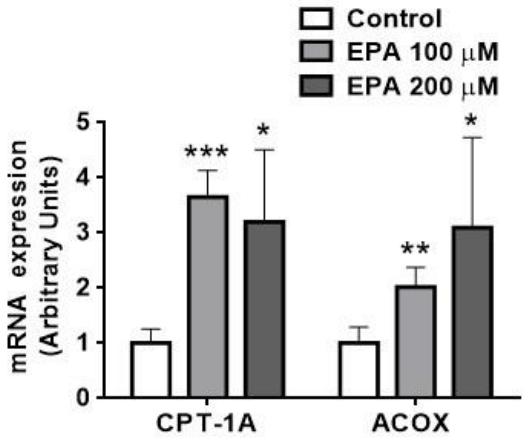
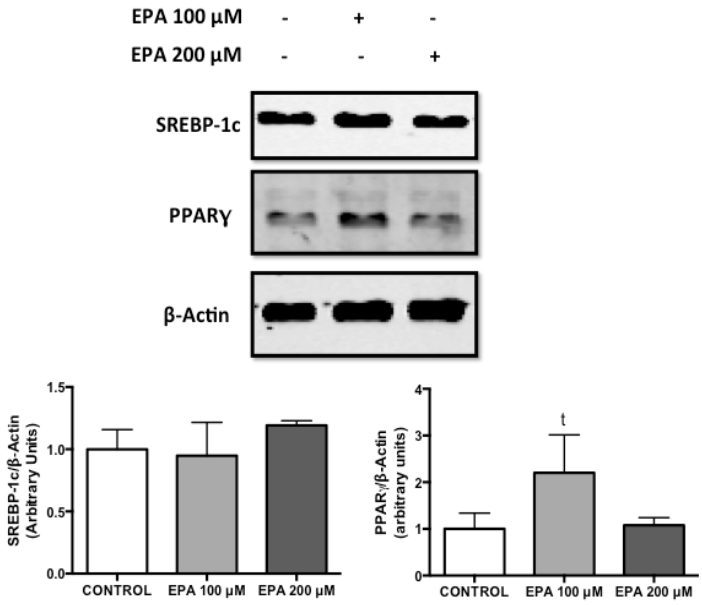


Fig 2. EPA stimulates the expression of genes involved in fatty acid oxidation. EPA (100 μM and 200 μM) actions on *CPT-1A* and *ACOX* were evaluated in subcutaneous abdominal adipocytes from overweight subjects. Gene expression ($2^{-\Delta\Delta CT}$) is expressed as fold change relative to control group considered as 1. Data are expressed as mean ± SEM. (n = 4). **P* <0.05, ***P* <0.01, ****P* <0.001 *vs*. control group.



Supplemental Fig.1 Effects of EPA (100 and 200 µM) on SREBP-1c and PPARγ protein expression in nuclear extracts from human subcutaneous adipocytes from overweight subjects. Data are expressed as mean ± SEM. (n = 4). ^{t}P =0.06 *vs*. control group.

EPA treatment increased mitochondrial biogenesis

In order to study if EPA was able to promote mitochondrial biogenesis, we first examined the mitochondrial content in adipocytes treated with EPA. MitoTracker Green FM fluorescent staining revealed that EPA-treated adipocytes showed a moderate (~20%) but statistically significant increase (P <0.01) in mitochondrial content (Fig. 3A), which was accompanied by a significant up-regulation (P <0.05) of *NRF-1* and *TFAM* mRNA expression, two transcription factors that stimulate mitochondrial biogenesis, as well as the gene expression levels of *COXIV* (Fig. 3B), which catalyses the final step in mitochondrial electron transfer chain, and is one of the major regulation sites for oxidative phosphorylation (Li *et al.* 2006).

We next evaluated the effects of EPA on SIRT1 and PGC-1α, which are considered master regulators of mitochondrial biogenesis. As shown in Fig. 3B, EPA (100 µM) up-regulated (P < 0.05) SIRT1 mRNA levels. Moreover, our data showed that SIRT1 activity was increased in a concentration-dependent manner in EPA-treated adipocytes (P <0.05 and P <0.001, for EPA 100 and 200 µM respectively) (Fig. 3C). While EPA did not modify the protein content of PGC-1α, it promoted (P < 0.05) PGC-1α deacetylation (Fig 3D), which is known to cause PGC-1α activation (Scarpulla *et al.* 2012). Because

AMPK is able to stimulate fatty acid oxidation and also to activate PGC-1α, the effect of EPA on AMPK (total and phosphorylated) was also tested. Our data revealed that EPA was also able to increase (~30%, $P < 0.05$) the phosphorylation levels of AMPK (Fig. 3E). Altogether, these data strongly suggest that EPA stimulates mitochondrial biogenesis in human subcutaneous adipocytes.

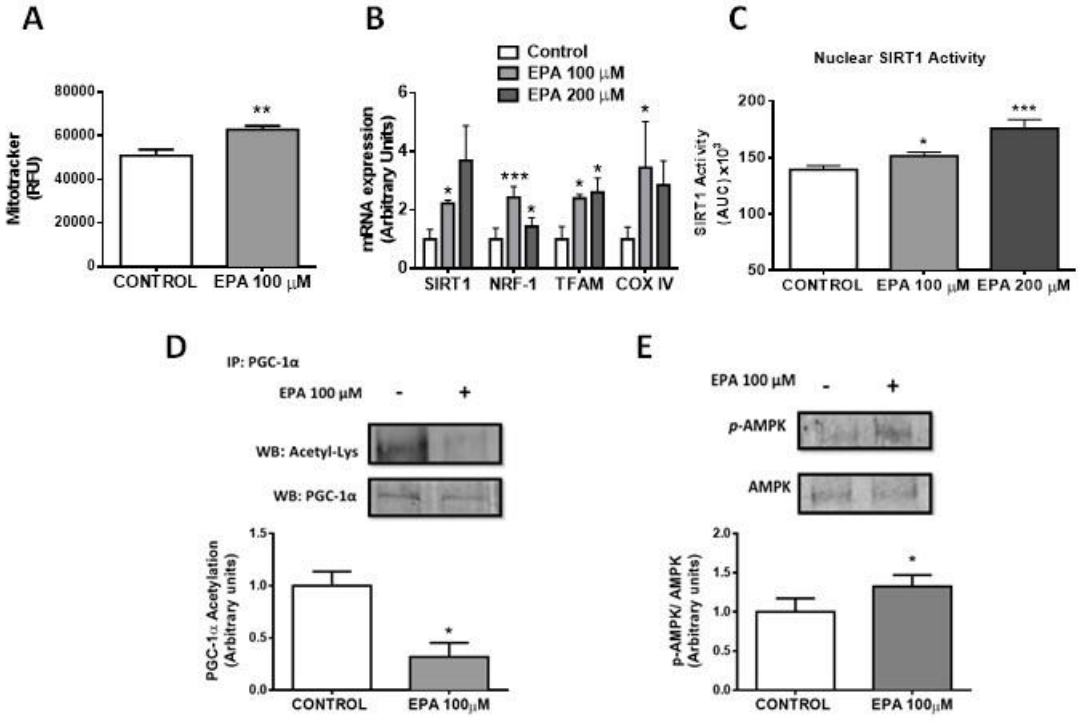


Fig. 3 EPA promotes mitochondrial biogenesis in human subcutaneous adipocytes. (A) Fluorescence intensity quantification of mitochondria stained with Mitotracker Green FM in control and EPA (100 μM)-treated adipocytes. (B) Effects of EPA (100 and 200 μM) on the expression of genes related with mitochondrial biogenesis. Gene expression ($2^{-\Delta\Delta CT}$) is expressed as fold change relative to control group considered as 1. (C) Nuclear SIRT1 activity in EPA-treated (100 and 200 μM) adipocytes. (D) PGC-1α deacetylation in EPA (100 μM)-treated adipocytes. (E) Effects of EPA (100 μM) on total and AMPK phosphorylation protein levels. Data are expressed as mean ± SEM (n=4). **P* <0.05, ***P* <0.01, ****P* <0.001 *vs*. control group.

EPA up-regulates beige adipocyte markers

In parallel with the increase in mitochondrial content, EPA treatment (100 μM) also promoted ($P < 0.05$) the expression of *PRDM16, CIDEA* and *UCP1* (Fig. 4A), which are genes associated to WAT beiging. Interestingly, genes expression analysis of reliable markers for beige human adipocytes (*TBX1, CD137* and *TMEM26*) revealed

that EPA treatment (100 μM) induced the expression of these genes, reaching statistical significance ($P < 0.05$) for *TBX1* and *CD137* (Fig. 4B), further suggesting that EPA may promotes beiging process in human subcutaneous adipocytes.

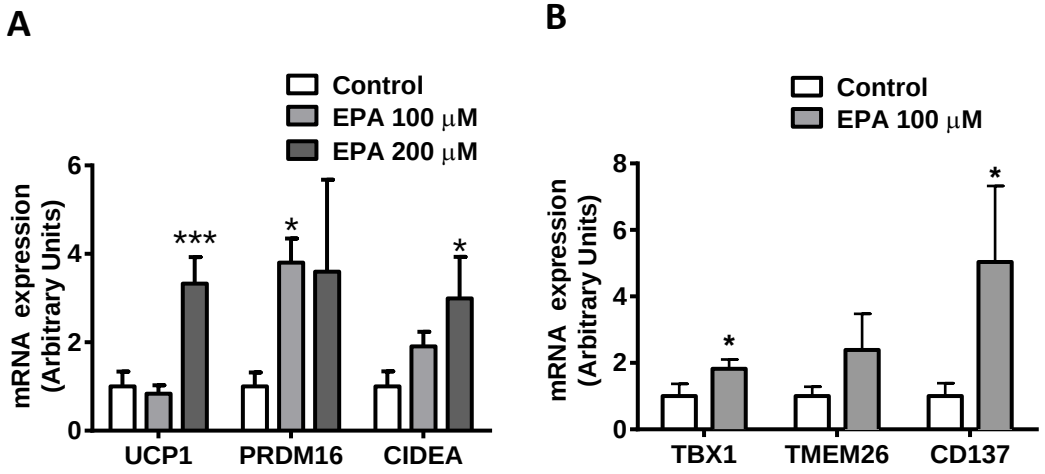


Fig. 4 EPA induces beige-adipocytes related genes in human subcutaneous adipocytes from overweight subjects. (A) Effects of EPA (100 and 200 μM) on brown-adipocyte characteristic genes. (B). EPA actions on beige-adipocytes specific markers. Gene expression ($2^{-\Delta\Delta CT}$) is expressed as fold change relative to control group considered as 1. Data are mean ± SEM. (n =4 -11). **P* <0.05, ****P* <0.001 *vs*. control group.

DISCUSSION

There is consistent and proved evidence about the beneficial effects of n-3 PUFAs, EPA and DHA in obesity-associated features such as cardiovascular disease and dyslipidaemia (Nestel *et al.* 2002, López-Alvarenga *et al.* 2010, Lorente-Cebrián *et al.* 2013, Calder 2015, Martínez-Fernández *et al.* 2015), although the particular mechanisms remain to be completely understood. In the present study, we investigated the effects of EPA on genes/proteins that regulate lipid metabolism pathways (catabolic and anabolic) in human subcutaneous adipocytes from overweight subjects.

Previous trials have reported that n-3 PUFAs (EPA and DHA) are able to reduce the expression of some lipogenic enzymes such as SCD-1 and FAS both in cultured murine

adipocytes when added along the differentiation process (Manickam *et al.* 2010, Barber *et al.* 2013), and also *in vivo* after dietary supplementation (Sebokova *et al.* 1996, Raclot *et al.* 1997). Interestingly, our current data revealed that EPA downregulates both *FAS* and *SCD-1* in mature adipocytes, suggesting that this n-3 PUFA could also be able to reduce *de novo* lipogenesis pathway in human adipocytes from overweight subjects. This agrees with the observation of a previous study from our group in cultured primary rat adipocytes showing that EPA treatment (200 µM, 96 h) reduced *de novo* lipogenesis from glucose (Pérez-Matute *et al.* 2005). On the other hand, our results have revealed that EPA also inhibits the expression of genes encoding for enzymes involved in the re-esterification of fatty acids in adipocytes such as *DGAT1* and *DGAT2*. Taken together, our current results support the ability of EPA to counteract lipid accumulation in human adipocytes by concomitant inhibition of both *de novo* lipogenesis and fatty acid re-esterification genes, which might contribute to the reduction of adipose tissue mass reported after EPA or/and DHA treatment in rodents (Pérez-Matute *et al.* 2007) and humans (Kunesova *et al.* 2006, Hill *et al.* 2007, Kabir *et al.* 2007, Rosqvist *et al.* 2014). In parallel with the reduction of genes favouring lipid accumulation, our data also suggest that EPA could promote fatty acid usage as energy fuel, based on the significant up-regulation of fatty acid oxidation-related genes *CPT1-A* and *ACOX*. Although this is in agreement with previously published *in vitro* and *in vivo* studies (Flachs *et al.* 2005, Guo *et al.* 2005, den Besten *et al.* 2015) in murine models, to our knowledge, the current trial is the first one addressing EPA effects on β-oxidation genes in human subcutaneous adipocytes from overweight subjects. It has been suggested that overweight/obese subjects may have decreased fatty acid oxidation ability due to lipid accumulation and mitochondrial dysfunction mainly in adipose tissue (Kusminski and Scherer 2012, Heinonen *et al.* 2015). Therefore, stimulation of β-oxidation in adipocytes might be a good strategy to reduce triglyceride accumulation and reduce obesity-related disorders (Malandrino *et al.* 2015). On the other hand, the lack of statistically significant effects of EPA on PPARγ and SREBP-1c at nuclear level suggest that the actions of EPA on lipogenic and fatty acid oxidation genes are not secondary to changes in the levels of these transcription factors.

AMPK plays a key role as an energy fuel sensor, because it controls metabolic switch between anabolic/catabolic pathways depending on ATP:AMP ratio. Phosphorylation of AMPK leads to activation of catabolic pathways in order to increase ATP levels. Activation of AMPK in adipocytes has been also associated to inhibition of fatty acid synthesis (Flachs *et al.* 2009, Hardie *et al.* 2012) and stimulation of fatty acid β-oxidation through CPT-1 stimulation (Fernández-Galilea *et al.* 2014). Previous studies have reported the ability of n-3 PUFAs to stimulate AMPK activation by phosphorylation both *in vitro* (3T3-L1 murine adipocytes) (Lorente-Cebrián *et al.* 2009) and after administration to rodents (González-Périz *et al.* 2009). Our current data also show that EPA promotes AMPK phosphorylation in human adipocytes, in parallel with the downregulation of lipogenic and the stimulation of fatty acid oxidation genes, suggesting that AMPK activation could be mediating these metabolic effects of EPA on mature human adipocytes.

Several of our observations support the notion that EPA promotes mitochondrial biogenesis in adipocytes of overweight subjects, including the increase in mitochondrial mass and the up-regulation of mitochondrial genes such as *NRF-1*, a transcription factor that activates expression of respiratory genes, and *TFAM*, which is a master transcription factor that regulates mitochondrial transcription initiation and mitochondrial DNA copy number (Campbell *et al.* 2012). This is in agreement with previously published data in rodents which described an up-regulation of NRF-1 levels after supplementation with n-3 PUFAs (Flachs *et al.* 2005). The stimulation of NRF-1 and TFAM and therefore mitochondrial biogenesis might be achieved via direct or indirect (i. e. through other transcriptional intermediates/factors) mechanisms: while no evidence has been found available about the potential role of n-3 PUFAs as direct NRF-1 ligands, there are several studies that have reported that long chain fatty acids, particularly, n-3 PUFAs, are activators of PGC-1α which is considered a master regulator of mitochondrial biogenesis (Flachs *et al.* 2005). PGC-1α controls the activity of several transcription factors involved in mitochondrial biogenesis including NRF-1 (Scarpulla 2006). SIRT1-mediated deacetylation and activation of PGC-1α is one of the pathways that are particularly important for mitochondrial biogenesis (Fernández-Galilea *et al.* 2015). In this context, our current data has revealed that treatment with EPA increased not only the gene expression levels, but also the activity of SIRT1, which

may activate PGC-1α by decreasing PGC-1α acetylation levels, as recognised in other studies involving molecules that stimulates mitochondrial biogenesis (Lagouge *et al.* 2006, Fernández-Galilea *et al.* 2015). Interestingly, AMPK is also able to promote mitochondrial biogenesis by activating PGC-1α (Wan *et al.* 2014). Thus, it could be feasible that EPA-induced mitochondrial biogenesis may be also secondary to EPA-stimulated AMPK phosphorylation. Despite the fact that some of the changes observed are moderate (20-30%), based in previous studies of our group and others in different models of adipocytes, it can be considered as physiologically relevant (Fernández-Galilea *et al.* 2015, Wang *et al.* 2015, Lone *et al.* 2016).

An interesting finding of our study is that EPA also up-regulates the expression of *PRDM16*, a transcriptional regulatory protein that participates in the development and function of classical brown and beige adipocytes (Cohen *et al.* 2014). Furthermore, EPA also increases the expression of brown-like adipocyte characteristic genes such as *CIDEA* and *UCP1*, which could be related to the *PRDM16* induction. Importantly, EPA induces the expression of beige specific markers such as *TBX1, TMEM26, CD137* (Wu *et al.* 2012, Shan *et al.* 2013), suggesting that this n-3 PUFA is able to promote the beiging process in subcutaneous adipocytes of overweight subjects. In this context, a recent study has also proposed the ability of EPA to promote browning of mouse inguinal fat adipocytes based on the observations that EPA not only increases mitochondrial DNA content and the expression of genes implicated in mitochondrial biogenesis (*Pgc-1α, Nrf-1* and *Coxiv*), but also stimulates *Ucp1, Ucp2, Ucp3* and *Cidea* mRNA during differentiation of adipocytes from stroma vascular cells of mouse inguinal fat. However, this effect was not found when EPA was added to mature inguinal mouse adipocytes, suggesting that EPA exerts the browning effects via recruiting brite adipocytes (Zhao and Chen 2014). This outcome is in contrast with our current observations, which support that EPA promotes the beiging process in mature human subcutaneous adipocytes, suggesting that EPA could trigger the interconversion of a white adipocyte into a beige-like phenotype, a process referred as transdifferentiation (Wu *et al.* 2012, Rosenwald and Wolfrum 2014). However, the ability of EPA to promote *de novo* beige adipocyte formation from human preadipocytes cannot be ruled out and deserves future research.

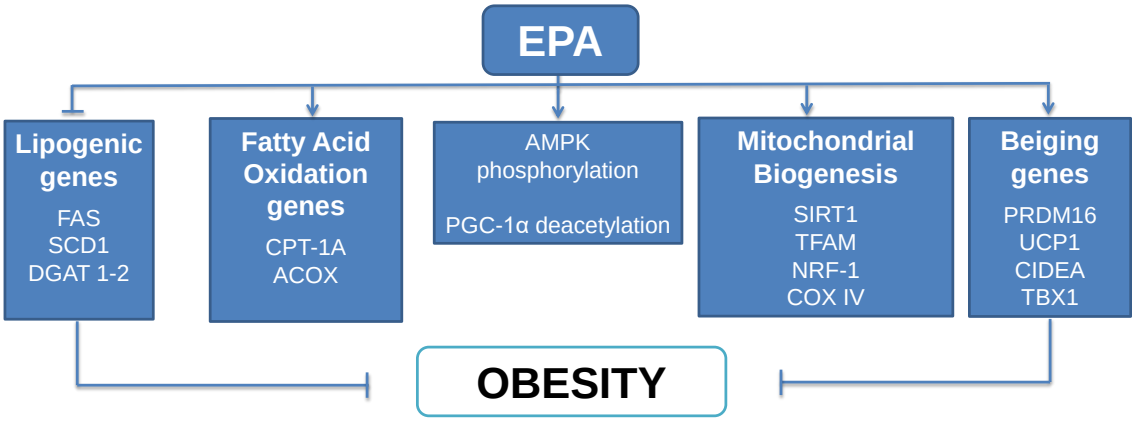
Interestingly, the study of Lodhi *et al.* (2012) suggested that inhibiting adipose tissue lipogenesis reprograms subcutaneous adipose tissue thermogenesis. In fact, such trial demonstrated that mice lacking FAS in adult adipose tissue show increased brown fat-like (brite) adipocytes in subcutaneous adipose tissue, characterized by increased expression of PRDM16, CIDEA and PGC-1α as well as CPT-1 and ACOX. In this context, these findings lead us to suggest that the inhibition induced by EPA on FAS and other genes involved in lipogenesis might also contribute to the ability of this fatty acid to promote beige adipocytes features.

The increased clearance and utilization of nutrients by brown and beige adipocytes could reduce the excess of triglycerides and glucose clearance, conferring beneficial metabolic effects or protection from obesity (Stanford and Goodyear 2013, Bartelt and Heeren 2014, Chondronikola *et al.* 2014), highlighting the importance of identifying bioactive molecules (including food components) that promote the acquisition of beige features by white adipocytes.

Conclusions

Our data suggest that EPA induces a switch from white to beige-like adipocytes features by inhibiting pathways that promote fat storage and inducing the stimulation of those routes involved in fatty acid oxidation, in parallel with a stimulation of mitochondrial biogenesis and the upregulation of genes typifying beige adipocytes phenotype and function. Thus, EPA-induced activation of SIRT1, AMPK and PGC-1α could underlie these EPA actions. Further studies are required in order to characterize the potential ability of n-3 PUFAs supplementation to promote a beige-like switch phenotype of white fat depots in humans, and the relationship with the metabolic effects of these fatty acids.

Graphical abstract



Conflicting interests

The authors declared no conflicts of interest.

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CHAPTER 2

Maresin 1 inhibits TNF-alpha-induced lipolysis and autophagy in 3T3-L1 adipocytes

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ABSTRACT

Obesity is associated with high pro-inflammatory cytokine levels like tumor necrosis factor-alpha (TNF-α), which promotes inflammation in adipose tissue. The omega-3 PUFAs, and their derived lipid mediators, such as Maresin 1 (MaR1) have anti-inflammatory effects on adipose tissue. This study aimed to analyse if MaR1 may counteract alterations induced by TNF-α on lipolysis and autophagy in mature 3T3-L1 adipocytes. Our data revealed that MaR1 (1, 10, 100 nM) inhibited TNF-α-induced glycerol release after 48 h, which may be related to MaR1 ability of preventing the decrease of the lipid droplet-coating protein perilipin and G0/G1 Switch 2 protein expression. MaR1 also reversed the decrease on total hormone sensitive lipase (total HSL), and the ratio of phosphoHSL at Ser-565/total HSL, while preventing the increased ratio of phosphoHSL at Ser-660/total HSL and phosphorylation of extracellular signal-regulated kinase 1/2 induced by TNF-α. Moreover, MaR1 counteracted the cytokine-induced decrease of p62 protein, a key autophagy indicator, and also prevented the induction of LC3II/LC3I, an important autophagosome formation marker. Current data suggest that MaR1 may ameliorate TNF-α-induced alterations on lipolysis and autophagy in adipocytes. This might also contribute to the beneficial actions of MaR1 on adipose tissue and insulin sensitivity in obesity.

Key words: Adipocytes, Autophagy, Lipolysis, Maresin 1, TNF-α.

INTRODUCTION

Obesity is considered as a chronic low-grade inflammatory disease (Sethi and Vidal-Puig 2007). In fact, increasing levels of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), are found within the adipose tissue in obesity (Hotamisligil *et al.* 1995, Hauner 2005). The misbalance of anti-inflammatory/pro-inflammatory cytokines that is established in obesity, could lead to adipose tissue dysfunction and systemic metabolic disorders such as dyslipidaemia and insulin resistance (Moller 2000). In fact, elevated levels of TNF-α, as observed in obesity, could alter adipocytes lipid metabolism, promoting increased lipolysis (Arner 2002). Although, this might seem beneficial in order to eliminate accumulated fat, it has been proved that increased levels of basal lipolysis and the subsequent high levels of circulating free fatty acids (FFA) (Guilherme *et al.* 2008, Morigny *et al.* 2016), may interfere with the insulin signaling, leading to insulin resistance systemically (Arner 2002, Guilherme *et al.* 2008).

Additionally, autophagy is a highly conserved catabolic process, which delivers cytoplasmic components to the lysosome for degradation and use as an energy source (Singh *et al.* 2009a). Actually, macroautophagy (hereafter referred to as autophagy) may be dysfunctional when an increase on pro-inflammatory cytokines occurs (Harris 2011, Maixner *et al.* 2012).

Interestingly, mice with systemic deletion of the autophagy-related genes *Atg5* or *Atg7* exhibit enhanced insulin sensitivity and are resistance to high fat diet-induced obesity (Singh *et al.* 2009b, Zhang *et al.* 2009). Although, several studies found increased autophagy levels in adipose tissue of overweight/obese patients with type 2 diabetes (Ost *et al.* 2010, Rodriguez *et al.* 2012, Kosacka *et al.* 2015), it remains unclear whether autophagy is repressed or stimulated in obese adipose tissue (Mizunoe *et al.* 2017). Moreover, autophagy and lipolysis may share similar functions, working as complementary pathways in order to provide energy to the cell during starvation (Singh *et al.* 2009a, Singh and Cuervo 2012, Kaushik and Cuervo 2015, Schweiger and Zechner 2015). Indeed, there is evidence that lipolysis and lipid droplet formation are important for autophagosome biogenesis (Shpilka *et al.* 2015). Besides, autophagy can modulate inflammation after oxidative stress in diabetes and autophagy dysfunction

may cause disturbances in diabetes and other diseases (Wang *et al.* 2013, Wilson *et al.* 2014).

In this context, it is important to identify nutritional/pharmacological candidates that could counteract the disturbed adipocyte lipid metabolism induced by chronic inflammation in obesity. There is widely accumulated evidence of the beneficial effects of marine omega-3 PUFAs (n-3 PUFAs) on inflammatory diseases (Lorente-Cebrián *et al.* 2013, Martínez-Fernández *et al.* 2015). Actually, n-3 PUFAs have been shown to attenuate white adipose tissue inflammation, in both humans and rodents, in obesity (Pérez-Matute *et al.* 2007, Pérez-Echarri *et al.* 2009, Kalupahana *et al.* 2010, Titos and Clària 2013, Huerta *et al.* 2016). We have previously reported that the n-3 PUFA eicosapentaenoic acid (EPA) inhibits TNF-α-induced lipolysis in murine cultured adipocytes (Lorente-Cebrián *et al.* 2012). Docosahexanoic acid (DHA) and EPA serve as substrates for the formation of specialized pro-resolving lipid mediators (SPMs), including resolvins (Rv), protectins (PD) and maresins (MaR), which not only exert anti-inflammatory effects but also have been hypothesized to mediate the beneficial metabolic effects attributed to these n-3 PUFAs in obesity (Spite *et al.* 2014). In this context, it has been proved that a deficit in the production of SPMs arises in obese adipose tissue, and that the restoration of endogenous levels by n-3 PUFAs dietary supplementation improves inflammation and ameliorates metabolic dysfunction (Clària *et al.* 2012, Clària *et al.* 2013, Neuhofer *et al.* 2013, Titos and Clària 2013, Martínez-Fernández *et al.* 2017). Moreover, treatments with 17-HDHA, RvD1, RvE1, and PDX have been shown to exert beneficial effects on inflammation, insulin sensitivity and lipid metabolism in adipose tissue, liver and muscle (Neuhofer *et al.* 2013, White *et al.* 2014, López-Vicario *et al.* 2016, Titos *et al.* 2016). Additionally, a recent study of our group found that Maresin 1 (MaR1), a SPM derived from DHA, reduces adipose tissue inflammation and improves insulin sensitivity in obese mice (Martínez-Fernández *et al.* 2017). However, to our knowledge, the actions of this DHA derivative on adipocytes lipid metabolism are still mainly unexplored. Thus, the aim of the present study was to characterize the ability of MaR1 to prevent TNF-α-induced alterations on lipolysis and autophagy pathways in 3T3-L1 adipocytes.

MATERIAL AND METHODS

Cell culture and differentiation of 3T3-L1 cells

Mouse embryo fibroblast 3T3-L1 cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM containing 25 mM glucose, 10% (v/v) calf bovine serum (Invitrogen, Carlsbad, CA), and 1% (v/v) penicillin/streptomycin (Invitrogen). Cells were conserved in an incubator set to 37 °C and 5% of CO_2. At confluence, pre-adipocytes were cultured for additional 48 h in DMEM (Invitrogen) containing 25 mM glucose, 10% fetal bovine serum (FBS, Invitrogen), penicillin/streptomycin, and supplemented with dexamethasone (1 mM; Sigma-Aldrich, St. Louis, MO), isobutylmethylxantine (0.5 mM; Sigma-Aldrich), and insulin (10 µg/mL; Sigma-Aldrich). Afterwards, cells were cultured with 10% FBS and insulin for 48 h and thereafter media were replaced with 10% FBS in DMEM and antibiotics without insulin. Media was changed every 2 days until day 8 post-confluence, when differentiated 3T3-L1 cells are considered as mature adipocytes since they display typical morphology and functional features of adipocytes (López-Yoldi *et al.* 2014).

Treatments and culture reagents

MaR1 was obtained from Cayman Chemical (Ann Arbor, MI, USA) and TNF-α was purchased from Sigma-Aldrich. All reagents/chemicals used were of analytical grade. All compounds were prepared as 1000X stock solutions and added to the culture medium at required concentration. Control and TNF-α-treated cells were incubated with the same amount of vehicle (ethanol) where MaR1 was diluted. Prior to the addition of the appropriate treatments, cells were serum starved for 4 h using the medium DMEM supplemented with 1% FBS. Then, cells were treated with or without MaR1 (1-100 ng/mL) in presence or absence of TNF-α (10 ng/mL) for 6, 24 and 48 h. Cell viability was determined by lactate dehydrogenase (LDH) activity, an indicator of cell membrane integrity, and therefore, as a measurement of cell necrosis/apoptosis (Laiglesia *et al.* 2016). Adipocytes treated with MaR1, at all the concentrations tested, did not shown differences with control or TNF-α cells, indicating that MaR1 did not alter cell viability (data not shown).

Determination of lipolysis in 3T3-L1 adipocytes

Lipolysis was evaluated by the biochemical determination of the amount of glycerol released into the culture media. Glycerol measurements were performed at basal conditions and after 6, 24 and 48 h of MaR1 and TNF-α treatment using the Pentra C200 autoanalyser (Roche Diagnostic, Basel, Switzerland), following manufacturer's instructions.

Western blot analysis

3T3-L1 cell lysates were obtained by the addition of a buffer containing 2 mM Tris HCl (pH 8), 137 mM NaCl, 2 mM EDTA, 1% protease inhibitor cocktail 1 (Sigma-Aldrich), 2 mM orthovanadate, and 1 mM PMSF). Samples were centrifuged and protein concentrations were determined by the BCA method according to the manufacturer's instructions (Pierce-Thermo Scientific, Rockford, IL). Then, equivalent amounts of total protein (25-35 μg) were electrophoretically separated by 10-15% SDS-PAGE in the presence of a reducing agent. Proteins were electroblotted from the gel to polyvinylidene difluoride (PVDF) membranes (GE Healthcare Europe GmbH, Barcelona, Spain). Following the transfer of proteins, the membranes were blocked (10% dry-milk) and then probed with specific primary antibodies (1:1000) against phospho-hormone sensitive lipase (p-HSL) (Ser-565), p-HSL (Ser-660), adipose triglyceride lipase (ATGL), perilipin, microtubule-associated protein 1A/1B-light chain 3 (LC3), autophagy-related protein (ATG7), phospho-extracellular signal-regulated kinase 1/2 (p-ERK1/2) (Cell Signaling Technologies, Danvers, MA), G0/G1 switch 2 (G0S2), comparative gene identification-58 (CGI-58), p62/SQSTM1 (Sigma-Aldrich, St. Louis, MO), proliferator-activated receptor gamma (PPARγ) (Cusabio, College Park, MD) and β-actin (Sigma-Aldrich). To determine total HSL and ERK 1/2, protein expression levels, membranes were stripped with an appropriate buffer (ReBlot Plus Mild Solution, Millipore, Temecula, CA) for 15 min at room temperature and shaking. Then, membranes were blocked and probed overnight with specific total HSL and ERK 1/2 antibody (Cell Signaling Technology) at 4 °C. After that, infrared fluorescent secondary anti-rabbit (Cell Signaling Technology) or anti-mouse (LI-COR Biosciences, Lincoln, USA) antibodies were used (diluted 1:15000) and incubated for one hour at room temperature.

Fluorescence signal was quantitated using an Odyssey® Sa infrared imaging system (LI-COR).

Statistical analysis

Statistical analyses were performed using the software GraphPad Prism 5.0 (GraphPad Software Inc, CA, USA). Data were expressed as mean ± SEM. Differences between two groups were assessed using t-Student test after testing for sample normality. Comparisons between more than two groups were analysed by one-way ANOVA followed by *post-hoc* of Tukey test for normal distribution variables or Kruskal–Wallis test when variables did not follow normal distribution. Differences between groups were set up as statistically significant at $P < 0.05$.

RESULTS

Effects of MaR1 on TNF-α-stimulated lipolysis

As expected, TNF-α induced a time-dependent increase on glycerol release to the media, being significant after 24 h ($P < 0.05$) and 48 h ($P < 0.001$). Interestingly, co-treatment with MaR1 (1-100 nM) was able to prevent the stimulatory effect of TNF-α on glycerol release in a time-dependent manner, reaching statistical significance ($P < 0.001$) after 48 h (Fig. 1), suggesting anti-lipolytic effects of MaR1 on TNF-α-induced lipolysis.

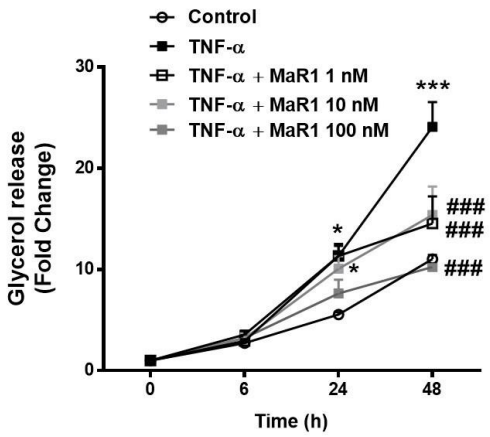

Fig 1. MaR1 prevents TNF-α-induced lipolysis in a time-dependent manner in differentiated 3T3-L1 adipocytes. Mature 3T3-L1 adipocytes were serum starved for 4 h and then treated with TNF-α (10 ng/mL) alone or in the presence of MaR1 (1, 10, 100 nM) for 6, 24 and 48 h. Control cells were treated with the same amount of vehicle for the same time points. Total amount of glycerol released into the media was measured as an index of lipolysis. Data are expressed as mean ± SEM (n = 3–6 independent experiments). **P* <0.05, ****P* <0.001 as compared to control group; $^{###}P$ <0.001 as compared with TNF-α group.

Effects of MaR1 on adipocyte lipases and perilipin

We next aimed to evaluate the effect of MaR1 on the alterations induced by TNF-α on the main adipocyte lipases and lipid droplet proteins controlling lipolysis. Although TNF-α induced a slight (non-significant) decrease in ATGL protein expression, MaR1 tended to partially counteract this effect (Fig. 2A, B) after 48 h of treatment. Since, ATGL activity is regulated by interaction with two proteins, CGI-58 (co-activator) and G0S2 (inhibitor), we tested whether MaR1 effects on TNF-α-induced lipolysis might be secondary to changes on ATGL regulators. MaR1 did not modify the expression levels of CGI-58 (Fig. 2A, D), although the inhibitory effect that TNF-α caused on G0S2 (*P* <0.05) was not observed in MaR1-treated adipocytes (Fig. 2A, C). These data suggest that MaR1 could counteract TNF-α-induced lipolysis by increasing G0S2 protein expression and therefore inhibiting ATGL activity.

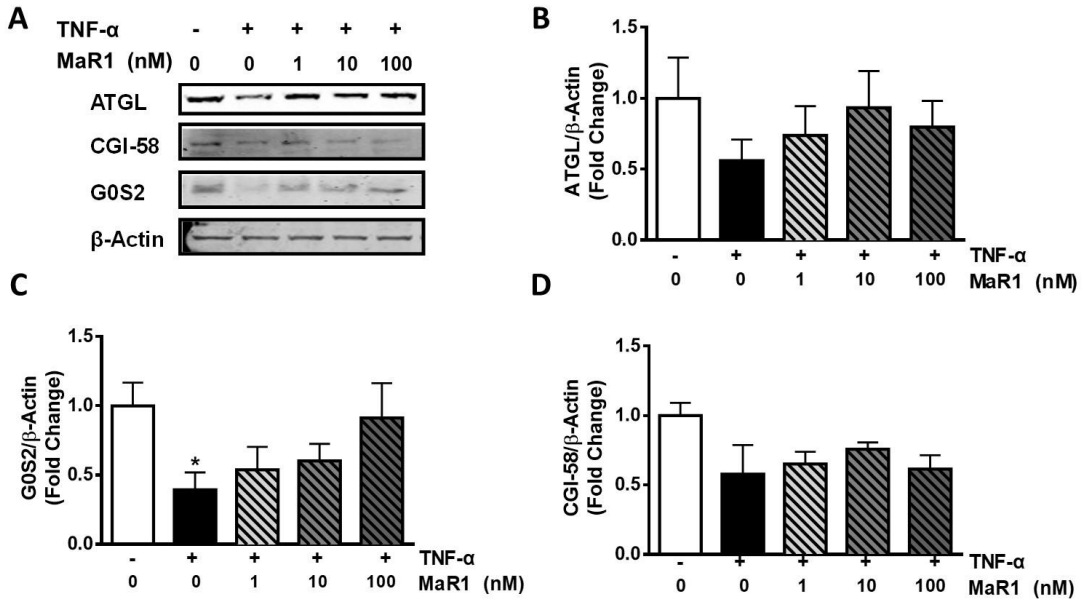

Fig 2. Effects of MaR1 on ATGL and its co-regulators (G0S2 and CGI-58). A: Representative Western blot of ATGL, G0S2 and CGI-58. B–D: densitometry analysis of ATGL (B), G0S2, inhibitor of ATGL (C) and CGI-58, activator of ATGL (D) in fully differentiated 3T3-L1 adipocytes treated with TNF-α (10 ng/mL) and/or MaR1 (1, 10 and 100 nM) for 48 h. Control cells were treated with the same amount of vehicle for 48 h. Band densities for ATGL, G0S2, and CGI-58 were normalized to β-actin. Data are expressed as mean ± SEM (n = 3). **P* <0.05 compared to control cells.

MaR1 (10-100 nM) was also able to prevent (*P* <0.05) the inhibitory effect of TNF-α on total HSL protein expression (Fig. 3A, B) after 48 h of treatment. HSL activity is regulated by several mechanisms including reversible phosphorylation at several serine residues. Our measurements revealed that chronic exposure to TNF-α (48 h) significantly increased the ratio of phosphorylated HSL at Ser-660/total HSL (*P* <0.05), while decreased the ratio of phosphorylated HSL at Ser-565/total HSL (*P* <0.01), showing a reciprocal regulation of HSL phosphorylation in stimulatory and inhibitory serine residues of HSL by this inflammatory cytokine (Fig. 3A, C, D). Interestingly, MaR1 was able to partially prevent the inhibitory actions of TNF-α on HSL phosphorylation at Ser-565 in a concentration-dependent manner (Fig. 3A, C). Moreover, the stimulatory effect of TNF-α on HSL phosphorylation at Ser-660/total HSL ratio was not observed when adipocytes were co-treated with MaR1 (Fig. 3A, D).

Furthermore, we assessed the actions of MaR1 on Perilipin, a lipid droplet-coating protein that, together with lipases, is one of the major regulators of adipocyte lipolysis.

While TNF-α potently inhibited Perilipin protein expression (*P* <0.01), co-treatment with MaR1 abrogated this inhibition on Perilipin content (*P* <0.05) after 48 h (Fig. 3A, E). All these findings support the capability of MaR1 to prevent TNF-α-induced actions on adipocyte lipolysis by regulating the activation of main lipases and lipid-droplet proteins involved in this process.

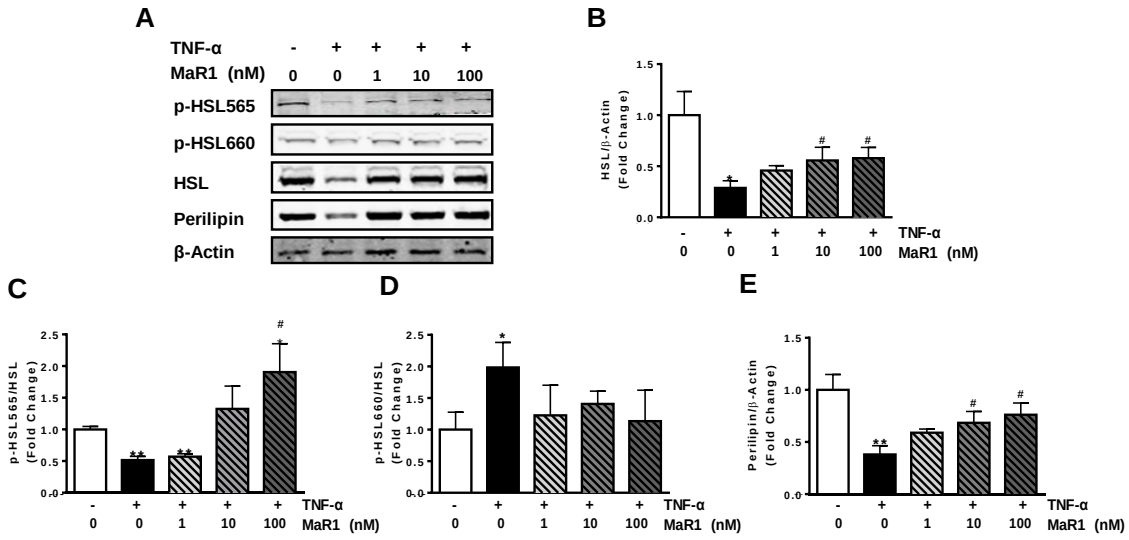

Fig 3. MaR1 modulates key lipolysis regulators Perilipin and HSL in TNF-α-treated 3T3-L1 adipocytes. A-E: Representative Western blot (A) and densitometry analysis of total HSL (B) and HSL phosphorylation at Ser565 (C), Ser660 (D) and Perilipin (E) in differentiated 3T3-L1 adipocytes. Band intensities were quantified and normalized to β-actin (Perilipin and HSL) and/or total HSL protein (p-HSL565 and p-HSL660). Control cells were treated with same amount of vehicle for 48 h. Data are expressed as mean ± SEM. (n = 3). **P* <0.05, ***P* <0.01 as compared to control group; #*P* <0.05 *vs.* TNF-α-treated adipocytes.

In parallel, MaR1 decreased the induction of ERK 1/2 phosphorylation stimulated by TNF-α (Fig. 4A, B). Additionally, we also tested whether these anti-lipolytic effects of MaR1 in adipocytes might be secondary to modifications in PPARγ, an adipogenic transcription factor. However, neither TNF-α nor MaR1 altered PPARγ protein content, suggesting that MaR1 effects on lipolysis are not reliant on changes on adipocyte differentiation.

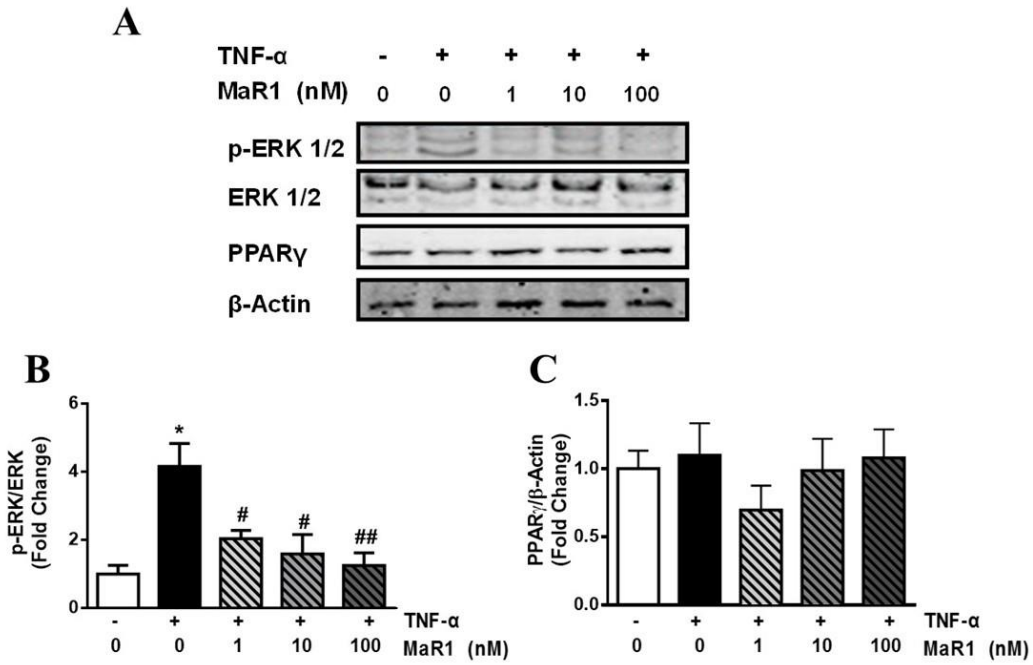

Fig 4. MaR1 counteracts TNF-α-induced ERK 1/2 phosphorylation and does not alter PPARγ protein expression in 3T3-L1 differentiated adipocytes. A-C: Representative Western blot (A) and densitometry analysis of ERK1/2 phosphorylation (B) and PPARγ (C). Band densities for ERK 1/2 phosphorylation were normalized by total ERK 1/2 protein expression while PPARγ was normalized to β-actin. Data are expressed as mean ± SEM (n = 3). **P* < 0.05 compared with control cells; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs*. TNF-α-treated adipocytes.

Effects of MaR1 on autophagy markers

The potential effects of MaR1 on changes induced by TNF-α on autophagy regulators were also evaluated. Adipocytes showed a significant reduction (*P* <0.01) on p62 protein expression (Fig. 5A, B) after 48 h of TNF-α treatment, suggesting an induction of autophagy by this cytokine. It is important to note that the decreased p62 levels are not related to down-regulation of *p62/ SQSTM1* gene expression (data not shown), supporting that the changes in p62 protein levels can be ascribed to its degradation through autophagy. Interestingly, MaR1 was able to prevent TNF-α-induced p62 reduction (*P* <0.05) in a concentration-dependent manner (Fig. 5A, B). Furthermore, the LC3II/LC3I ratio, an important marker of autophagosome formation, was increased (*P* <0.05) upon 48 h of TNF-α treatment. Additionally, incubation in the presence of MaR1 reduced LC3II/LC3I ratio to similar levels as observed for control adipocytes, being significant (*P* <0.05) at 100 nM (Fig. 5A, C). However, neither TNF-α

nor MaR1 induced significant changes on ATG7, which is involved in the autophagy pathway, inducing the formation and elongation of the autophagosome (Fig. 5A, D).

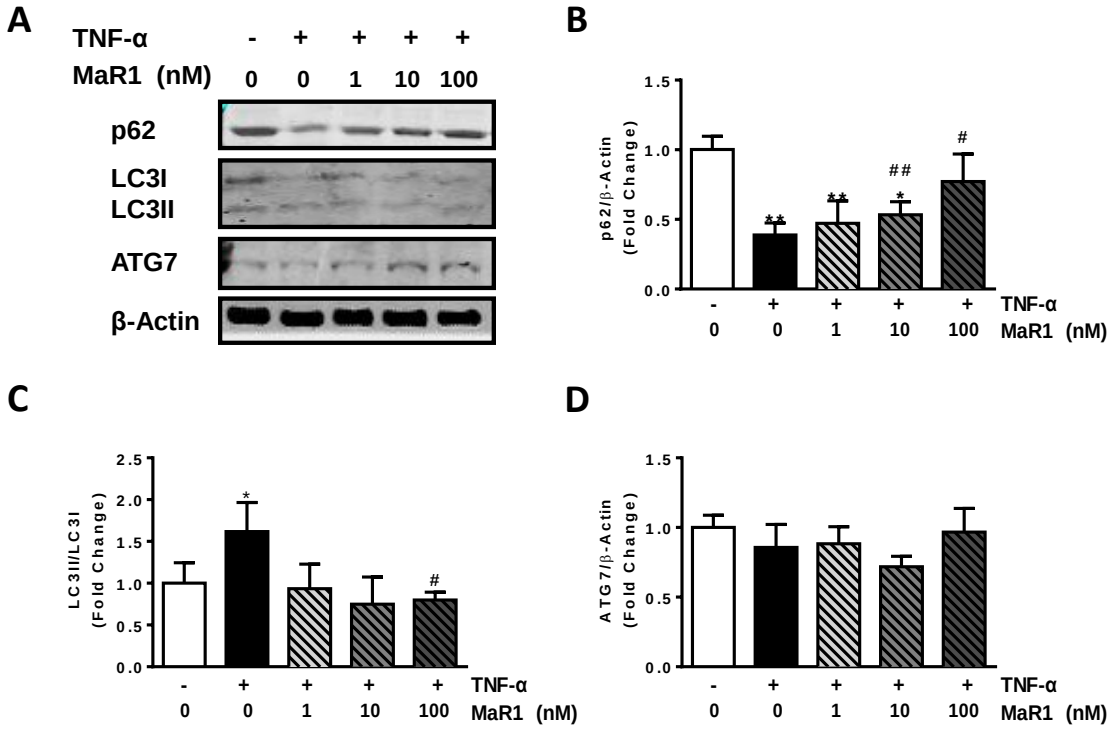

Fig 5. MaR1 modulates TNF-α actions on key autophagy related-proteins in 3T3-L1 adipocytes. Mature adipocytes were treated with TNF-α in the presence or absence of MaR1 for 48 h. A: Representative Western blot of autophagy-related proteins and B–D: densitometry analysis of p62 autophagosome substrate (B), LC3II/LC3I ratio (C) and ATG7 (D). Band densities for p62 and ATG7 were normalized to β-actin. Data are expressed as mean ± SEM (n = 3). **P* <0.05, ***P* <0.01 compared to control cells; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs.* TNF-α -treated adipocytes.

DISCUSSION

It is well known that the microenvironment that occurs in obesity within the adipose tissue is often prominently altered. Adipose tissue becomes hypoxic, with increased oxidative stress and pro-inflammatory cytokines production, such as TNF-α (Hosogai *et al.* 2007, O'Rourke *et al.* 2011). In fact, increased levels of TNF-α strongly induce lipolysis (Ryden *et al.* 2002, Souza *et al.* 2003) and autophagy (Rodriguez *et al.* 2012) in adipose tissue. In this study, we described potential benefits of the DHA-derived pro-resolving lipid mediator, MaR1 on ameliorating TNF-α-induced alterations

on adipocyte lipolysis and autophagy. Although previous studies have demonstrated the ability of n-3 PUFAs to prevent inflammatory-cytokine effects in adipocytes (Kang and Weylandt 2008, Calder 2015), including adipocyte lipolysis (Lorente-Cebrián *et al.* 2012), this is, to our knowledge, the first study addressing the direct effects of MaR1, on cytokine-induced lipolysis. Our findings show that the ability of MaR1 to decrease TNF-α induced lipolysis might rely on specific changes at lipases and key lipolysis regulators. As previously reported, total HSL protein expression is reduced by TNF-α (Ryden *et al.* 2004), while this effect is strongly counteracted by MaR1. Although it might be surprising that MaR1 stimulates HSL protein expression despite reduction in glycerol release (and lipolysis) (Laurencikiene *et al.* 2007), it is well known that HSL activity is mainly regulated by specific phosphorylation at different serine residues. In this sense, HSL phosphorylation at Ser-565, inhibits HSL activity by allosteric impediment and when the phosphorylation occurs at Ser-660, HSL activity is increased (Watt *et al.* 2006). Indeed, MaR1 was able to hinder the lipolytic actions of TNF-α probably by decreasing HSL activity through restoring the ratio of HSL phosphorylation specifically at Ser-565 in a concentration-dependent manner. This preferential regulation of HSL phosphorylation at Ser-565 by MaR1 seems to be functionally relevant since stimulation of "inactivating" residue prevents further activation of "stimulating" residues, which all together contribute to the inhibition of adipocyte lipolysis induced by this lipid mediator. In this regard, the increase in the ratio p-HSL (Ser660)/total phosphorylation observed in TNF-α-treated adipocytes was not observed after co-treatment with MaR1. Moreover, MaR1 also decreased the ERK1/2 phosphorylation induced by TNF-α, which has been reported to promote HSL activation by phosphorylation at Ser-600 (Watt *et al.* 2006). It is important to mention that neither the lipolytic effect of TNF-α nor MaR1 seem to be a result of alterations on adipocyte differentiation, since no significant differences were found in PPARγ protein content.

Additionally, no statistical significant effects of TNF-α in the presence or absence of MaR1 were found on ATGL or its co-regulator, CGI-58 protein expression. This fact contrasts with other studies that have shown that TNF-α decrease ATGL expression (Kim *et al.* 2006, Bezaire *et al.* 2009). However, in agreement with our current data, it has been demonstrated that TNF-α increases ATGL activity by decreasing the levels of

its co-repressor G0S2 levels (Yang *et al.* 2011, Jin *et al.* 2014). In this context, our current findings suggest that MaR1 could also prevent the lipolytic effect of TNF-α by diminishing cytokine-induced G0S2 inhibition and therefore, decreasing ATGL activity.

Furthermore, the ability of MaR1 to prevent TNF-α-induced down-regulation of the lipid droplet coating protein perilipin, could also contribute to protect lipid droplets content from being accessed by lipases and therefore, reduce triglycerides breakdown. Supporting this hypothesis, it has been demonstrated that high levels of perilipin protein expression reduce lipolysis rate (Guilherme *et al.* 2008, Greenberg *et al.* 2011) and that over-expression of perilipins inhibits TNF-α-induced lipolysis (Souza *et al.* 1998).

On the other hand, it has been suggested that accessibility to lipid droplet content might contribute to autophagic vacuoles formation at lipid droplet surface (lipophagy) (Singh and Cuervo 2012). Indeed, there is evidence suggesting a complementary regulation of both catabolic pathways (lipolysis and autophagy) (Singh *et al.* 2009a, Singh and Cuervo 2012, Kaushik and Cuervo 2015, Schweiger and Zechner 2015).

In this context, our results show the ability of MaR1 to reverse TNF-α effects on specific autophagy-related proteins, including p62 and LC3I and II. It is well known that autophagy maintains homeostatic levels of p62, whereas a decrease in autophagy leads to accumulation of p62 and vice versa (Komatsu *et al.* 2007). Our data suggest that chronic exposure to TNF-α promotes autophagy, based on the observations that the cytokine reduced p62 protein levels (which is degraded by autophagy) and increases the LC3II/LC3I ratio, considered as a marker of monitoring autophagy (Ichimura *et al.* 2000). In this sense, there is evidence that some molecules (ghrelin and cardiotrophin-1) might increase autophagy in parallel with an increase in LC3II/LC3I ratio (Castaño *et al.* 2014, Yuan *et al.* 2016). Interestingly, co-treatment with MaR1 counteracted the changes induced by TNF-α on both p62 and LC3II/LC3I ratio, supporting the ability of MaR1 to prevent TNF-α-induced autophagy.

During autophagy, members of the ATG family of proteins mediate membrane rearrangements, which lead to the engulfment and degradation of cytoplasmic cargo (Fullgrabe *et al.* 2016). A previous study (Rodriguez *et al.* 2012) pointed out that TNF-α treatment (24 h) increases *Atg5* and *Atg7* mRNA expression in visceral adipocytes. In contrast, we found that longer period of treatment with TNF-α (48 h) did not modify

ATG7 protein levels. This apparent discrepancy could be related to differences of the origin of the adipocytes, length of the treatment and/or post-transcriptional modifications. On the other hand, the observation that TNF-α decreases p62 and increases LC3II/I ratio with no changes on ATG7, suggest that TNF-α could induce Atg7-independent alternative autophagy. In this context, mouse cells lacking Atg7 can still form autophagosomes and perform autophagy-mediated protein degradation when subjected to certain stressors (Nishida *et al.* 2009).

In summary, the current investigation reveals for the first time the ability of MaR1 to reverse the alterations induced by the pro-inflammatory cytokine TNF-α on autophagy and lipolysis machinery in adipocytes. Indeed, MaR1 counteracts the changes induced by TNF-α on some of the main lipases and lipid droplets proteins controlling lipolysis (perilipin, HSL and G0S2) as well as on proteins regulating autophagy (p62 and LC3). These observations suggest that MaR1 might represent a promising therapeutic agent to counteract the alterations induced by inflammation in adipose tissue.

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The authors have declared no conflict of interest.

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CHAPTER 3

Maresin 1 mitigates liver steatosis in *ob/ob* and diet-induced obese mice

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ABSTRACT

The aim of this study was to characterize the effects of Maresin 1 (MaR1) in obesity-related liver steatosis and the potential mechanisms involved. MaR1 effects on fatty liver disease were tested in *ob/ob* (2-10 μg/kg i.p., 20 days) and in diet-induced obese (DIO) mice (2 μg/kg, i.p., or 50 μg/kg, oral gavage for 10 days), as well as in primary cultured hepatocytes. In *ob/ob* mice, MaR1 reduced liver triglycerides (TG) content, fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 protein expression, while increased acetyl-CoA carboxylase (ACC) phosphorylation and LC3II protein expression, in parallel with a drop in p62 levels. Similar effects on hepatic TG, ACC phosphorylation, p62 and LC3II were observed in DIO mice after MaR1 i.p. injection. Interestingly, oral gavage of MaR1 also decreased serum transaminases, reduced liver weight and TG content. MaR1-treated mice exhibited reduced hepatic lipogenic enzymes content (FAS) or activation (by phosphorylation of ACC), accompanied by upregulation of carnitine palmitoyltransferase (*Cpt1a*), acyl-coenzymeA oxidase (*Acox1*) and autophagy-related proteins 5 and 7 (*Atg 5-7*) gene expression, along with increased number of autophagic vacuoles and reduced p62 protein levels. MaR1 also induced AMP-activated protein kinase (AMPK) phosphorylation in DIO mice and in primary hepatocytes, and AMPK inhibition completely blocked MaR1 effects on *Cpt1a, Acox1, Atg5* and *Atg7* expression. In conclusion, MaR1 ameliorates liver steatosis by decreasing lipogenic enzymes, while inducing fatty acid oxidation genes and autophagy, which could be related to AMPK activation. Thus, MaR1 may be a new therapeutic candidate for reducing fatty liver in obesity.

Keywords: Maresin, autophagy, liver metabolism, non-alcoholic fatty liver disease, obesity.

INTRODUCTION

Obesity is considered as an epidemic worldwide disease, which has been related to higher prevalence of developing non-alcoholic fatty liver disease (NAFLD) (Lazo and Clark 2008, WHO 2011). NAFLD plays a key role in the development of metabolic complications associated to obesity, such as insulin resistance and type 2 diabetes (Jornayvaz and Shulman 2012). NAFLD is a multilayered syndrome that encompasses different stages ranging from 1) steatosis to 2) non-alcoholic steatohepatitis (NASH), 3) fibrosis and 4) cirrhosis (Van De Wier *et al.* 2017). Ectopic lipid accumulation in liver occurs from an imbalance between lipid synthesis (increased *de novo* lipogenesis) and lipid breakdown (decreased fatty acid oxidation and/or impaired lipophagy) (Musso *et al.* 2009, Tilg and Moschen 2010, Polyzos *et al.* 2012). In this sense, dysregulated hepatic autophagy in liver seems to play a causative role in liver steatosis (Singh *et al.* 2009, Singh and Cuervo 2012). Indeed, defective autophagy has been observed in liver of obese insulin-resistance mice (Liu *et al.* 2009, Yang *et al.* 2010).

On the other hand, the current therapy for patients suffering from NAFLD is focused mainly on weight loss strategies. However, the compliance of these patients with dietary programs is relatively low and the identification of new therapeutic agents that could ameliorate liver steatosis is highly encouraged. Bioactive pro-resolving lipid mediators (SPMs) such as resolvins, protectins and maresins, which are derived from omega-3 polyunsaturated fatty acids (n-3 PUFAs), play an active role in the resolution of inflammation and have been pointed out as potential pharmacological agents for acute and chronic inflammatory diseases (González-Périz and Clària 2010, Serhan 2014, White *et al.* 2014). In fact, obesity and metabolic-related complications such as insulin resistance have been related to decreased production of these lipid mediators in adipose tissue and thus, restoration of endogenous levels ameliorated metabolic dysfunction (Neuhofer *et al.* 2013, Titos and Clària 2013). In addition to these anti-inflammatory properties, SPMs such as resolvin E1 (RvE1), resolvin D1 (RvD1) and protectin DX (PDX) also regulate insulin signaling, glucose homeostasis, and key metabolic regulators such as PPARs, adiponectin and AMPK in different tissues in obesity (González-Périz *et al.* 2009, Hellmann *et al.* 2011, White *et al.* 2014). Recently, a study from our group has demonstrated similar actions of Maresin 1 (MaR1), a DHA-derived SPM, in adipose tissue suggesting that MaR1 might represent a novel

therapeutic candidate for controlling inflammation and obesity-related metabolic disorders (Martínez-Fernández *et al.* 2017). Some previous studies have also pointed out to potential antisteatotic actions of RvE1 and RvD1 (González-Périz *et al.* 2009, Rius *et al.* 2014). A recent investigation has also shown that MaR1 acts in liver mitigating carbon tetrachloride-induced injury in mice (Li *et al.* 2016). However, the role of MaR1 on NAFLD remains to be clarified.

Therefore, the aim of the current study was to investigate the potential ability of MaR1 to alleviate liver steatosis in *ob/ob* and diet-induced (DIO) mice. Moreover, we also elucidated the actions of MaR1 on master regulators of autophagy and lipid metabolism pathways, including lipogenesis and fatty acid oxidation in liver of obese animals. Finally, the potential involvement of AMPK on MaR1 actions was tested in primary cultured mice hepatocytes.

MATERIALS AND METHODS

Animal models and experimental design

Genetically obese (*ob/ob*) mice

Eight-week old *ob/ob* mice obtained from Janvier Laboratories (Le Genest St. Isle, France), were housed in plastic cages under controlled conditions (22 ± 2 °C, with a 12 h light–dark cycle, relative humidity, 55 ± 10%). Animals were fed with a standard mouse pelleted chow diet (13% of kcal from fat, 67% from carbohydrates and 20% from protein) from Harlan Teklad Global Diets (Harlan Laboratories, IN, USA) for an adaptation period of 4 days. Thereafter, *ob/ob* mice were separated into three experimental groups, the control group (n=6), which received a daily intraperitoneal (i.p.) injection of vehicle (100 µl of sterile saline-0.1% ethanol), and two groups treated (i.p.) with MaR1 (Cayman, MI, USA) at 2 µg/kg (n=7) and 10 µg/kg (n=7) of body weight (BW) for 20 days, respectively. BW and food intake were registered daily (Martínez-Fernández *et al.* 2017). Animals were sacrificed after 20 days of treatment, and blood, subcutaneous and visceral white adipose tissue (WAT) and liver were collected, processed, and stored at -80 °C for subsequent analysis.

Diet-induced obese (DIO) mice

Seven-week old C57BL/6J mice were purchased from Harlan Laboratories (Barcelona, Spain) and housed in plastic cages under same controlled conditions described above. After a 7 days acclimation period, mice were fed *ad libitum* with a high-fat diet (HFD, 60% of kcal from fat, 20% from carbohydrates and 20% from protein), provided by ResearchDiets (New Brunswick, NJ, USA) for 3 months. Two different trials were then performed.

In trial 1, DIO mice were separated into two sub-groups: one sub-group (n=11) that received i.p. injection of vehicle (100 µl of sterile saline-0.1% ethanol) and other sub-group (n=11) that received daily i.p. injection of MaR1 (2 µg/kg BW) for 10 days. In trial 2, DIO mice were assigned into other two sub-groups that received a daily oral gavage of the vehicle (n=8) or MaR1 (50 µg/kg BW, n=8) for 10 days.

Then, mice were sacrificed and blood and tissues were collected, processed and kept at -80 °C for further analysis. All experimental procedures were performed under protocols approved by the University Ethics Committee for the use of laboratory animals, according to the National and Institutional Guidelines for Animal Care and Use (Protocols 029-12 and 047-15).

Biochemical analysis

Serum biochemical analyses were performed after a 12 h fasting period. Cholesterol, high-density lipoprotein cholesterol (HDL-chol), triglycerides (TG), alanine amino transferase (ALT), aspartate amino transferase (AST) and β-hydroxybutyrate serum levels were determined using a Pentra C200 autoanalyser (Roche Diagnostic, Basel, Switzerland), following manufacturer's instructions. The values of low-density lipoprotein cholesterol (LDL-chol) were calculated using the Friedewald equation defined as LDL-cholesterol = Total-cholesterol – HDL-cholesterol – TG/5, as described elsewhere (Friedewald *et al.* 1972).

Determination of liver triglycerides content

Liver pieces (100 mg) were homogenized in phosphate buffer 0.1 M pH=7, to determine lipid content. As indicated, Folch method (Folch *et al.* 1957) was used to extract lipids and TG content was determined by Infinity Triglycerides Liquid Stable

Reagent (Thermo Electron Corporation, CO, USA) following manufacturer's instructions. TG content was normalized to mg of protein.

Light microscopy analysis

Liver pieces were fixed in 10% neutral formalin (pH=7.4) for 24 h, dehydrated and embedded in paraffin. 5 µm thick sections were deparaffinized and stained with hematoxylin-eosin. Liver sections were visualized with a Nikon Eclipse E800 microscope (Nikon Instruments Europe, Amsterdam, Netherlands) and corresponding images (magnification 100x) were taken with a camera Nikon Eclipse E800 (Nikon Instruments Europe). Moreover, Fat-red stain was performed in frozen sample and images (magnification 100x) were taken with a Digital Pathology Slide Scanner Leica Aperio CS2 (Leica Biosystems, Wetzlar, Germany). Relative areas of lipid accumulation were quantified by ImageJ software (National Institute of Health, MD, USA).

Ultrastructural analysis of hepatic autophagic vacuoles

Small pieces of liver were prefixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer during 7 h at 4 °C and post-fixed in 1% osmium tetroxide during 2.5 h at 4 °C, followed by uranyl acetate for 1 h at room temperature. Ultra-thin sections were examined with a transmission electron microscope (TEM) Libra 120 (Zeiss GMgH, Oberkochen, Germany). Images (magnification 4000x) were obtained using iTEM 5.1 (Olympius Soft Imaging Solutions GmbH, Münster, Germany). Ultrastructural examination of autophagic vacuoles was carried out using standard criteria (Singh *et al.* 2009, Bejarano *et al.* 2014); autophagic vacuole was considered when they exhibited one or two membranes. The micrographs were independently examined by two people and the number of autophagic vacuoles showed was referred to each hepatocyte observed (n=3, 10 hepatocytes *per* mice) (Singh *et al.* 2009, Bejarano *et al.* 2014).

Primary culture of hepatocytes and *in vitro* treatments

C57BL/6J mice (aged 6 months) fed with a standard chow diet or with a HFD (45% of kcal from fat, 35% from carbohydrates and 20% from protein, provided by ResearchDiets) for 3 months were used. Hepatocytes were isolated by liver

collagenase perfusion via portal vein as previously described (Rodriguez-Ortigosa *et al.* 2014). Then two centrifugations with cold William's E Medium, GlutaMAX Supplement Medium (Gibco, Thermofisher, MA, USA) enriched with 10% Fetal bovine serum (FBS) were carried out. Cell viability was determined with Trypan Blue dye. Thereafter, hepatocytes were cultured in Williams Medium enriched with 10% FBS, 1% of non-essential aminoacids (Invitrogen, CA, USA) and antibiotics (1% Penicillin/Streptomycin) for two hours at 37 °C in an humidity atmosphere of 5% CO_2. Then, media was replaced with fresh William's E Medium 0.5% FBS.

Primary hepatocytes were pre-treated for 1 h with specific AMP-activated protein kinase (AMPK) inhibitor, Compound C (Sigma-Aldrich) at 20 µM. After pre-incubation time, MaR1 (1 nM) was added to the media, and incubated for additional 6-24 h. Hepatocytes were treated with the same amount of the corresponding vehicle (ethanol and/or DMSO).

Analysis of protein expression by Western blot

Total liver and primary hepatocytes lysates were obtained as previously described (López-Yoldi *et al.* 2014). Total proteins (30-50 µg) were electrophoretically separated on 10-17% SDS-PAGE. Then, proteins were electroblotted from the gel to polyvinylidene difluoride membranes (GE Healthcare Europe GmbH, Barcelona, Spain). Efficient protein transfer was monitored by Ponceau S stain. Next, membranes were blocked (1% BSA) for 1 h at room temperature and probed with specific primary antibodies against fatty acid synthase (FAS, rabbit), Acetyl-CoA Carboxylase (ACC, rabbit), phospho-ACC (p-ACC, rabbit), AMP-activated protein kinase (AMPK, rabbit), phospho-AMPK (mouse), Microtubule-associated protein 1A/1B-light chain 3 (LC3, rabbit) (Cell Signaling Technology, MA, USA), p62 (rabbit) and β-Actin (mouse) (Sigma-Aldrich), and stearoyl-CoA desaturase (SCD1, rabbit) (Abcam, Cambridge, UK) overnight at 4 °C. Thereafter, infrared fluorescent secondary antibodies anti-rabbit (Cell Signaling Technology) and anti-mouse (LI-COR Biosciences, Lincon, USA) were used and quantitated using an Odyssey® Sa infrared imaging system (LI-COR).

Analysis of mRNA expression levels

Total RNA was isolated from livers or primary hepatocytes and then reverse transcribed to cDNA as previously described (López-Yoldi *et al.* 2014). Carnitine palmitoyltransferase (*Cpt1a*), acyl-coenzyme A oxidase (*Acox1*), autophagy related 5 (*Atg5*), autophagy related 7 (*Atg7*) mRNA levels were determined using predesigned Taqman® Assays-on-Demand and Taqman Universal Master Mix (Applied Biosystems, CA, USA). Amplification and detection of specific products were analysed with the ABI PRISM 7900HT Fast Sequence Detection System (Applied Biosystems). Cyclophilin A (*Ppia*) was used as housekeeping gene. Relative expression of the specific genes was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

Statistical analyses were carried out with GraphPad Prism 5 software (Graph-Pad Software Inc., CA, USA). Data are presented as mean ± SEM. Differences between groups were set up as statistically significant at $P < 0.05$. Comparisons between the values for different variables were analysed by one-way ANOVA followed by Bonferroni *post-hoc* tests or by Student's *t*-tests or Mann-Whitney U-tests once the normality had been screened using Kolmogorov-Smirnoff and Shapiro-Wilk tests.

RESULTS

Maresin 1 alleviates liver steatosis in *ob/ob* and diet-induced obese (DIO) mice

Treatment of *ob/ob* mice with MaR1 (2-10 μg/kg, i.p.) for 20 days did not modify food intake (data not shown), body weight or WAT depots weights (Table 1). However, MaR1 administration significantly *(P* <0.05) reduced serum TG and AST levels at the highest dose tested (10 μg/kg) (Table 1), suggesting that MaR1 could ameliorate fatty liver in obesity. Indeed, MaR1 caused a concomitant reduction of TG accumulation in liver in *ob/ob* mice (Fig. 1A, B). Interestingly, MaR1 treatment decreased the levels of several hepatic lipogenic enzymes, such as FAS (*P* <0.001) and SCD1 (*P* <0.05) (Fig. 1C). In parallel, MaR1 also induced a significant (*P* <0.05) dose-dependent increase in ACC phosphorylation (Fig. 1C), clearly suggesting an inhibition of lipid synthesis in liver of MaR1-treated *ob/ob* mice.

TABLE 1. Effects of MaR1 (2 and 10 μg/kg i.p. for 20 days) on body, liver and adipose tissue weights and serum biochemistry in *ob/ob* mice.

Parameter	*ob/ob* + Vehicle (n=6)	*ob/ob* + MaR1 (2 μg/kg) (n=7)	*ob/ob* + MaR1 (10 μg/kg) (n=7)
Body weight (g)	47.32 ± 0.61	47.77 ± 0.68	46.71 ± 0.91
Visceral fat weight (g)	5.93 ± 0.13	6.51 ± 0.40	5.52 ± 0.19
Subcutaneous fat weight (g)	2.99 ± 0.23	3.28 ± 0.22	2.81 ± 0.19
Liver weight (g)	1.96 ± 0.04	1.99 ± 0.11	1.98 ± 0.07
Cholesterol (mg/dL)	153.00 ± 11.58	162.60 ± 10.76	149.10 ± 10.50
HDL-Chol (mg/dL)	68.41 ± 4.22	70.19 ± 3.07	66.76 ± 3.76
LDL-Chol (mg/dL)	61.12 ± 7.21	68.15 ± 6.96	61.69 ± 6.77
Triglycerides (mg/dL)	117.30 ± 5.63	121.10 ± 7.16	103.40 ± 4.36*
ALT (U/L)	278.00 ± 23.33	298.50 ± 40.07	283.10 ± 26.15
AST (U/L)	471.80 ± 88.48	413.70 ± 40.63	367.00 ± 16.11*
β-Hydroxybutyrate (mmol/L)	3.88 ± 0.41	4.29 ± 0.72	2.68 ± 0.47

Data are means ± SEM. High-density lipoprotein cholesterol (HDL-Chol), Low-density lipoprotein cholesterol (LDL-Chol), Alanine amino transferase (ALT), Aspartate amino transferase (AST); * P <0.05 *vs. ob/ob* (vehicle-treated) group.

Moreover, liver of MaR1-treated mice also exhibited a significant reduction (P <0.05) of p62 protein levels (Fig. 1D), while LC3II protein expression was significantly (P <0.05) up-regulated after MaR1 (10 μg/kg) administration (Fig. 1D). These suggest that MaR1 might also prevent lipid accumulation in liver through up-regulation of autophagy.

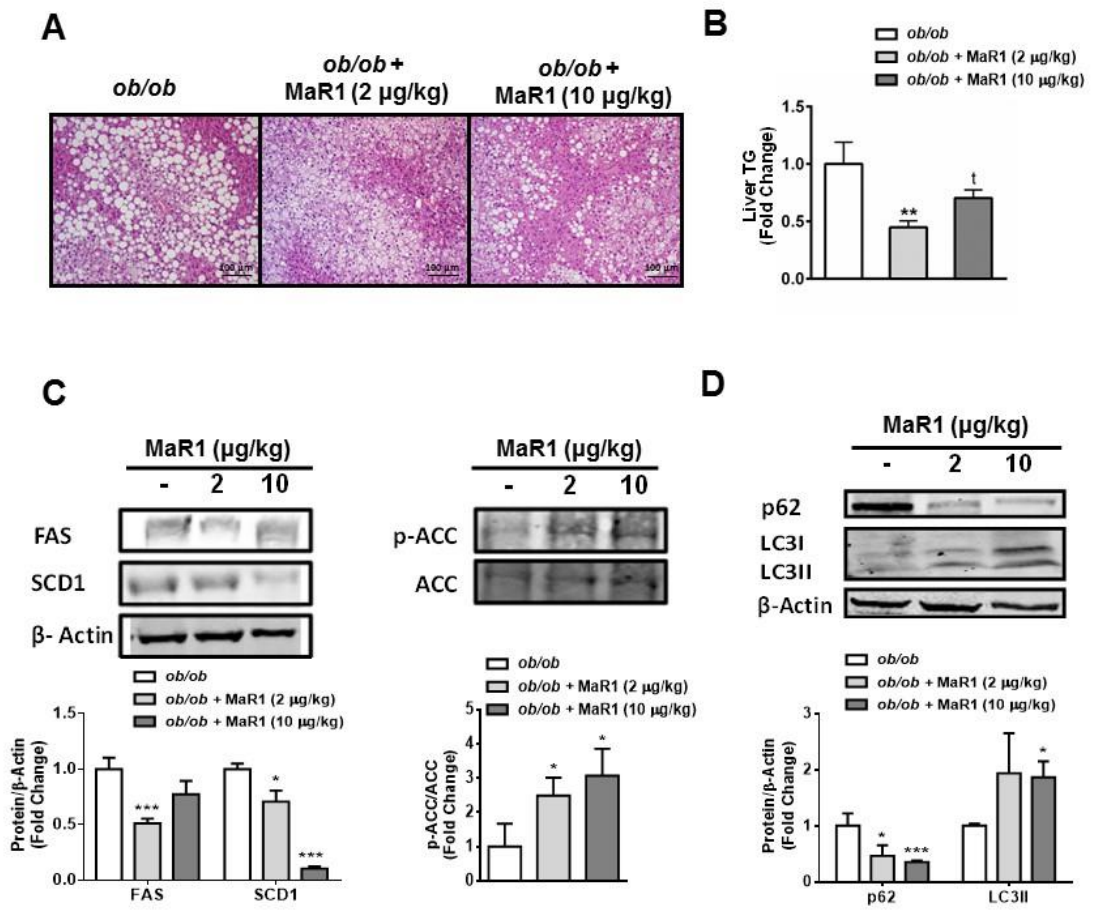


Fig 1. MaR1 ameliorates liver steatosis in *ob/ob* mice. Eight weeks-old *ob/ob* mice were treated with MaR1 (2-10 µg/kg) or vehicle by intraperitoneal injection for 20 days. (A) Representative photomicrographs (magnification 100x) of liver sections stained with hematoxylin-eosin. (B) Liver triglyceride (TG) content normalized to total protein, and expressed as fold change *vs.* vehicle-treated *ob/ob* mice. (C) Representative Western Blot (top panel) and densitometry analysis (bottom panel) of hepatic lipogenic enzymes, fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1) and Acetyl-CoA Carboxylase (ACC), phosphorylated and total; band densities of FAS and SCD1 were normalized to β-actin, while phosphorylated ACC was normalized by total ACC. (D) Representative Western Blot (top panel) and densitometry analysis (bottom panel) of p62 and microtubule-associated protein 1A/1B-light chain 3 (LC3 I and II). Band densities of p62 and LC3II were normalized to β-actin. Data are expressed as mean ± SEM (n = 6-7). **P* < 0.05, ***P* < 0.01, ****P* < 0.01, ^{t}P =0.06 *vs.* vehicle-treated *ob/ob* mice.

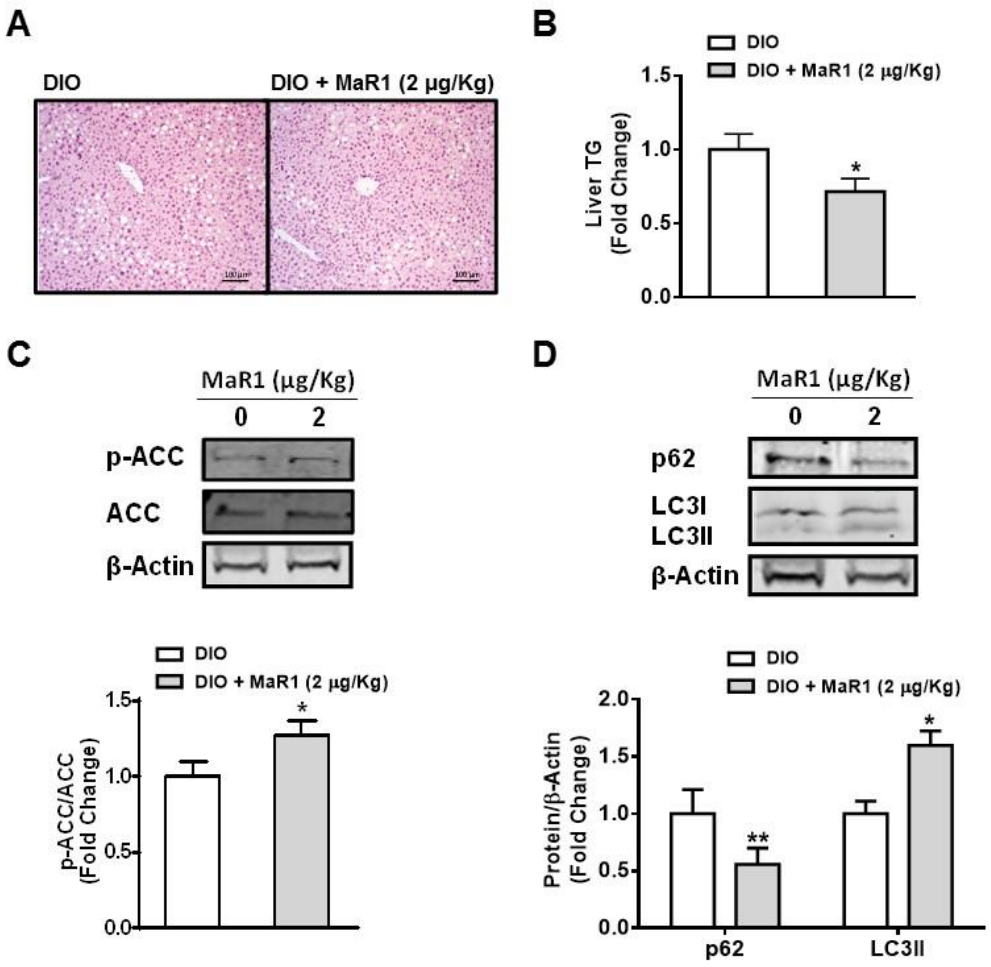
Considering the interesting effects of MaR1 in *ob/ob* mice and pre-existing physiological limitations due to absence of leptin in *ob/ob* mice, we further evaluated whether MaR1 effects could be also observed in a high-fat diet-induced obesity mice model. Thus, MaR1 (2 µg/kg) was administered by i.p. injection to DIO mice for 10 days, after 3 months on a HFD. Remarkably, in DIO mice MaR1 also decreased (*P* <0.05) serum TG and ALT levels (Supplementary Table 1) and induced a strong

reduction (*P* <0.05) of total liver TG content (Supplemental Fig. 1A, B). Similar outcomes on ACC phosphorylation, p62 and LC3II protein expression were also observed after i.p. injection of MaR1 to DIO mice, as compared to *ob/ob* mice (Supplemental Fig. 1C, D), supporting the effectiveness of MaR1 to alleviate HFD-induced fatty liver disease.

SUPPLEMENTAL TABLE 1. Effects of intraperitoneal administration of MaR1 (2 μg/kg, 10 days) on body, liver and adipose tissue weights and serum biochemistry in DIO mice.

Parameter	DIO + Vehicle (n=11)	DIO + MaR1 (2 μg/kg) (n=11)
Body weight (g)	46.80 ± 0.70	45.80 ± 0.70
Visceral fat weight (g)	2.37 ± 0.20	2.54 ± 0.16
Subcutaneous fat weight (g)	2.41 ± 0.10	1.97 ± 0.07 **
Liver weight (g)	1.52 ± 0.07	1.36 ± 0.11
Cholesterol (mg/dL)	198.60 ± 4.40	185.20 ± 6.50
HDL-Chol (mg/dL)	78.00 ± 2.70	76.00 ± 2.50
LDL-Chol (mg/dL)	104.70 ± 5.75	90.80 ± 5.63
Triglycerides (mg/dL)	109.60 ± 4.70	97.30 ± 5.10 *
ALT (U/L)	196.70 ± 23.40	124.20 ± 20.90 *
AST (U/L)	281.80 ± 26.80	252.90 ± 18.60
β-Hydroxybutyrate (mmol/L)	1.20 ± 0.30	1.40 ± 0.20

Data are means ± SEM. High-density lipoprotein cholesterol (HDL-Chol), Low-density lipoprotein cholesterol (LDL-Chol), Alanine amino transferase (ALT), Aspartate amino transferase (AST); **P* <0.05; ***P* <0.01 *vs.* DIO (vehicle-treated) group.



Supplemental Fig 1. Intraperitoneal injection of MaR1 reduces diet-induced liver steatosis. Twenty weeks-old DIO mice were treated with MaR1 (2 µg/kg) or vehicle by intraperitoneal injection for 10 days. (A) Representative photomicrographs (magnification 100x) of liver sections stained with hematoxylin-eosin. (B) Liver triglyceride (TG) content expressed as fold change *vs*. vehicle-treated DIO mice. (C) Representative Western Blot (top panel) and densitometry analysis (bottom panel) of Acetyl-CoA Carboxylase, ACC phosphorylated and total. (D) Representative Western Blot (top panel) and densitometry analysis (bottom panel) of p62 and microtubule-associated protein 1A/1B-light chain 3 (LC3 I and II). Band densities of p62 and LC3II proteins were normalized to β-actin, while phosphorylated ACC was normalized by total ACC. Data are expressed as mean ± SEM (n = 8). **P* < 0.05; ***P* < 0.01 *vs.* vehicle-treated DIO mice.

Due to the therapeutic potential of MaR1 in this pathology, we tested whether the beneficial effects of this SPM on liver steatosis could be also observed after oral administration, which represents an easier route for drug administration in humans. Importantly, our data unravel that MaR1 (50 µg/kg, oral gavage, 10 days) induced a significant (*P* <0.05) reduction in serum AST (~ 40%, *P* <0.05) and ALT (~ 50%, *P* <0.01) levels (Table 2).

TABLE 2. Effects of oral gavage of MaR1 (50 μg/kg, 10 days) on body, liver and adipose tissue weights and serum biochemistry in DIO mice.

Parameter	DIO + Vehicle (n=8)	DIO + MaR1 (50 μg/kg) (n=8)
Body weight (g)	41.13 ± 0.80	39.28 ± 0.70
Visceral fat weight (g)	3.44 ± 0.27	3.44 ± 0.18
Subcutaneous fat weight (g)	1.75 ± 0.18	1.71 ± 0.18
Liver weight (g)	1.44 ± 0.07	1.23 ± 0.07 *
Cholesterol (mg/dL)	225.50 ± 19.29	219.50 ± 13.51
HDL-Chol (mg/dL)	59.66 ± 11.60	66.90 ± 6.72
LDL-Chol (mg/dL)	127.00 ± 19.59	114.70 ± 13.66
Triglycerides (mg/dL)	194.00 ± 29.20	189.50 ± 21.38
ALT (U/L)	411.30 ± 58.33	193.70 ± 27.54 **
AST (U/L)	967.10 ± 143.70	596.60 ± 73.84 *
β-Hydroxybutyrate (mmol/L)	5.63 ± 2.24	3.52 ± 1.77

Data are means ± SEM. High-density lipoprotein cholesterol (HDL-Chol), Low-density lipoprotein cholesterol (LDL-Chol), Alanine amino transferase (ALT), Aspartate amino transferase (AST); * P <0.05; ** P <0.01 *vs.* DIO (vehicle-treated) group.

Moreover, MaR1 caused a decrease on total liver weight (P <0.05) in parallel with a marked reduction (P <0.05) of total liver fat infiltration (Fig. 2A and Supplemental Fig. 2) and TG content (Fig. 2B), which unequivocally support the effectiveness of oral gavage of MaR1 to counteract obesity-associated NAFLD. This was further reinforced by the observation that the administration of MaR1 through this route was also able to reduce FAS protein expression (P <0.05) and induce ACC phosphorylation (P <0.05) (Fig. 2C). Additionally, oral gavage of MaR1 also induced a significant up-regulation (P <0.05) of fatty acid oxidation genes *Cpt1a* and *Acox1* (Fig. 2D). Furthermore, similarly to what was observed after i.p. administration, several outcomes support that gavage of MaR1 also seems to promote autophagy in liver: 1) the reduction of p62 protein (P <0.05) (Fig. 2E), probably as a consequence of degradation in autophagosomes, and not subsequent to a decreased transcription, since *p62/SQSTM1* gene expression remained unchanged (data not shown); 2) the increased expression of LC3II protein (Fig. 2E); 3) the up-regulation (P <0.05) of key autophagy-related (*Atg*) genes *Atg5* and *Atg7* (Fig. 2F); 4) the higher number of autophagic vacuoles observed in hepatocytes of

MaR1-treated DIO mice (Fig. 2G). Because AMPK is able to modulate fatty acid oxidation and autophagy, the effect of MaR1 on AMPK (total and phosphorylated) was tested. Our data revealed that MaR1 induced (P <0.05) AMPK phosphorylation in liver of DIO mice (Fig. 2H).

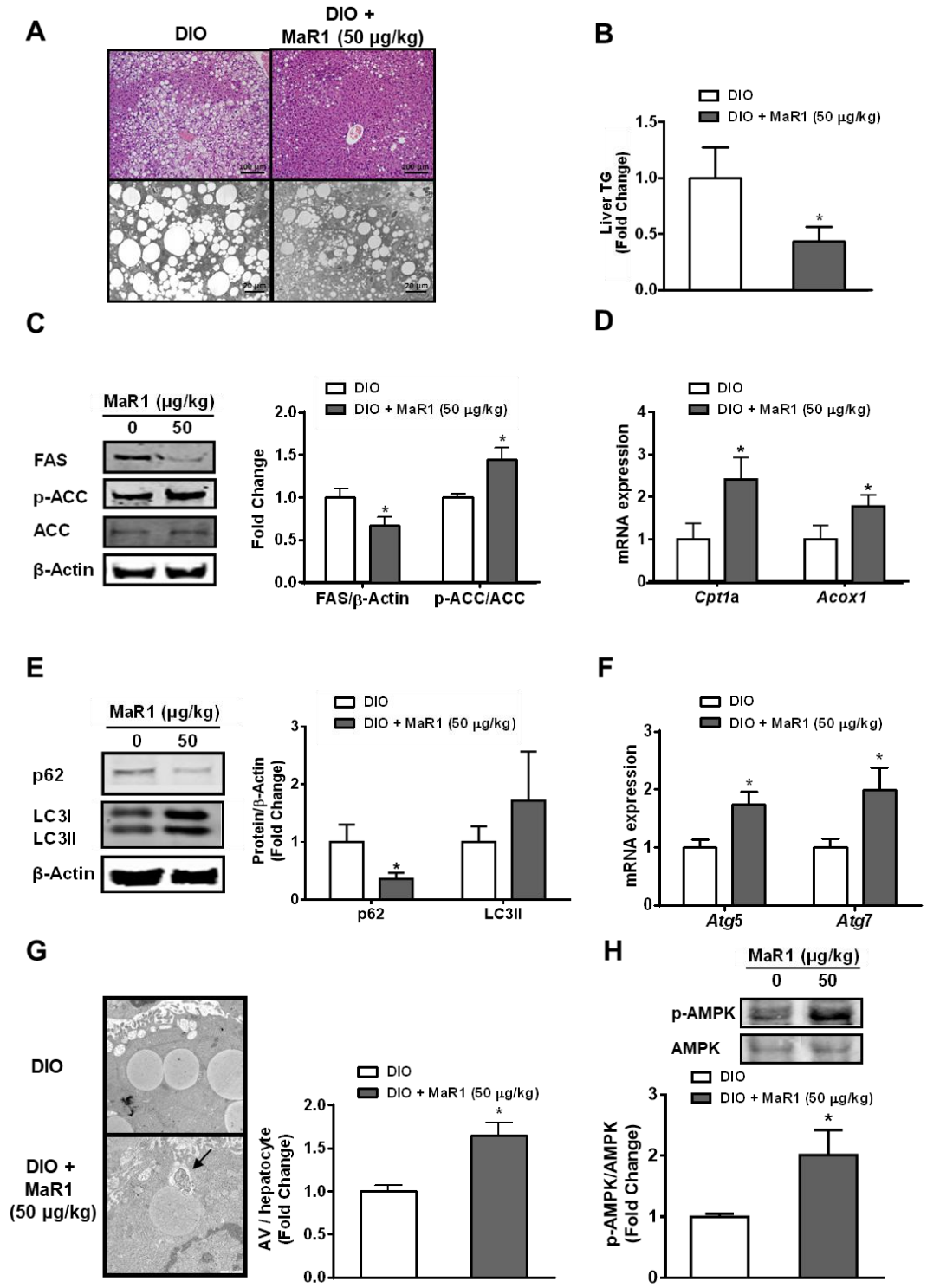
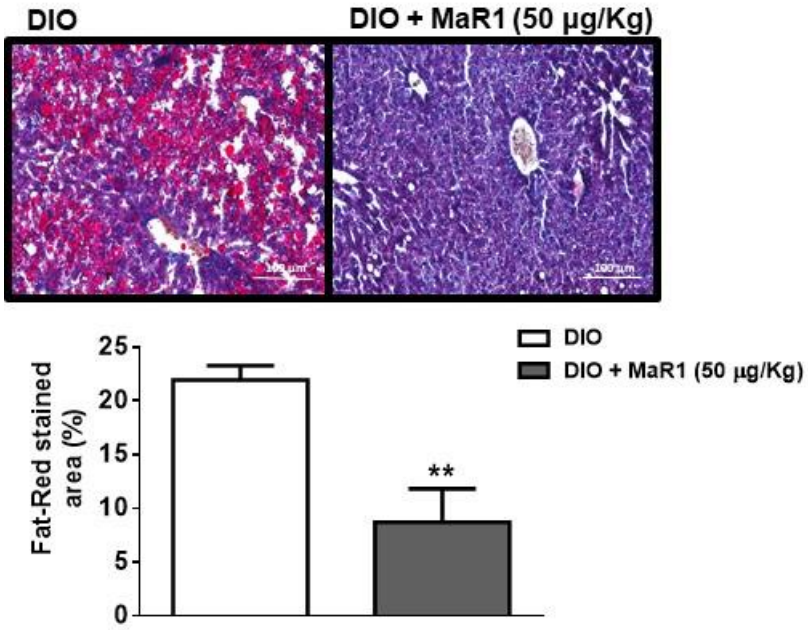


Fig 2. Administration of MaR1 by oral gavage reduces diet-induced liver steatosis in DIO mice. Twenty weeks-old DIO mice were treated with MaR1 (50 µg/kg) or vehicle by oral gavage for 10 days. (A) Representative photomicrographs (magnification 100x/63x) of liver sections stained with hematoxylin-eosin dye. (B) Liver triglycerides (TG) content was normalized to total protein, and expressed as fold change versus vehicle-treated DIO mice. (C) Representative Western Blot (left panel) and densitometry analysis (right panel) of hepatic lipogenic enzymes fatty acid synthase (FAS) and Acetyl-CoA Carboxylase (ACC) phosphorylated and total; band densities of FAS were normalized to β-actin, while phosphorylated ACC was normalized by total ACC. (D) mRNA expression levels of fatty acid oxidation enzymes, carnitine palmitoyltransferase-1A (*Cpt1a*) and acyl-CoA oxidase (*Acox1*). Data are expressed as fold change relative to vehicle-treated DIO mice considered as 1. (E) Representative Western Blot (left panel) and densitometry analysis (right panel) of p62 and microtubule-associated protein 1A/1B-light chain 3 (LC3 I and II). Band densities of p62 and LC3II were normalized to β-actin. (F) Gene expression levels of autophagy-related 5 and 7 (*Atg5-7*). Data are expressed as mean ± SEM (n = 4-8). (G) Ultrastructural study of autophagic vacuoles (AV) in liver tissue of MaR1-treated mice. Representative electron micrographs (magnification 4000x) of hepatocyte cytoplasm AV free in vehicle-treated mice (top-left panel) and showing AV (black arrow) in MaR1-treated mice (bottom-left panel). Corresponding quantification of AV in DIO and DIO + MaR1 (right panel) (n=3). Values = number of AV *per* hepatocyte calculated from 10 hepatocytes *per* liver. (H) Representative Western Blot of AMP-activated protein kinase (AMPK) phosphorylated and total (top panel) and densitometry analysis (bottom panel); band densities of phosphorylated AMPK were normalized by total AMPK. **P* < 0.05 *vs.* vehicle-treated DIO mice.



Supplemental Fig 2. Administration of MaR1 (50 µg/kg, 10 days) by oral gavage reduces liver fat accumulation in DIO mice. Representative photomicrographs (top panel) (magnification 100x) of liver sections stained with Fat-Red dye. Fat-Red stained area is expressed as percentage of total area (bottom panel). Data are expressed as mean ± SEM (n = 8). ***P* < 0.01 *vs.* DIO mice.

MaR1 regulates fatty acid oxidation and autophagy-related genes through AMPK in primary cultured hepatocytes

Interestingly, incubation of primary cultured hepatocytes with MaR1 (1 nM) also promoted AMPK phosphorylation (*P* <0.05), which was prevented (*P* <0.05) by the addition of the specific AMPK inhibitor, Compound C (Fig. 3A). More importantly, preincubation with Compound C completely (*P* <0.05) abrogated MaR1 up-regulation on fatty acid oxidation genes *Cpt1a* (*P* <0.05) and *Acox1* (*P* <0.01), and autophagy-related genes *Atg5* (*P* <0.05) and *Atg7* (*P* <0.05) (Fig. 3B, C). These data suggest that AMPK is involved in the regulation of MaR1 actions on fatty acid oxidation and autophagy pathways in primary hepatocytes.

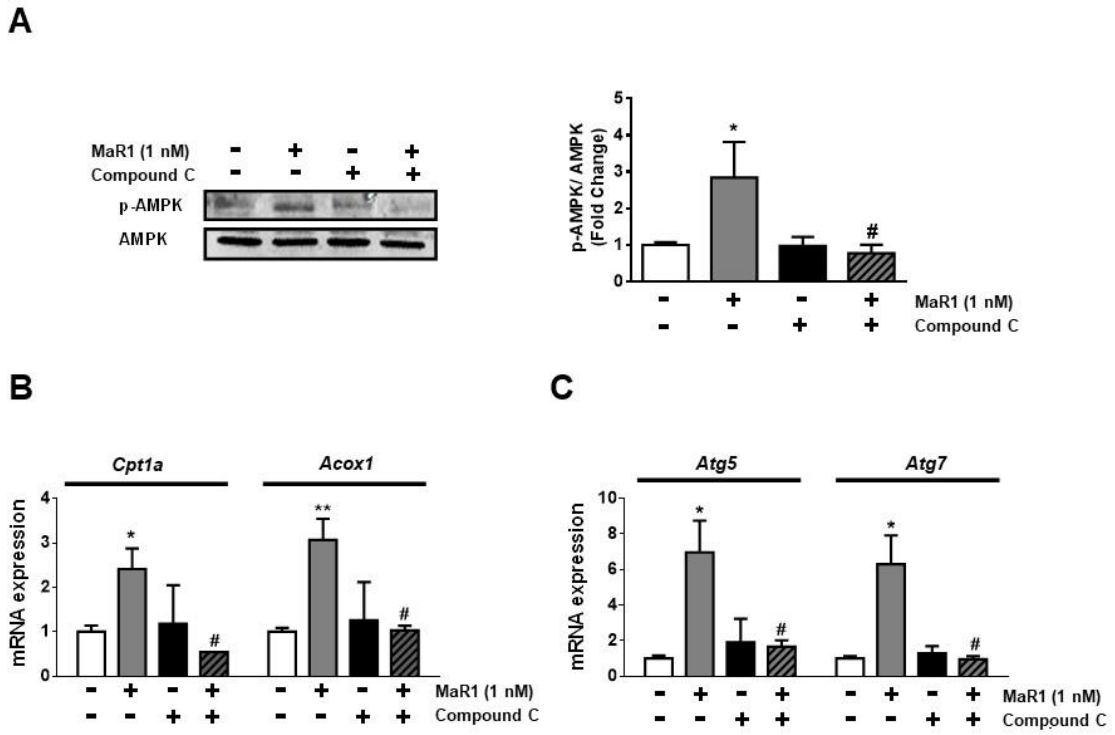


Fig 3. MaR1 up-regulates the expression of fatty acid oxidation and autophagy-related genes through AMPK in primary cultured hepatocytes. Hepatocytes from control and high-fat diet (HFD) fed mice (3 months of HFD, 45% kcal from fat) were pre-treated for 1 h in the presence or absence of the AMP-activated protein kinase (AMPK) inhibitor, Compound C (20 μM) and then with MaR1 (1 nM) for 6 h. (A) Representative Western Blot (left panel) and densitometry analysis (right panel) of AMPK phosphorylated and total. Band densities of phosphorylated AMPK were normalized by total AMPK. (B) Gene expression levels of carnitine palmitoyltransferase-1A (*Cpt1a*) and acyl-CoA oxidase (*Acox1*). (C) Gene expression levels of autophagy-related 5 (*Atg5*) and 7 (*Atg7*). Data (mean ± SEM) are expressed as relative fold change of vehicle-treated hepatocytes. Results are representative of at least, three independent experiments. **P* <0.05; ***P* <0.01 *vs.* vehicle-treated hepatocytes; $^{\#}P$ <0.05 *vs.* MaR1-treated hepatocytes.

DISCUSSION

Our results demonstrate for the first time the ability of MaR1, a SPM derived from DHA, to attenuate liver steatosis in obesity. This fact is supported on the consistent findings of reduced transaminases levels and liver TG content induced by MaR1 in obese mice. Indeed, the anti-steatosis actions of MaR1 on liver seem to rely on its capacity to: 1) decrease protein expression of key enzymes involved in hepatic *de novo* lipogenesis; 2) up-regulate expression of genes-related to fatty acid oxidation; 3) increase autophagy markers.

A decrease on hepatic TG content has also been described for other n-3 PUFAs-derived lipid mediators alone (RvE1), accompanied with lower ALT serum levels (González-Périz *et al.* 2009) and/or in combination with caloric restriction (RvD1) (Rius *et al.* 2014). Similarly, Lipoxin A4, the anti-inflammatory SPM derived from arachidonic acid, also reduced ALT serum levels along with liver weight and fat content (Borgeson *et al.* 2015).

Our current data suggest that the antisteatotic effect of MaR1 is in part mediated by the reduction of content or activation of key lipogenic enzymes such as FAS and ACC. In this context, different dietary treatments with n-3 PUFAs (fish oil or with pure EPA or DHA) have shown to be effective in reducing hepatic FAS expression in NAFLD in obese rodents (González-Périz *et al.* 2009, Pérez-Echarri *et al.* 2009, Bargut *et al.* 2014, de Castro *et al.* 2015a and 2015b). However, it should be pointed out that MaR1 displays similar effects at noteworthy lower doses as compared to its n-3 PUFAs precursors. SCD1 content was also decreased in *ob/ob*, but not in DIO mice treated with MaR1, which could also contribute to the reduced liver fat accumulation observed in these animals (Dobrzyn *et al.* 2004). This differential outcome regarding SCD1 between *ob/ob* and DIO mice might be due to the leptin deficiency of *ob/ob* mice. However, other differences such as treatment length and administration route should not be ruled out. In both models, MaR1 treatment clearly promoted the phosphorylation of ACC, which causes enzyme inactivation. In fact, inhibition of ACC prevents the conversion of acetyl-CoA to malonyl-CoA, a strong inhibitor of CPT1A. Therefore, the inactivation of ACC will reduce the inhibition on CPT1A leading to mitochondrial fatty acid oxidation (Ceddia 2013). Interestingly, it has been probed that an overexpression of *Cpt1a* induces fatty acid oxidation and reduces liver steatosis in

obese rodents (Orellana-Gavalda *et al.* 2011, Monsenego *et al.* 2012). In this line, the finding that MaR1 up-regulated not only *Cpt1a* but also *Acox1* mRNA suggest that MaR1 antisteatotic properties could be also related to increased mitochondrial and peroxisomal fatty acid oxidation. Therefore, these data suggest that the previously described ability of n-3 PUFAs (DHA and EPA) to promote hepatic *Cpt1a* gene expression and decrease fat accumulation in liver (de Castro *et al.* 2015) could be also mediated through the production of n-3 PUFAs-derived SPMs.

Defective autophagy seems to play an important role in fatty liver disease (Singh and Cuervo 2012). In fact, in both genetic and dietary-induced obese insulin resistance mice, fatty liver is accompanied by a robust decrease on hepatic autophagy makers (Liu *et al.* 2009, Codogno and Meijer 2010, Yang *et al.* 2010), and impaired autophagic flux occurs in patients with NAFLD (González-Rodriguez *et al.* 2014). A previous study has shown that n-3 PUFAs (EPA and DHA) induced hepatic autophagy by increasing LC3II levels in cultured hepatocytes (Chen *et al.* 2015). Importantly, our data strongly suggest that *in vivo* administration of MaR1 promotes autophagy since increased autophagy markers (LC3II protein levels and *Atg5-7* gene expression), accompanied with decreased p62 levels were found. Therefore, considering the higher number of autophagic vacuoles observed in hepatocytes of MaR1-treated DIO mice, it could be suggested an induction of lipophagy, which might also contribute to the reduction of fat accumulation in liver. In this line, other n-3 PUFAs derivatives mediators, such as n-3 PUFAs epoxides are also capable to modulate liver autophagy, by reducing p62 protein expression and also increasing LC3II (López-Vicario *et al.* 2015).

AMPK is a key energy sensor in main metabolic tissues. In liver, AMPK activation modulates metabolism by switching on catabolic pathways, such as autophagy (Alers *et al.* 2012) and fatty acid oxidation (Fernández-Galilea *et al.* 2014), and switching off anabolic pathways such as lipid synthesis (Zhou *et al.* 2001). Consequently, the increased phosphorylation levels of AMPK by MaR1 and the subsequent inhibition of MaR1 effects on fatty acid oxidation (*Acox1* and *Cpt1a*) and autophagy related genes (*Atg5-7*) by concomitant treatment with Compound C, a well-known AMPK inhibitor (Zhou *et al.* 2001), strongly suggest that MaR1 effects on these genes are directly mediated through AMPK activation. Indeed, it has been demonstrated that AMPK activation is also essential for n-3 PUFAs ability to decrease liver steatosis (Jelenik *et al.*

2010). It has also been recently reported that Protectin DX, other DHA derivate, induces AMPK phosphorylation in hepatocytes, reversing HFD-induced deleterious effects in liver (Jung *et al.* 2017).

In conclusion, our study indicates that MaR1 treatment ameliorates liver steatosis by decreasing TG accumulation and *de novo* lipogenesis enzymes, inducing fatty acid oxidation genes and regulating autophagy. These beneficial effects seem to be in part mediated through AMPK activation. Importantly, our data demonstrate the effectiveness of oral administration of MaR1, a non-invasive often safer route, which could be of relevance for its potential therapeutic use in obesity-related fatty liver disease.

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Conflict of interests

No potential conflict of interests.

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CHAPTER 4

Maresin 1 induces brown adipose tissue activation and promotes browning of white adipose tissue in diet-induced obese mice

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ABSTRACT

Maresin1 (MaR1) is a specialized proresolving lipid mediator derived from DHA with insulin-sensitizing properties in obese mice. The aim of the present study was to analyse if MaR1 actions are related to promotion of brown adipose tissue (BAT) activation and the browning of white adipose tissue. Here, we report that oral gavage of MaR1 (50 μg/kg, 10 days) to diet-induced obese (DIO) mice increased BAT UCP1 levels and upregulated other thermogenic-related genes such as *Pgc1α, Prdm16* and *Dio2,* in parallel with an increase in the mRNA levels of glucose transporters and fatty acid oxidation-related genes. Indeed, in cultured brown adipocytes MaR1 promoted glucose uptake and fatty acid utilization along with the upregulation of thermogenic genes and oxygen consumption rate. Interestingly, microPET studies with ^{18}F-FDG revealed that acute treatment with MaR1 potentiates cold-induced BAT activation in mice. Moreover, MaR1 induced beige adipocyte markers (*Ucp1*, *Pgc1α*, *Tmem26* and *Tbx1*) in subcutaneous WAT of DIO mice as well as in human mesenchymal cells (hMSC)-derived adipocytes treated along the differentiation process. Additionally, MaR1 promoted fatty acid oxidation in mature hMSC adipocytes. These data reveal MaR1 as a novel agent able to promote BAT activation and subcutaneous WAT browning, which could contribute to MaR1 insulin-sensitizing properties in obesity.

Keywords: Maresin, brown adipose tissue, browning, obesity.

INTRODUCTION

Obesity and its comorbidities such as insulin resistance and type 2 diabetes have reached epidemic proportions (WHO 2011). Indeed, diabesity not only represent a burden for patient health, but also causes important medical costs (Li *et al.* 2013). Obesity is characterized by an excess of fat accumulation derived from a positive misbalance between energy intake and energy expenditure. White adipose tissue (WAT) function as an energy storage organ and WAT-derived factors (fatty acids and adipokines) play crucial roles in the development of obesity and systemic insulin resistance, key events in the pathophysiology of the metabolic syndrome (Choe *et al.* 2016). Indeed, dysfunctional WAT metabolism and secretory function in obesity leads to the development of metabolic alterations (Bluher 2013). In this line, a chronic low-grade inflammatory state occurs in obese WAT, which also takes an important role in the development of insulin resistance (Kloting and Bluher 2014). In contrast to WAT, brown adipose tissue (BAT) is a thermogenic tissue which main function is energy dissipation through heat production, and therefore an important contributor to energy balance (Cannon and Nedergaard 2004, Peirce *et al.* 2014). BAT activation has been brought to attention, since it provides a protective mechanism against excessive body weight and fat mass accumulation (Yoneshiro *et al.* 2013). Recent investigations have suggested emerging roles of BAT activation in the regulation of metabolism, including control of triglyceride clearance (Bartelt *et al.* 2011), glucose homeostasis and insulin sensitivity (Chondronikola *et al.* 2014, Townsend and Tseng 2014), and BAT has been identified as a secretory organ (Villarroya *et al.* 2017). Therefore, therapeutic strategies to increase BAT activity and subsequently, fat-burning and improvements on insulin sensitivity could be promising to counteract obesity and type 2 diabetes (Betz and Enerback 2015). Beige/brite adipocytes have similar morphological and functional features of brown adipocytes, but co-locate within WAT depots (Wu *et al.* 2012). Since beige adipocytes can also dissipate energy as heat, white-to-beige conversion has also been pointed out as a potential therapy against obesity and insulin resistance (Jeremic *et al.* 2017). BAT activation and adipose tissue browning occurs in response to certain stimuli such as β_3-adrenergic stimulation and chronic cold exposure (Villarroya and Vidal-Puig 2013). However, their important side effects and uncomfortableness

respectively, limit their therapeutic applications. Consequently, the identification of new therapeutic agents capable to activate BAT and promote browning of WAT to ameliorate obesity-associated type 2 diabetes and insulin resistance is highly encouraged.

Omega-3 polyunsaturated fatty acids (n-3 PUFAs), mainly those of marine origin such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been deeply studied due to their anti-inflammatory actions in different metabolic disturbances (Lorente-Cebrián *et al.* 2013 and 2015). Accordingly, there is wide evidence that n-3 PUFAs administration can regulate WAT inflammation and both glucose and lipid metabolism pathways, *in vitro* and *in vivo* (Martínez-Fernández *et al.* 2015). Recently, it has been described that EPA promotes BAT activation (Quesada-López *et al.* 2016) and induces browning in human (Laiglesia *et al.* 2016) and mice (Zhao and Chen 2014) adipocytes. N-3 PUFAs-derived specialized proresolving lipid mediators (SPMs), including resolvins (Rv), protectins (PD) and maresins, actively participate in the resolution of inflammation and have much more potent anti-inflammatory actions that their n-3 PUFAs precursors (Spite *et al.* 2014). Interestingly, the production of these SPMs is reduced in obese white adipose tissue, and supplementation with n-3 PUFAs can restore SPMs endogenous levels improving both inflammation and obesity-linked metabolic dysfunction (Clària *et al.* 2013, Neuhofer *et al.* 2013). Several recent studies have evidenced the therapeutic potential of some of these SPMs, such as PDX, PD1, RvD1 and RvE1 to improve insulin sensitivity acting at doses of several orders of magnitude lower than those required for n-3 PUFAs (González-Périz *et al.* 2009, Hellmann *et al.* 2011, White *et al.* 2014). MaR1 is a DHA-derived SPM that was first identified in macrophages and more recently in several tissues (Serhan *et al.* 2009). It has been shown that M2 macrophages produce more MaR1 than M1 macrophages. Moreover, MaR1 and its precursor promote the shift of macrophage phenotype toward an M2 profile (Dalli *et al.* 2013). Recently, we have reported that MaR1 reduced WAT inflammation by regulating macrophage infiltration and phenotype along with improvements of insulin sensitivity in animal models of obesity (Martínez-Fernández *et al.* 2017). It has been described that immune-adipose interactions could be key regulators to increase fat thermogenesis (Qiu *et al.* 2014).

Based on these previous findings, we hypothesized that the insulin-sensitizing effects of MaR1 could also rely on the potential induction of BAT activity and WAT browning.

Therefore, the aim of the present study was to analyse the ability of MaR1 to promote BAT activation and browning of inguinal WAT (iWAT) in diet-induced obese (DIO) mice. Moreover, MaR1 effects on these processes were also tested in murine brown adipocytes and in human mesenchymal stem cells (hMSC)-derived adipocytes.

RESEARCH DESIGN AND METHODS

Animals and experimental design

Seven-week old C57BL/6J mice were obtained from Harlan Laboratories (Barcelona, Spain), and housed in plastic cages (three-four animals *per* cage) under controlled conditions (22 ± 2 °C, with a 12 h light–dark cycle, relative humidity, 55 ± 10%). Animals were fed with a standard mouse pelleted chow diet (13% of kcal from fat, 67% from carbohydrates and 20% from protein) from Harlan Teklad Global Diets (Harlan Laboratories, IN, USA) for an adaptation period of 7 days. Then, one group (control, n=7) was fed *ad libitum* with standard mouse chow diet, and a second group (DIO, n=16) with a high-fat diet (60% of kcal from fat, 20% from carbohydrates and 20% from protein) provided by ResearchDiets (New Brunswick, USA) for three months. Thereafter, the control group received daily oral gavage of the vehicle (100 µl of sterile saline-0.1% ethanol) for 10 days. Additionally, DIO mice were assigned into two sub-groups that received a daily oral gavage of the vehicle (n=8) or MaR1 (50 µg/kg body weight, n=8, Cayman, Ann Arbor, Michigan, USA) for 10 days.

Then, mice were sacrificed and blood was extracted. Brown and white subcutaneous inguinal and visceral adipose tissue depots were also immediately harvested, weighed and snap-frozen in liquid nitrogen, and then kept at -80 °C for further determinations. All experimental procedures were performed under protocols approved by the University Ethics Committee for the use of laboratory animals, according to the National and Institutional Guidelines for Animal Care and Use (Protocol number: 047-15).

Biochemical analyses and insulin tolerance test (ITT)

Plasma insulin was measured using commercially available ELISA kit (Mercodia, Uppsala, Sweden). Fasting and postprandial glucose levels were determined by a standard glucometer (Accu-Check Advantage blood glucose meter, Roche, Manheim, Germany). ITT was performed one day prior sacrifice. After 8 h of fasting, baseline glucose levels were determined with a glucometer, and then mice were intraperitoneally injected with 0.375 mU/g BW human insulin (NovoRapid, Novo Nordisk A/S, Bagsvaerd, Denmark). Blood glucose levels were measured at 30, 60, 120 and 180 min after insulin injection.

Histological analysis of brown adipose tissue

Brown adipose tissue pieces were fixed in 10% neutral formalin (pH 7.4) for 24 h, dehydrated, and embedded in paraffin. Five µm thick sections were deparaffinized and stained with hematoxylin-eosin (H-E). Adipose tissue sections were visualized with Nikon Eclipse E800 microscope (Nikon Instruments Europe, Amsterdam, Netherlands) and representative images (magnification 100x) were taken with the camera Nikon Eclipse E800 (Nikon Instruments Europe).

***In vivo* study of BAT activation by positron emission tomography (PET)**

BAT activation after MaR1 acute treatment was studied *in vivo* by PET with the radiotracer ^{18}F-fluorodeoxyglucose (^{18}F-FDG). MaR1 (50 µg/kg) was intraperitoneally injected to male C57BL/6J mice (n=5) and control animals (n=5) received the same amount of saline. Thirty minutes after MaR1 or saline administration, BAT was stimulated by cold exposure during 1 hour at 4 °C (Wu *et al.* 2011). After cold exposure, ^{18}F-FDG (10.1±0.9 MBq) was injected through the tail vein. PET static images were acquired 1, 2 and 4 h post ^{18}F-FDG injection in a small animal PET scanner (Mosaic, Philips). For each study, animals were anesthetized with 2% isoflurane in 100% O_2 gas.

All studies were exported and analysed using PMOD software (PMOD Technologies Ltd., Adliswil, Switzerland). For semiquantitative analysis, ^{18}F-FDG uptake by BAT was evaluated drawing volume-of-interest (VOIs) on coronal PET images including the interscapular BAT. From each VOI, maximum standardized uptake value

(SUVmax) was calculated using the formula SUV = [tissue activity concentration (Bq/cm^3)/injected dose (Bq)] × body weight (g).

Brown adipocytes culture and treatments

Primary brown adipocytes obtained from the interscapular BAT of neonatal mice and immortalized as previously described (Ortega-Molina *et al.* 2012), and were kindly provided by Dr. Valverde. Brown pre-adipocytes were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 20 nM insulin and 1 nM Triiodothyronine (T3) until 90% of confluence at 37 °C in a humidity atmosphere of 5% CO_2. Then, cells were stimulated with a differentiation medium (10% FBS, 20 nM insulin, 1 nM T3, 0.5 µM dexamethasone, 0.125 µM indomethacin, 0.5 mM isobutylmethylxanthine (IBMX) and 1 µM rosiglitazone) for 36 h. Afterwards, cells were cultured with DMEM (10% FBS, 20 nM insulin and 1 nM T3) until day 6 of differentiation, when cells reached typical features of mature brown adipocytes (Boutant *et al.* 2015). For treatments, brown mature adipocytes were treated with MaR1 (0.1 and 1nM) for 24 h. Control cells were treated with the same amount of vehicle (0.1 % ethanol) under same conditions.

Human mesenchymal stem cells (hMSC) culture and treatments

hMSC obtained from subcutaneous abdominal adipose tissue of subjects undergoing elective laparoscopic surgery were provided by Dr. Arbones-Mainar (Torres-Pérez *et al.* 2015). All donors signed the written consent, and the study was approved by the local Institutional Review Board (CEIC-A) and the Ethics Committee of the University of Navarra. hMSC were isolated as previously described (Pérez-Diaz *et al.* 2017) and maintained in 10% fetal bovine serum (FBS) low-glucose DMEM at 37 °C and 5% CO_2. Once hMSC reached confluence were differentiated into adipocytes by adding an adipogenic cocktail (500 mM IBMX, 1.67 mM insulin, 1 mM dexamethasone, 1 mM rosiglitazone, and 10% FBS high-glucose DMEM) for 3 days. Then, the medium was removed, and new adipogenic cocktail was added. After 6 days of differentiation, cells were incubated with 10% FBS high-glucose medium for 3 additional days when cells displayed typical features of mature white adipocytes.

The effects of MaR1 were tested along the differentiation process or in mature differentiated adipocytes. To study the effects of MaR1 treatment during the differentiation process, MaR1 (1, 10 and 100 nM) was added on confluent un-differentiated hMSC cells at the same time of the adipogenic cocktail and then added to fresh medium ever after. The effects of MaR1 (1 and 10 nM) were also tested on mature adipocytes for 24 h. Control cells were treated with the same amount of vehicle (0.1 % ethanol) under the same conditions.

2-Deoxy-D-glucose (2DG) uptake in brown adipocytes

In order to determine the effects of MaR1 on glucose uptake, mature brown adipocytes treated with vehicle (0.1 % ethanol) or MaR1 (0.1-1 nM) for 24 h, were incubated for 2 h with DMEM without serum or glucose at 37 °C prior the experiments. Some cells were treated with 0.5 nM insulin during the last 15 minutes of deprivation. Then, plates were incubated with 0.1 mM 2DG (Sigma-Aldrich) and 2 µM ^{14}C-2DG (Moraveck Biochemicals, Brea, CA) diluted in DMEM for 15 min. After incubation at 37 °C and 5% CO_2, 2DG uptake was stopped by adding ice-cold phosphate buffered saline with calcium and magnesium (PBS, Sigma-Aldrich). Adipocytes were again washed twice with ice-cold buffer to eliminate non-specific radioactivity fixation and were finally lysed in 500 µL 1% Triton X-100 in 0.1 M NaOH. Samples (100 µL) were taken to measure radioactivity by liquid scintillation counting, HIDEX 300 SL scintillation counter (Hidex Oy, Turku, Finland). The results were normalized to total protein content of cell lysates.

Free Fatty acids uptake in brown adipocytes

For free fatty acids uptake, mature brown adipocytes treated with vehicle or MaR1 (0.1-1 nM) for 24 h, were incubated in Krebs-Ringer buffer without glucose, containing 125 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.25 mM KH_2PO_4, 1.25 mM $MgSO_4$ x $7H_2O$, 25 mM $NaHCO_3$ and 3% fatty acid-free bovine serum albumin (BSA) pH= 7.8, during 50 min, according to Fernández-Galilea *et al.* (2015). Then, 80 µM palmitic acid (Sigma-Aldrich), 2 mM L-carnitine and the non-metabolizable analogue 20 µM ^{14}C-2-Bromopalmitic acid (Moraveck Biochemicals) were added to the media and incubated for 10 min. After that, culture plates were rinsed twice with ice-cold PBS. Adipocytes

were scraped in 0.05 M NaOH, and ^{14}C-2-Bromopalmitic acid uptake was measured by liquid scintillation counting of cell lysates in HIDEX 300 SL (Hidex Oy). The results were normalized to total protein content of cell lysates.

Fatty acid oxidation determination

Fatty acid oxidation was determined by the sum of $^{14}CO_2$ liberated to the media and ^{14}C-acid soluble metabolites as previously described (Fernández-Galilea *et al.* 2015). For that purpose, mature hMSC-derived adipocytes treated with vehicle or MaR1 (1-10 nM) for 24 h, and mature brown adipocytes treated with vehicle or MaR1 (0.1-1 nM) treated for 24 h were used. Adipocytes were incubated in Krebs-Ringer buffer as described above, also containing 2 mM L-carnitine, 80 µM palmitic acid (Sigma-Aldrich), and 20 µM ^{14}C-palmitic acid (Perkin Elmer, Waltham, MA) during 4 h. Then, $^{14}CO_2$ and ^{14}C-acid soluble metabolites were measured as previously described (Fernández-Galilea *et al.* 2015) by scintillation counting HIDEX 300 SL (Hidex Oy). Results were normalized to total protein content of cell extracts.

Analysis of adipocyte oxygen consumption

Oxygen consumption in mature brown adipocytes (treated with vehicle or MaR1 0.1 nM for 24 h) was measured using Seahorse Extracellular Flux (XF) 24 Analyser (Agilent Technologies, CA, USA) with the aim to measure the oxygen consumption rate (OCR), an indicator of mitochondrial respiration. Briefly, adipocytes were incubated in XF24 microplates. 1 h prior the assay, the cells were washed thoroughly with assay medium (unbuffered DMEM supplemented with 50 mM glucose and 1 mM sodium pyruvate) and incubated in a CO_2-free incubator at 37 °C. After incubation, microplates were loaded into the XF24 Analyser. During the assay, oligomycin (1 µg/mL), carbonylcyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; 0.6 µM), and a mixture of rotenone and antimycin A (2 µM of each one), were sequentially injected for OCR measurement to obtain the values for the basal mitochondrial respiration, ATP-linked, proton leak, maximal respiratory capacity, reserve capacity and non-mitochondrial respiration. The results were normalized to total protein content.

Analysis of protein levels by western blot

White and brown adipose tissue lysates were obtained as previously described (López-Yoldi *et al.* 2014). Total protein (30-40 μg) were electrophoretically separated by 12% SDS-PAGE. Proteins were electroblotted from the gel to polyvinylidene difluoride membranes (GE Healthcare Europe GmbH, Barcelona, Spain). Efficient protein transfer was observed by Ponceau S stain. Afterwards, the membranes were blocked (BSA 1 %) and probed with specific primary antibodies against uncoupling protein 1 (UCP1) (rabbit, Abcam, Cambridge, UK) and β-actin (mouse, Sigma-Aldrich) overnight. Thereafter, infrared fluorescent secondary antibodies anti-rabbit (Cell Signaling Technology) and anti-mouse (LI-COR Biosciences, Lincon, USA) were used and quantitated using an Odyssey® Sa infrared imaging system (LI-COR).

Analysis of mRNA levels

Total RNA was isolated from fully differentiated hMSC-derived adipocytes, brown adipocytes, and subcutaneous and brown adipose tissue, and then reverse transcribed to cDNA as previously described (López-Yoldi *et al.* 2014). Acyl-coenzyme A oxidase (*Acox1*), adiponectin (*Adipoq*), adipose triglyceride lipase (*Atgl*), C-C motif chemokine ligand 2 (*Ccl2*), CD11 Antigen-Like Family Member C (*Cd11c*); carnitine palmitoyltransferase (*Cpt1a*), cell death-inducing DFFA-like effector A (*Cidea*), fatty acid synthase (*Fasn*), fibroblast growth factor 21 (*Fgf21*), free fatty acid receptor 4 (*Gpr120*), hormone-sensitive lipase (*Hsl*), interleukin 6 (*Il6*), interleukin 10 (*Il10*), iodothyronine deiodinase 2 (*Dio2*), mitochondrial transcription factor A (*Tfam*), nuclear respiratory factor 1 (*Nrf1*), perilipin (*Plin*), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1a*), PR domain containing 16 (*Prdm16*), sirtuin 1 (*Sirt1*), solute carrier family 2 member 1 (*Glut1*), solute carrier family 2 member 4 (*Glut4*), T-box 1 transcription factor (*Tbx1*), transmembrane protein 26 (*Tmem26*) and uncoupling protein 1 (*Ucp1*) mRNA levels were determined using predesigned Taqman® Assays-on-Demand and Taqman Universal Master Mix (Applied Biosystems). Amplification and detection of specific products were done using the ABI PRISM 7900HT Fast Sequence Detection System (Applied Biosystems). Cyclophilin A (*Ppia*) and 18S were used as housekeeping genes. Relative expression of the genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

GraphPad Prism 5 software (Graph-Pad Software Inc., San Diego, CA) was used for statistical analyses and significant (*P <0.05*) differences between groups were reported. Data are presented as mean ± SEM. Comparisons between the values for different variables were analysed by one-way ANOVA followed by Bonferroni *post hoc* tests or by Student's *t*-tests or Mann-Whitney U-tests once the normality had been screened using Kolmogorov-Smirnoff and Shapiro-Wilk tests.

RESULTS

Maresin 1 induces brown adipose tissue activation in DIO mice

Although MaR1 did not induce significant changes either in food intake (data not shown), body weight or fat mass of DIO mice, MaR1-treated animals displayed a reduction on fasting hyperglycaemia (*P* <0.01) and hyperinsulinemia (*P* <0.05) induced by the HFD. Moreover, the area under the curve (AUC) of the ITT reflected an improvement (*P* <0.01) in insulin sensitivity in MaR1-treated DIO mice (Table 1).

Table 1. Effects of MaR1 on body and adipose tissue weights and serum biochemical measurements in DIO mice.

Parameter	Control (n=7)	DIO (n=8)	DIO + MaR1 (50 μg/kg) (n=8)
Body weight (g)	24.44 ± 0.60	41.13 ± 0.80 ***	39.28 ± 0.73 ***
Subcutaneous fat weight (g)	0.20 ± 0.01	1.75 ± 0.18 ***	1.71 ± 0.18 ***
Retroperitoneal fat weight (g)	0.17 ± 0.05	1.52 ± 0.16 ***	1.29 ± 0.20 ***
Epididymal fat weight (g)	0.32 ± 0.04	1.92 ± 0.16 ***	2.15 ± 0.14 ***
Brown fat weight (g)	0.06 ± 0.01	0.13± 0.01 ***	0.15 ± 0.01 ***
Fasting glucose (mg/dL)	67.14 ± 3.91	123.80 ± 6.27 ***	99.75 ± 4.65 ***,##
Fasting insulin (μg/L)	0.13 ± 0.01	0.64 ± 0.26 **	0.23 ± 0.04 #
ITT (AUC)	13,828.00 ± 616.80	25,243.00 ± 1892.00***	18,891.00 ± 999.00 **,##

Data are mean ± SEM. Insulin tolerance test (ITT), area under the curve (AUC). ** *P* <0.01, ***P* <0.001 *vs.* Control group; #*P* <0.05, ##*P* <0.01 *vs.* DIO group.

Since BAT activation has been pointed out as a potential therapy for obesity and diabetes, serving as a glucose clearance organ, we next evaluated the effects of MaR1

on BAT metabolism and function. The histological examination of BAT revealed that MaR1-treated DIO mice exhibited smaller lipid droplets as compared to DIO untreated mice and recovered typical morphological features of BAT observed in control animals (Fig. 1A). Interestingly, MaR1-treated DIO mice presented a significant increase (P <0.05) on UCP1 protein expression, the main protein involved in non-shivering thermogenesis (Fig. 1B). Moreover, MaR1 treatment was able to reverse (P <0.05) the downregulation of *Prdm16* and *Pgc1a* mRNA levels observed in BAT of DIO mice (Fig. 1C). Complementary, MaR1 up-regulated gene expression level of *Sirt1* (P <0.05), *Dio2* (P <0.001), *Fgf21* (P <0.001) and *Gpr120* (P <0.05), a lipid sensor that has been recently shown to promote BAT activation (Fig. 1C). Taken together these observations suggest that MaR1 treatment could promote BAT recruitment and activity.

To further understand the role of MaR1 on BAT activation, we studied the effects of this lipid mediator on genes involved in regulation of lipid and glucose metabolism in BAT. In this sense, MaR1-treated DIO mice exhibited increased expression of some genes related to lipolysis (*Atgl*, P <0.01) and fatty acid oxidation (*Acox1,* P <0.001) as compared to vehicle-treated DIO mice (Fig. 1D). Moreover, MaR1 further up-regulated (P <0.05) the mRNA levels of both glucose transporters *Glut1* and *Glut4* (Fig. 1E). Since SPMs are known to have potent anti-inflammatory and proresolutive properties, the effects of MaR1 on the expression of key pro-inflammatory and anti-inflammatory cytokines/markers were also analysed in BAT. MaR1 treatment reversed HFD-induced gene expression of *Ccl2* (P <0.01) and *Cd11c* (P <0.05), and reduced (P <0.001) *IL6* mRNA expression. In contrast, an increase on the anti-inflammatory genes *Adipoq* (P <0.001) and *Il10* (P <0.01) was also observed as compared to control mice, but not significant changes were observed in comparison to DIO mice (Fig.1E).

In order to better characterize the BAT-stimulating properties of MaR1, we evaluated the effects of acute MaR1 administration on cold-induced BAT activation using PET scanning. Interestingly, the PET data revealed that MaR1-treated mice exhibited greater ^{18}F-FDG uptake by BAT after cold exposure, compared to control mice (Fig. 2A). Interestingly, this improvement of BAT response to cold by MaR1 was maintained (P <0.05) during at least 4 h after cold intervention (Fig. 2A, 2B), and accordingly, the AUC of SUVmax was increased (P <0.05) after MaR1 treatment (Fig. 2C). These data strongly support the ability of MaR1 to promote BAT activation.

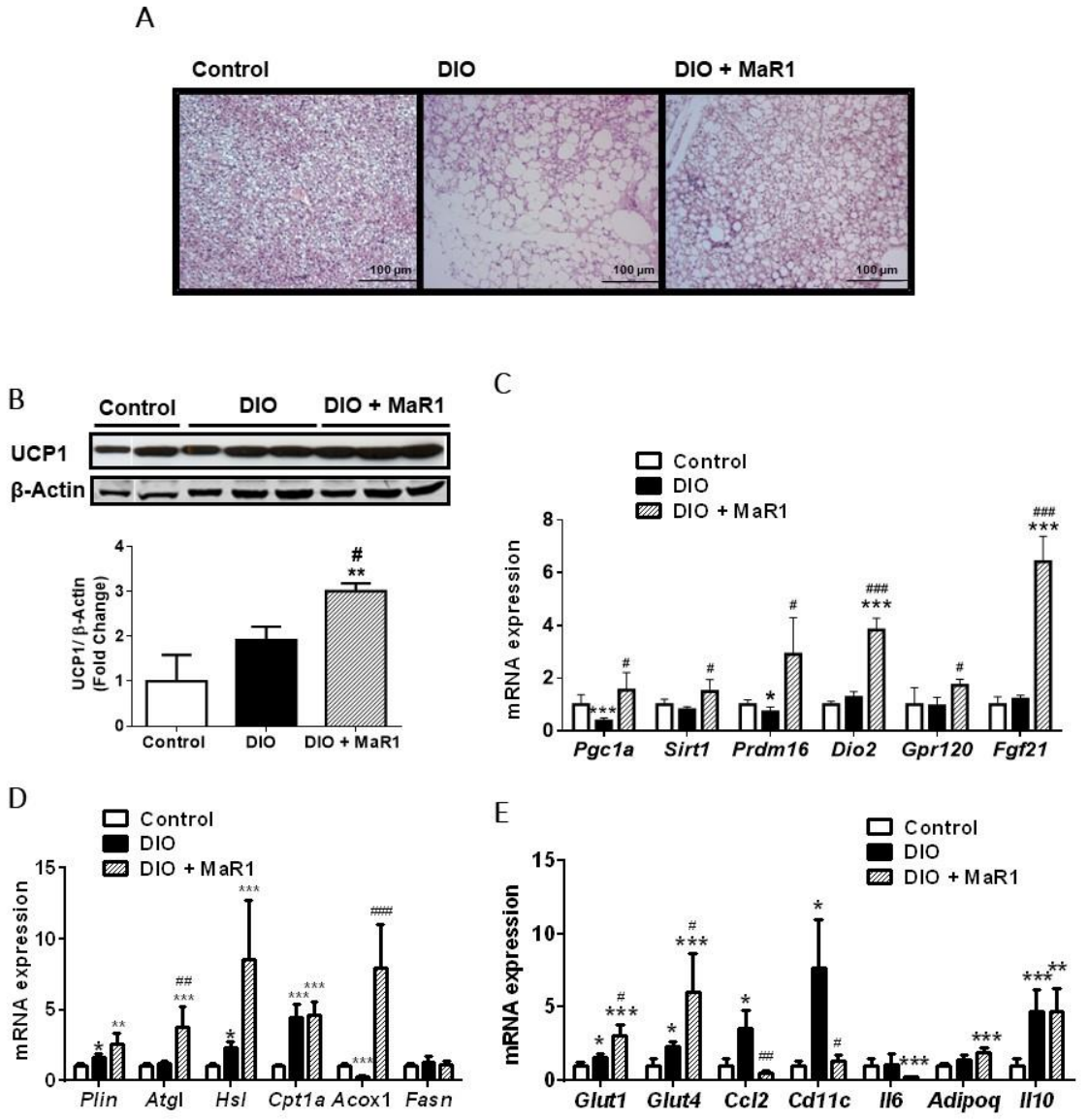

Fig 1. MaR1 promotes brown adipose tissue (BAT) activation in DIO mice. Twenty weeks-old DIO mice were treated either with MaR1 (50 µg/kg) or vehicle by oral gavage for 10 days. (A) Representative photomicrographs (magnification 100x) of BAT sections stained with hematoxylin-eosin dye. (B) Representative Western Blot (top panel) and densitometry analysis (bottom panel) of BAT uncoupling protein 1 (UCP1); band densities of UCP1 were normalized to β-actin. (C) mRNA expression levels of master mitochondrial biogenesis regulators and BAT activators genes (*Pgc1a*, *Sirt1*, *Prdm16*, *Dio2, Gpr120* and *Fgf21*). (D) mRNA expression levels of genes involved in BAT lipid metabolism (*Plin*, *Atgl*, *Hsl*, *Cpt1a*, *Acox1* and *Fasn*). (E) mRNA expression levels of glucose transporters (*Glut1* and *Glut4*) and pro-inflammatory (*Ccl2*, *Cd11c* and *Il6)* and anti-inflammatory (*Adipoq* and *Il10*) markers. Data (mean ± SEM) are expressed as fold change relative to Control (vehicle-treated) mice considered as 1 (n=7-8). **P* < 0.05; ***P* < 0.01; ****P* < 0.001 *vs.* vehicle-treated Control mice; $^{\#}P$ < 0.05; $^{\#\#}P$ < 0.01; $^{\#\#\#}P$ < 0.001 *vs.* DIO (vehicle-treated) mice.

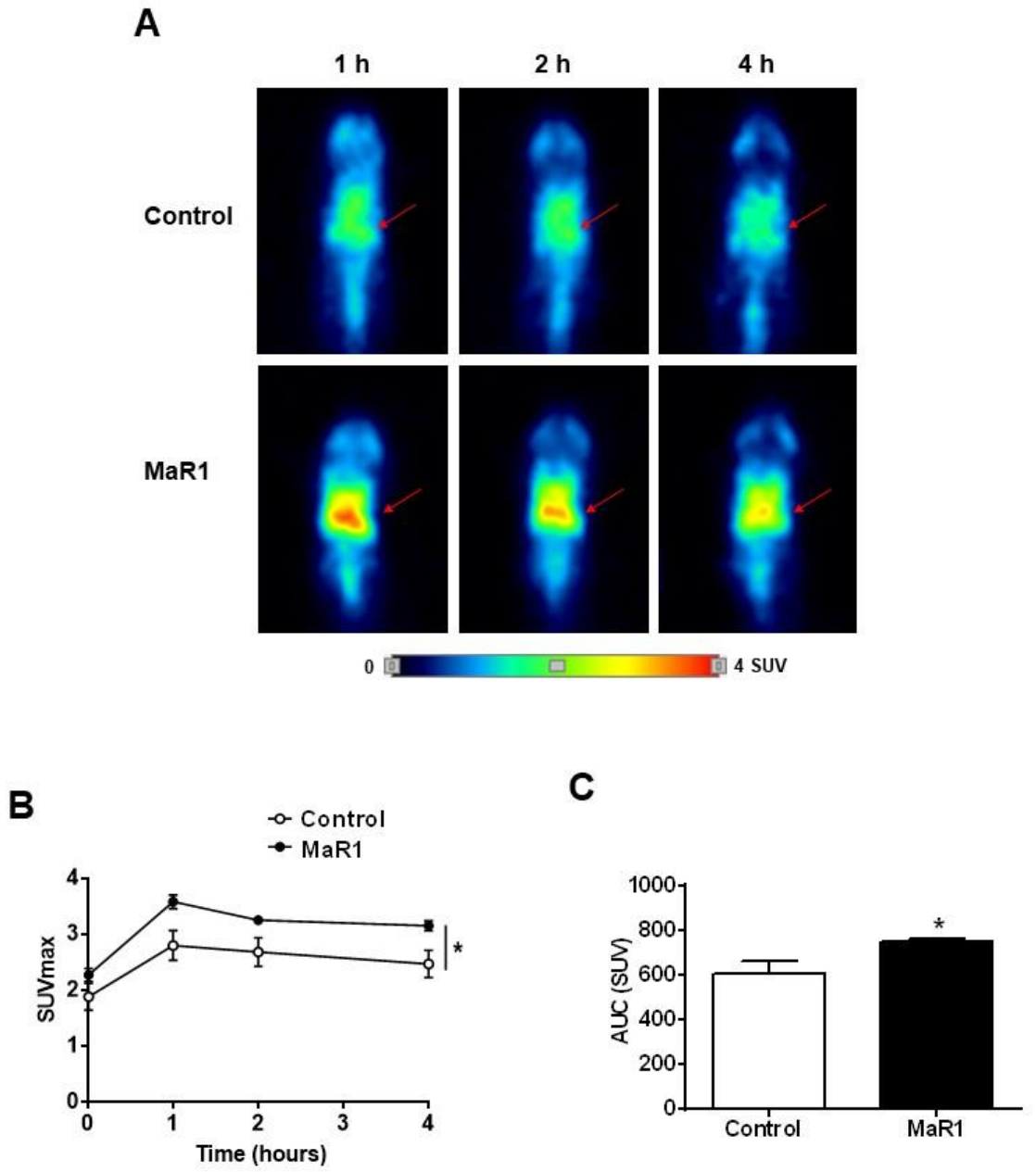

Fig 2. Acute treatment with MaR1 potentiates cold-induced BAT activation in mice. Eight weeks-old mice were treated with MaR1 (50 μg/kg) or vehicle by intraperitoneal injection, and then exposure for 1 h at 4 °C, prior injection of ^{18}F-fluorodeoxyglucose (^{18}F-FDG). BAT activity was assessed by measuring ^{18}F-FDG uptake using microPET at basal, 1, 2 and 4 h after ^{18}F-FDG injection. (A) Interscapular BAT in control and MaR1-treated mice. Each set of images corresponds to coronal sections of the same animal over time. Red arrow: interscapular BAT pads. (B) Time-course of maximum standardized uptake value (SUVmax). Statistical analysis was performed by repeated-measures two-way ANOVA with Bonferroni posttest. (C) Area under the curve (AUC) of SUVmax. Data are expressed as mean ± SEM (n = 5). *P < 0.05 *vs.* control (vehicle-treated) mice.

MaR1 promotes glucose uptake, fatty acid utilization, thermogenic genes and oxygen consumption in cultured brown adipocytes

We next tested the direct effects of MaR1 (0.1-1 nM) on brown adipocytes activity and metabolism. Similarly, to what was observed after *in vivo* administration, MaR1-treated brown adipocytes exhibited a significant up-regulation (P <0.05) of thermogenic genes (*Ucp1, Fgf21* and *Gpr120*), as well as of *Glut4* and genes related to mitochondrial biogenesis (*Pgc1a, Tfam, Nrf1*) and fatty acid oxidation (*Cpt1a* and *Acox1*) (Fig. 3A). Remarkably, MaR1 induced both fatty acid uptake (P <0.01) and fatty acid oxidation (P <0.01) by murine brown adipocytes (Fig. 3B). Moreover, MaR1-treated brown adipocytes presented higher (P <0.05) glucose uptake than controls to a similar extent that those treated with insulin (Fig. 3C). In parallel, basal oxygen consumption rate (OCR), the maximal respiratory capacity and the proton leak were significantly higher (P <0.05) in adipocytes treated during 24 h with MaR1 (Fig. 3D). These data strongly support the ability of MaR1 to activate brown adipocytes function.

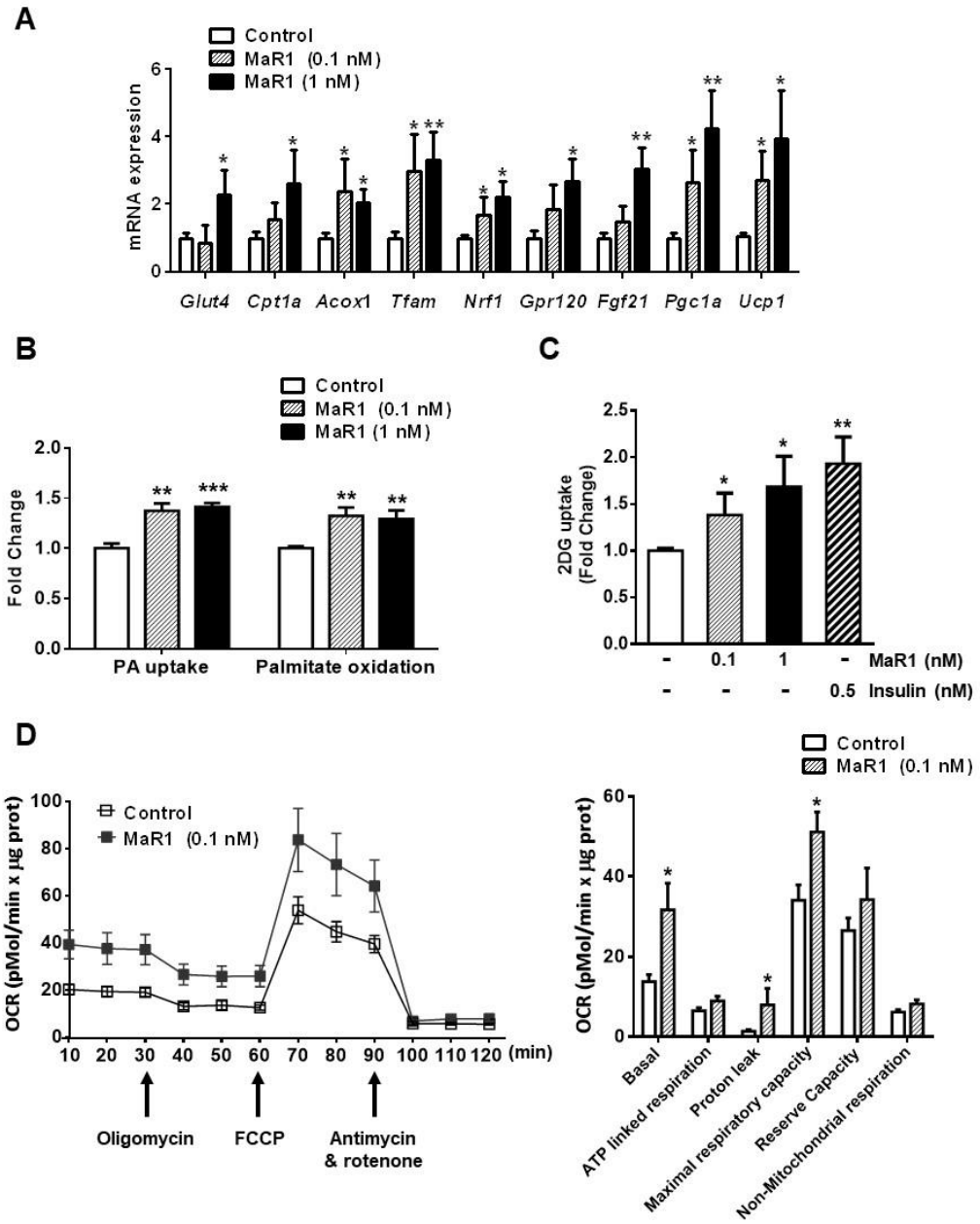

Fig 3. MaR1 promotes glucose uptake, fatty acid utilization and upregulates thermogenic genes in cultured brown adipocytes. Mature murine brown adipocytes were treated with MaR1 (0.1 and 1 nM) for 24 h. (A) mRNA expression levels of genes involved in glucose transport (*Glut4*), fatty acid oxidation (*Cpt1a* and *Acox1*), mitochondrial biogenesis and thermogenesis regulation (*Tfam*, *Nrf1*, *Gpr120*, *Fgf21*, *Pgc1a* and *Ucp*1). Data are expressed as fold change relative to Control (vehicle-treated) brown adipocytes considered as 1. (B) Palmitic acid (PA) uptake and Palmitate oxidation rates in MaR1-treated brown adipocytes. (C) Effects of MaR1 and insulin on 2-Deoxy-D-glucose (2-DG) uptake in brown cultured adipocytes. (D) Oxygen consumption rate (OCR) was measured in mature brown adipocytes (control and treated with 0.1 nM MaR1 during 24 h) on a Seahorse Analyser under basal conditions and during successive addition of 1 µg/mL oligomycin, 0.6 µM FCCP and a mixture of rotenone and antimycin A (2 µM of each one), to obtain the values for the basal mitochondrial respiration, ATP-linked OCR, maximal respiration, and spare respiratory capacity. Right panel shows a representative experiment. Left panel: Bioenergetics parameters were inferred from the OCR traces. Data are expressed as mean ± SEM (n = 3-5). **P* < 0.05; ***P* < 0.01; ****P* < 0.001 *vs.* vehicle-treated brown adipocytes.

Maresin 1 induces beige adipocyte markers in subcutaneous white adipose tissue of DIO mice and in hMSC-derived adipocytes

Due to the identification of MaR1 as an agent able to promote BAT activation, we further analysed if MaR1 was able to induce browning of subcutaneous WAT depots. In this sense, chronic MaR1 administration increased (P <0.05) UCP1 protein expression (Fig. 4A), and up-regulated (P <0.05) the expression of genes related to mitochondrial biogenesis (*Nrf1*, *Tfam*, *Pgc1a*, *Sirt1*) and mitochondrial fatty acid oxidation (*Cpt1a*) (Fig. 4B) in iWAT compared to vehicle-treated DIO mice. Additionally, iWAT from MaR1-treated mice exhibited increased (P <0.05) expression levels of genes that typify beige adipocytes such as *Tbx1, Tmem26* and *Prdm16,* as well as *Gpr120* and *Fgf21*, which have been related with browning process (Fig. 4C). All these data strongly suggest that MaR1 treatment could also switch on the promotion of the beiging process of subcutaneous iWAT in DIO mice.

Next, the ability of MaR1 to directly promote the browning program was tested in cultured hMSC during the differentiation process to adipocytes. Indeed, the induction of the adipogenic process in the presence of MaR1 increased (P <0.05) the expression of several beige adipocyte-related genes including *UCP1, CIDEA TMEM26*, *TBX1,* and also *PGC1α* (Fig. 4C). This suggests that MaR1 promotes the development of brite adipocytes from hMSC of subcutaneous white fat. However, it is important to mention that, this effect was not observed when MaR1 treatment was performed on mature hMSC-derived adipocytes for 24 h (data not shown). Nevertheless, the mature adipocytes treated with MaR1 (1-10 nM) for 24 h exhibited higher (P <0.01) fatty acid oxidation rate (Fig. 5A), in parallel with increased (P <0.01) *CPT1A* gene expression at the highest concentration tested, and up-regulation of genes related to mitochondrial biogenesis such as *PGC1α* (P <0.05)*, SIRT1* (P <0.001) and *TFAM* (P <0.05) (Fig. 5B).

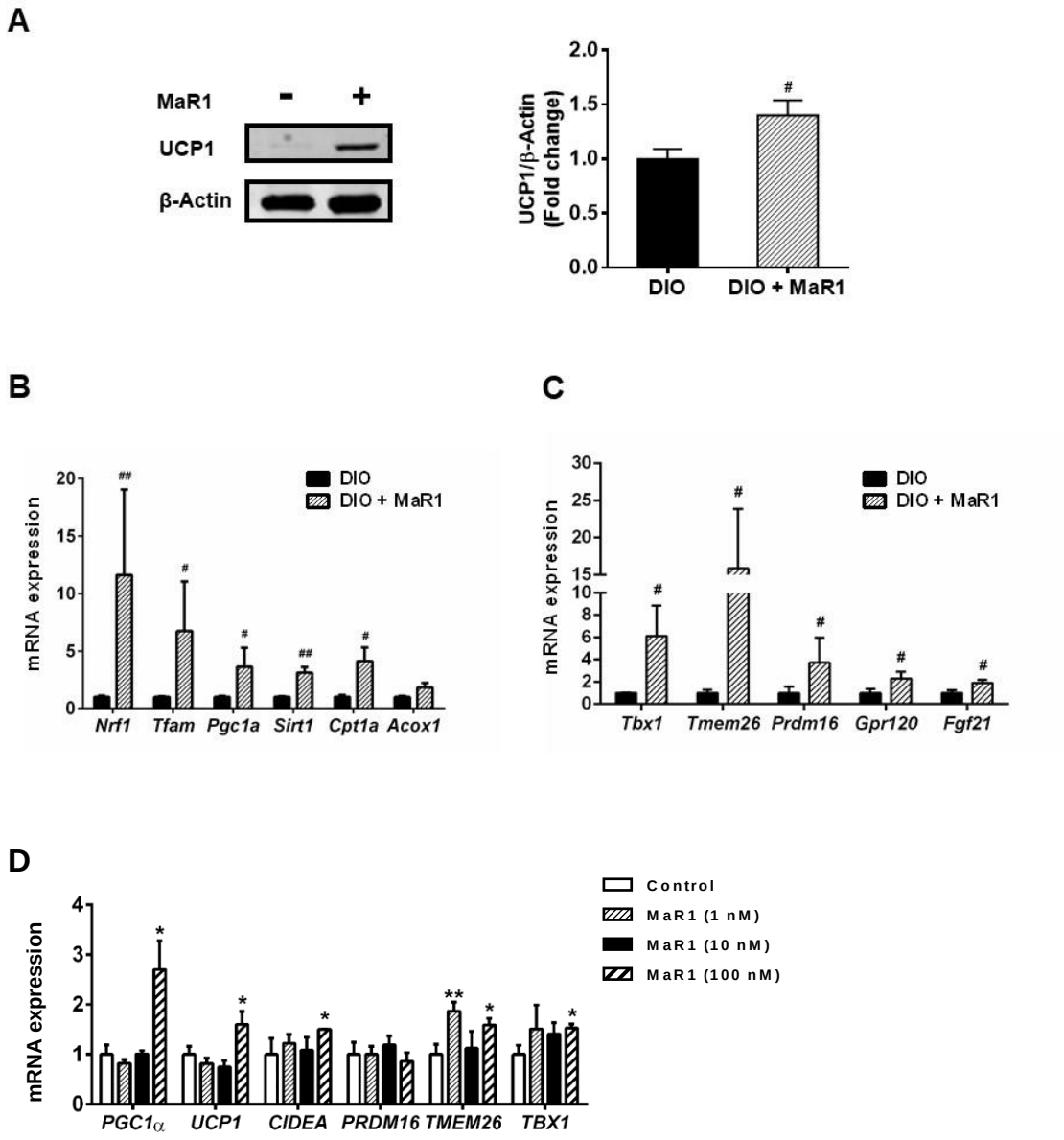

Fig 4. MaR1 induces beige adipocyte markers in subcutaneous white adipose tissue of DIO mice and in human mesenchymal cells (hMSC)-derived adipocytes. *A-C:* Beige adipocyte markers in subcutaneous WAT from 20 weeks-old DIO mice treated with MaR1 (50 µg/kg) or vehicle by oral gavage for 10 days. A: Representative Western Blot (left panel) and densitometry analysis (right panel) of uncoupling protein 1 (UCP1); band densities of UCP1 were normalized to β-actin. *B:* mRNA expression levels of key regulators of mitochondrial biogenesis (*Nrf1*, *Tfam*, *Pgc1a* and *Sirt1*); and fatty acid oxidation genes (*Cpt1a* and *Acox1*). *C:* mRNA expression levels of beige characteristic and browning-related genes (*Tbx1*, *Tmem26*, *Prdm16*; *Fgf21* and *Gpr120*). Data are expressed as fold change relative to DIO (vehicle-treated) mice considered as 1 (n=8). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ *vs.* DIO (vehicle-treated) mice. *D*: hMSC were treated along the differentiation process with MaR1 (1, 10, 100 nM) and determinations were carried out in fully differentiated hMSC-derived adipocytes. mRNA expression levels of genes characteristics of beiging process (*PGC1A*, *UCP1*, *CIDEA*, *PRDM16*, *TMEM26* and *TBX1*). Data are expressed as fold change relative to vehicle-treated hMSC-derived adipocytes considered as 1. Data are expressed as mean ± SEM (n = 3-4). $^{*}P < 0.05$; $^{**}P < 0.01$ *vs.* vehicle-treated adipocytes.

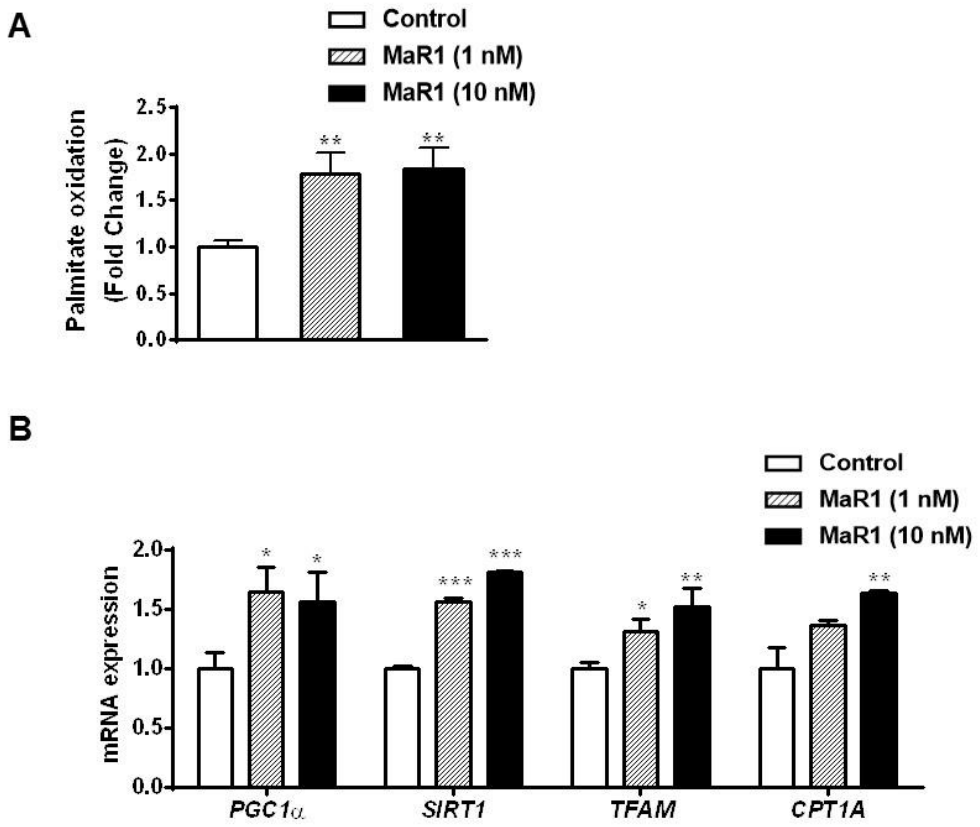

Fig 5. MaR1 promotes fatty acid oxidation in mature hMSC adipocytes. Mature adipocytes derived from hMSC were treated with MaR1 (1 and 10 nM) for 24 h. *A:* Palmitate oxidation rates in control and MaR1-treated adipocytes. *B:* mRNA levels of genes involved in mitochondrial biogenesis and fatty acid oxidation (*PGC1A, SIRT1*, *TFAM* and *CPT1A)*. Data (mean ± SEM) are expressed as fold change relative to control adipocytes considered as 1. (n = 3-4 independent experiments). **P* < 0.05; ***P* < 0.01; ****P* < 0.001 *vs.* control (vehicle-treated) adipocytes.

DISCUSSION

The current research revealed for the first time that MaR1, a specialized proresolving lipid mediator derived from DHA, is a molecule able to activate BAT and promote subcutaneous WAT browning, which could contribute to its glucose-lowering and insulin-sensitizing properties in obesity (Martínez-Fernández *et al.* 2017).

Several observations strongly support the effectiveness of chronic MaR1 administration in the promotion of BAT recruitment and activity, including: 1) the upregulation of *Prdm16*, a transcription factor that promotes brown fat development, 2) the increase in UCP1 levels, the main thermogenic protein as well as 3) the up-regulation of other thermogenic-related genes including *Pgc1a* and *Dio2*, which are essential for adaptive thermogenesis in brown adipocytes (de Jesus *et al.* 2001, Uldry

et al. 2006). All these data are strongly supported by the observation that acute MaR1 treatment potentiates cold-induced BAT functional activation in mice.

Previous studies have already demonstrated that n-3 PUFAs promote BAT activation along with the upregulation of UCP1, *Pgc1a* and *Prdm16* (Takahashi and Ide 2000, Bargut *et al.* 2016, Kim *et al.* 2016, Quesada-López *et al.* 2016). It has been recently discovered that the lipid sensor GPR120 plays a key role in the activation of BAT through the induction of FGF21, and that GPR120 is required for the EPA-induced brown adipocytes activation (Quesada-López *et al.* 2016). Interestingly, MaR1 treatment also upregulated both *Gpr120* and *Fgf21* gene expression, which could also mediate MaR1 effects on BAT activation. On the other hand, n-3 PUFAs have been shown to activate BAT via the sympathetic nervous system (SNS) (Kim *et al.* 2015). Our current data show that MaR1 is able to activate brown adipocytes in culture, suggesting that at least in part MaR1 can activate BAT independently of SNS stimulation. However, further research is required to characterize if the SNS activation is involved in the promotion of BAT function observed after *in vivo* administration of MaR1.

BAT activation requires fuel supply, which includes glucose and fatty acids. Indeed, cold exposure and insulin promote BAT glucose uptake in correlation with BAT activation (Orava *et al.* 2011, Townsend and Tseng 2014). Here, we show that acute MaR1 administration further increased cold-induced BAT glucose uptake. Moreover, the upregulation of BAT thermogenic genes/proteins observed in MaR1-chronic treated mice was accompanied by an increase in the expression of glucose transporters *Glut1* and *Glut4*. Furthermore, our studies in cultured brown adipocytes clearly demonstrate that MaR1, similarly to insulin, directly stimulates glucose uptake. On the other hand, MaR1-treated brown adipocytes also exhibited increased fatty acid uptake and fatty acid oxidation, in parallel with increased expression of genes involved in fatty acid oxidation and mitochondrial biogenesis, as well as oxygen consumption. In this context, a recent study has described that 12,13-diHOME, a newly recognized cold-induced lipokine, promotes fatty acid transport and activates BAT (Lynes *et al.* 2017).

It has been recently described emerging roles of BAT activation to tackle obesity and its comorbidities, including hypertriglyceridemia, glucose homeostasis and insulin

sensitivity (Bartelt *et al.* 2011, Stanford and Goodyear 2013). Regarding the relevance that MaR1-induced BAT activation could have in obesity-linked metabolic complications, it should be mentioned that although MaR1 did not induce significant body weight or fat mass changes, caused a relevant reduction in fasting hyperglycaemia and hyperinsulinemia induced by the high fat feeding, and clearly improved insulin sensitivity. These data strongly suggest that BAT activation could significantly account for these beneficial effects of MaR1 in glucose homeostasis.

The insulin-sensitizing properties of MaR1 and other SPMs have been related to the amelioration of inflammation in WAT (González-Périz *et al.* 2009, Hellmann *et al.* 2011, White *et al.* 2014, Martínez-Fernández *et al.* 2017). It has been demonstrated that HFD-induced BAT inflammation and insulin resistance can lead to impairment of BAT function (Roberts-Toler *et al.* 2015), suggesting that the anti-inflammatory effects of MaR1 on BAT may also contribute to the improvement observed on BAT function and metabolism.

The current studies also suggest the capability of MaR1 to promote a remodelling of iWAT towards a more thermogenic beige pattern, including the increase of UCP1 protein levels and the upregulation of *Prdm16*, and genes involved in mitochondrial biogenesis as well as beige-specific markers such as *Tbx1, Tmem26* (Wu *et al.* 2012) in DIO mice. The ability of MaR1 to switch on genes typifying the beiging process was also observed in hMSC-derived adipocytes incubated with MaR1 across the differentiation process. The fact that this effect was not observed when MaR1 treatment was performed on mature hMSC-derived adipocytes, suggests that MaR1 could exert its browning effect via recruiting brite adipocytes and not by promoting transdifferentiation from mature white to beige adipocytes. Nevertheless, mature white adipocytes treated acutely with MaR1 exhibited increased fatty acid oxidation rate, suggesting that although MaR1 seems not to be able to induce conversion from already mature white to a more thermogenic brite adipocytes, is promoting lipid burning in mature hMSC adipocytes.

Several studies have previously observed that in addition to BAT activation, EPA also induces mitochondrial oxidation and beiging of subcutaneous adipocytes (Fleckenstein-Elsen *et al.* 2016, Laiglesia *et al.* 2016). It should be mentioned that BAT activation and thermogenic effects of EPA were not observed for DHA (Fleckenstein-

Elsen *et al.* 2016, Quesada-López *et al.* 2016, Pahlavani *et al.* 2017). Therefore, our current data suggest differential effectiveness on the regulation of BAT function and brite adipogenesis for MaR1, a DHA-derived autacoid, and its n-3 PUFA precursor.

Another important consideration is the fact that effects of MaR1 of brown and white fat take place at much lower doses than those required for n-3 PUFAs (nM *vs*. µM range).

It has been described that immune-adipose interactions may be key regulators to increase fat thermogenesis (Qiu *et al.* 2014). In fact, M1:M2 macrophages balance seems to play a role in white adipose tissue browning. Nevertheless, it is important to note that there is some controversial recent observations about the direct role of alternatively activated macrophages in adaptive thermogenesis (Fischer *et al.* 2017). Our current and previous data have shown that MaR1 reduced inflammation in BAT and WAT (Martínez-Fernández *et al.* 2017) of DIO mice, characterized by a decrease in M1 macrophages markers. Therefore, this suggests that the browning properties of MaR1 could be in part related to its actions on immune cells in fat depots. Further research is requested to better explore this possibility and to find out if the BAT and beige activating properties of MaR1 are also shared by other SPMs or are specific for macrophage-derived SPMs such as Maresins.

In summary, our results provide first evidence of MaR1 acting as a novel inducer of BAT activity and browning in WAT, which could contribute to its insulin-sensitizing properties in obesity. Therefore, MaR1 might represent a promising therapeutic agent to tackle obesity comorbidities such as insulin resistance and type 2 diabetes.

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V. GENERAL DISCUSSION

N-3 PUFAs have been widely studied as anti-inflammatory agents in several chronic and acute inflammatory diseases. Interestingly, their derivatives bioactive lipid mediators (resolvins, protectins and maresins) play a key role in the resolution of inflammation and have potent inflammatory properties (López-Vicario *et al.* 2016, Clària *et al.* 2017). In this context, n-3 PUFAs (EPA and DHA) and some of these lipid mediators, including RvE1, RvD1 and PDX, have been proposed as good candidates for the treatment of obesity and insulin resistance (Spite *et al.* 2014, Martínez-Fernández *et al.* 2015). In line with these observations, a recent study of our group has revealed the ability of MaR1 to reduce WAT inflammation and improve systemic insulin sensitivity in obese mice (Martínez-Fernández *et al.* 2017).

In the present study, we provide novel effects and mechanism of actions of both EPA and MaR1, which could be involved in their ability to reduce obesity-related metabolic disorders. One of these findings is the capability of MaR1, a DHA-derived lipid mediator, to attenuate TNF-α-induced lipolysis and autophagy in murine adipocytes. MaR1 inhibited the TNF-α-induced lipolysis by regulating key lipases and lipid droplet proteins involved in the control of TG hydrolysis in adipocytes. In our experimental conditions, TNF-α in the presence or absence of MaR1 did not induce statistically significant changes on ATGL or to its co-regulator CGI-58 protein expression. This is in contrast with other studies that have observed that TNF-α decreased, ATGL expression (Kim *et al.* 2006, Bezaire *et al.* 2009). However, MaR1 may affect ATGL activity secondarily to its ability to hinder the decrease of the ATGL inhibitor G0S2 observed in TNF-α-treated adipocytes. Indeed, this co-repressor protein has been proposed to be reduced by TNF-α and thus, increase ATGL activity (Yang *et al.* 2011, Jin *et al.* 2014). Therefore, the inhibition of this action by MaR1 may be in part responsible of lower lipolysis rates, as can be demonstrated in the reduction on glycerol release into the media.

As previously described, TNF-α reduced HSL protein expression (Ryden *et al.* 2004), which is counteracted by MaR1 concomitant treatment. However, increasing the lipase content might seem controversial, since MaR1 reduces TNF-α-induced lipolysis. It is well known that HSL activity is tightly regulated by reversible phosphorylation at different Serine residues. Thus, HSL phosphorylation at Ser-660, increases HSL activity while the phosphorylation at Ser-565 inhibits HSL activity by

allosteric impediments (Watt *et al.* 2006). The effects of MaR1 seem to be preferential towards a downregulation of HSL activity by restoring the ratio of HSL phosphorylation at Ser-565/total HSL and inhibiting the increased ratio of phosphoHSL at Ser-660/total HSL observed in TNF-α-treated adipocytes. Moreover, MaR1 also decreased the TNF-α-induced phosphorylation of ERK1/2, which has also been shown to stimulate HSL activation by phosphorylation of HSL at Ser-600 (Watt *et al.* 2006). All these actions may lead to a reduction on HSL activity that could be the main responsible for MaR1 capability to reduce TNF-α-induced lipolysis. These preventive actions of MaR1 on TNF-α-induced lipolysis may lead to improvements on insulin sensitivity (Morigny *et al.* 2016).

An interesting finding of the present study is that MaR1 also prevented TNF-α-induced autophagy in adipocytes. Indeed, lipolysis and autophagy may share parallel roles, being complementary routes to provide energy to the cell during starvation (Singh *et al.* 2009, Singh and Cuervo 2012, Kaushik and Cuervo 2015, Schweiger and Zechner 2015). Indeed, lipolysis and lipid droplet formation have been related to autophagosome biogenesis (Shpilka *et al.* 2015). In this context, the reduction of p62 protein content by TNF-α was blocked by MaR1 concomitant treatment. This effect suggests a reduction of cytokine-induced autophagy, since p62 is mainly degraded by autophagosomes, and it is known that is accumulated in cells with lower autophagy (Komatsu *et al.* 2007). It is worth to mention that the effects on p62 by either TNF-α or MaR1 seem to be related to its degradation through autophagy, since p62 mRNA levels were not reduced. In addition, MaR1 was also able to partially inhibit the increased LC3II/LC3I ratio observed in cytokine-treated adipocytes, suggesting that MaR1 is reducing TNF-α-induced autophagy by regulating LC3I conversion to its lipidated form LC3II, widely used for monitoring autophagy (Ichimura *et al.* 2000, Tanida *et al.* 2008). However, neither the effect of TNF-α nor the actions of MaR1 were related to changes on ATG7 protein expression, suggesting that TNF-α could promote Atg7-independent alternative autophagy. In this context, it has been demonstrated that mouse cells lacking of Atg7 can also form autophagosomes and achieve autophagy properly (Nishida *et al.* 2009).

Interestingly, MaR1 not only acts locally in adipocytes, since the current studies have clearly demonstrated beneficial effects of *in vivo* MaR1 treatment in obesity-associated metabolic disorders.

Indeed, one of the main findings of this Thesis is the identification of MaR1 as an agent able to reduce liver steatosis in animal models of obesity, both genetic (*ob/ob)* and diet-induced obese (DIO) mice. Previous studies have already described antisteatotic effects of both n-3 PUFAs and some of their derived lipid mediators, including RvD1 and RvE1 (González-Périz *et al.* 2009, Rius *et al.* 2014, de Castro *et al.* 2015a and 2015b). It is important to note that these SPMs are able to reduce fatty liver acting at much lower doses than their n-3 PUFAs precursors. In all the previous trials, these n-3 PUFAs-derived SPMs were administered intraperitoneally. A relevant advantage of this study was the finding that MaR1 is also effective in reducing fatty liver when administered through oral gavage, which represents a non-invasive often safer administration route for potential pharmacological therapy in obesity-related fatty liver disease.

Indeed, in these MaR1-treated DIO mice, a reduction of liver weight occurred in parallel to the drop on both AST and ALT serum levels and reduced liver TG content, strongly supporting the hepatoprotective properties of oral MaR1 treatment in obesity. Our findings suggest that these anti-steatotic properties of MaR1 seems to be mediated through the reduction or inhibition of enzymes involved in hepatic *de novo* lipogenesis, while increasing expression of genes related to fatty acid oxidation and promoting lipophagy in liver. Indeed, MaR1 reduced lipogenic enzymes content such as FAS, while increased ACC phosphorylation, thus inhibiting ACC. In this line, it has been proved that reduced hepatic lipogenic enzymes content leads to a reduction on fat accumulation in liver (Paglialunga and Dehn 2016). Some differential outcomes such as the effect of MaR1 on SCD1, were observed between *ob/ob* and DIO mice, which could be due to the lack of leptin in the *ob/ob* mice, or as a result of other differences between treatments, such as treatment length and administration route. Moreover, our data point towards an induction of FA oxidation by MaR1, based on the upregulation of both *Cpt1a* and *Acox1*, two genes involved in mitochondrial and peroxisome FA oxidation respectively. In addition, FA oxidation could be also induced indirectly through ACC phosphorylation (Ceddia 2013), which causes enzyme

inactivation, and thus preventing the conversion of acetyl-CoA to malonyl-CoA, a strong inhibitor of CPT1A. Therefore, the phosphorylation of ACC will reduce the inhibition on CPT1A leading to mitochondrial fatty acid oxidation (Ceddia 2013).

Interestingly, autophagy seems to have a significant role in fatty liver disease (Singh and Cuervo 2012). Indeed, in obese mice, steatotic liver presents a reduction of autophagy markers (Liu *et al.* 2009, Codogno and Meijer 2010, Yang *et al.* 2010), and impaired autophagic flux occurs in NAFLD patients (González-Rodriguez *et al.* 2014). In this context, our results also uncovered a role for MaR1 in promoting autophagy in obesity-induced liver steatosis. This effect seems to be mediated by inducing *Atg5-7* gene expression, which are involved in autophagosome formation and increasing LC3II protein expression, which is recruited to autophagosomal membranes (Tanida *et al.* 2008). MaR1-treated DIO mice also exhibit higher number of autophagic vacuoles, and reduced levels of p62 protein, which is mainly degraded by autophagy. Therefore, taken together all these observations, strongly suggest that the anti-steatotic properties of MaR1 are also mediated by an induction of lipophagy. In this context, a previous study revealed that n-3 PUFAs induced autophagy by increasing LC3II levels in cultured hepatocytes (Chen *et al.* 2015). Moreover, n-3 PUFAs epoxides also modulate liver autophagy, by reducing p62 protein expression and also increasing LC3II (López-Vicario *et al.* 2015).

Our data also suggest that AMPK activation could also contribute to the anti-steatotic properties of MaR1 in obese mice, since AMPK activation by phosphorylation switches on hepatic catabolic pathways such as fatty acid oxidation (Castaño *et al.* 2014). It has been also suggested that AMPK activation could induce autophagy (Hardie 2011). Increased phosphorylation levels of AMPK by MaR1 were also observed in primary cultured hepatocytes. Interestingly, pre-treatment of hepatocytes with compound C, a well-known AMPK inhibitor (Zhou *et al.* 2001), clearly abrogated the stimulatory effects of MaR1 on both fatty acid oxidation (*Cpt1a* and *Acox1*) and autophagy (*Atg5-7*) related genes, strongly supporting the involvement of AMPK activation in MaR1 actions on hepatocytes. This is in agreement with other study that has demonstrated that AMPK activation is also essential for n-3 PUFAs capability to ameliorate liver steatosis (Jelenik *et al.* 2010). Additionally, other DHA-derivate,

Protectin DX also reverses HFD-induced harmful actions in liver by increasing AMPK phosphorylation (Jung *et al.* 2017).

It has been suggested that NAFLD plays an important role in the development of metabolic complications associated to obesity, including insulin resistance and type 2 diabetes (Jornayvaz and Shulman 2012). Therefore, it could be hypothesized that the amelioration of NAFLD by MaR1 could also contribute to its previously described insulin-sensitizing effects (Martínez-Fernández *et al.* 2017).

Additionally, the ability of MaR1 and other n-3 PUFAs-derived SPMs to improve insulin sensitivity have been related to their anti-inflammatory effects in WAT (González-Périz *et al.* 2009, Hellmann *et al.* 2011, White *et al.* 2014, Martínez-Fernández *et al.* 2017).

It has been shown that high fat feeding also induces BAT inflammation and insulin resistance leading to impaired BAT function (Roberts-Toler *et al.* 2015). Recently, BAT has been considered of great interest in metabolic research, since it has been observed that BAT is an active tissue in adult humans, and that BAT activity is negatively correlated to obesity and insulin resistance (Betz and Enerback 2015). Another remarkable finding of this study was the identification of MaR1 as an inducer of BAT recruitment and activity, as evidenced by the significant increase in the thermogenic protein UCP1, along with the up-regulation of other genes (*Pgc1a, Dio2)* essential for adaptive thermogenesis (de Jesus *et al.* 2001, Uldry *et al.* 2006) and/or brown fat development (*Prdm16*) (Harms *et al.* 2014). It has been recently discovered that the lipid sensor GPR120 plays a key role in the activation of BAT through the induction of FGF21 (Quesada-López *et al.* 2016). Interestingly chronic administration of MaR1 to DIO mice also caused a relevant increase in *Gpr120* and *Fgf21* mRNA levels in BAT.

BAT activation requires energy supply such as glucose and fatty acids. Indeed, insulin and cold exposure promote BAT glucose uptake, which is associated to BAT activation (Orava *et al.* 2011, Townsend and Tseng 2014). Here, we report that chronic administration of MaR1 to DIO mice upregulated BAT expression of glucose transporters *Glut1* and *Glut4,* suggesting increased glucose uptake. Indeed, our studies in cultured brown adipocytes clearly showed that MaR1 promoted glucose uptake in a similar extent to insulin. Additionally, MaR1 also promoted fatty acid uptake and fatty acid oxidation rates, along with upregulated expression of genes involved in fatty acid

oxidation, mitochondrial biogenesis and thermogenesis. All these data clearly evidenced that MaR1 activates brown adipocytes both *in vivo* and *in vitro*. These outcomes were further reinforced with the results from PET studies, which clearly demonstrated that acute MaR1 administration further stimulated cold-induced BAT glucose uptake, as indicator of BAT functional activation in mice.

There are strong evidences that BAT could have a relevant physiological role as a glucose and triglycerides clearance organ (Townsend and Tseng, 2014; Bartlet et al., 2011). Indeed, BAT activation has been proposed as a strategy against obesity and also to improve glucose homeostasis and insulin sensitivity (Stanford and Goodyear 2013, Bartelt and Heeren 2014). Our current data suggest that the higher BAT activation induced by MaR1 could also account for the glucose lowering and insulin-sensitizing properties of this lipid mediator. Probably, longer periods of treatment with MaR1 would be required in order to observe body weight changes as a consequence of increased BAT activity.

The present study also reveal that MaR1 is able to induce a more thermogenic beige pattern in WAT, by increasing UCP1 protein levels and the upregulation of master regulators genes involved in mitochondrial biogenesis in parallel with beige-specific markers in DIO mice. Furthermore, MaR1 promoted expression of genes typifying the beiging process in hMSC-derived adipocytes incubated with MaR1 along the differentiation process. However, mature hMSC-derived adipocytes treated acutely did not display these effects, suggesting that MaR1 induces a recruiting of brite adipocytes to promote browning and not by stimulation of mature white to beige adipocytes transdifferentiation. Nevertheless, fully differentiated hMSC-derived adipocytes acutely treated with MaR1 presented greater fatty acid oxidation rates, proposing that MaR1 promotes lipid burning in mature hMSC adipocytes.

Additionally, other important finding of this research it is the fact that the n-3 PUFA, EPA may also promote browning in WAT. This study provides evidence that EPA induces a remodeling of adipocyte metabolism downregulating fat storage genes while promoting fatty acid oxidation genes, mitochondrial biogenesis and importantly, beige-like markers in human subcutaneous adipocytes from overweight subjects. In line with our observations, several recent manuscripts have also demonstrated that treatment with EPA (both *in vitro* and *in vivo*) induced white fat browning and brown

adipocytes activation (Fleckenstein-Elsen *et al.* 2016, Quesada-López *et al.* 2016, Pahlavani *et al.* 2017). Nevertheless, it should be mentioned that the browning and thermogenic properties of EPA were not observed for DHA (Fleckenstein-Elsen *et al.* 2016, Quesada-López *et al.* 2016, Pahlavani *et al.* 2017). Therefore, our current data suggest differential effectiveness on the regulation of BAT function and brite adipogenesis for MaR1, a DHA-derived lipid mediator, and its n-3 PUFA precursor. Another important consideration is the fact that the effects of MaR1 on brown and white fat takes places at much lower doses than those required for n-3 PUFAs (nM *vs*. µM range). **Figure 27** summarized the main observations regarding the metabolic actions of MaR1.

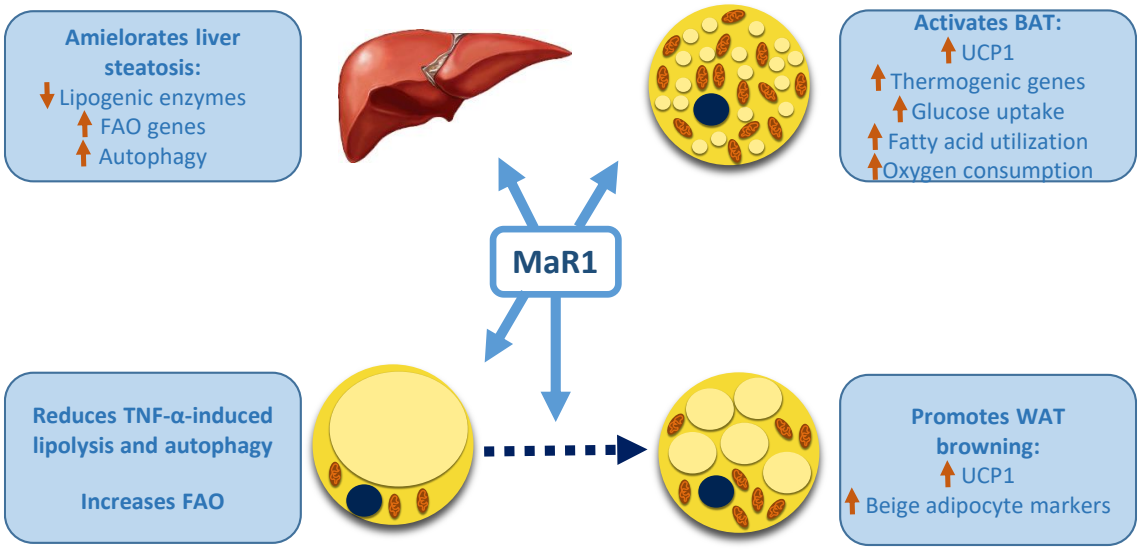

Figure 27. Summary of MaR1 actions in liver and adipose tissue.

Furthermore, the findings of this thesis open up several other avenues of research, including the characterization of the potential involvement of the SNS activation in the promotion of BAT function observed after *in vivo* administration of MaR1. Further research is also deserved to better characterize the MaR1 role in the regulation of immune-adipose interactions, which have been described as key regulators to increase fat thermogenesis (Qui *et al.* 2014). Moreover, it is important to find out if the BAT and beige activating properties of MaR1 are also shared by other

SPMs or are specific for macrophage-derived SPMs such as Maresins. Furthermore, it would be important to characterize if the risk to develop insulin resistance and NAFLD is related with lower levels of MaR1 or other SPMs. It would be also relevant to fully elucidate other mechanisms potentially involved in the beneficial actions of MaR1 on NAFLD and other obesity-associated comorbidities such as ER stress.

In summary, the main outcomes of the studies described in this Doctoral thesis revealed that: 1) EPA causes a remodeling of white adipocytes characterized by increased fatty acid oxidation, mitochondrial biogenesis and induction of beiging markers. 2) MaR1 counteracts TNF-α-induced alterations on lipolysis and autophagy in white adipocytes. 3) MaR1 ameliorates liver steatosis by decreasing lipogenic enzymes, while inducing fatty acid oxidation genes and autophagy, which could be related to AMPK activation. 4) MaR1 promotes BAT activation and switch on WAT browning, which could contribute to its insulin-sensitizing properties in obesity. All these findings strongly suggest that MaR1 might be a novel therapeutic agent to counteract obesity-associated inflammation and other metabolic disorders such as insulin resistance, type 2 diabetes and fatty liver disease.

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VI. CONCLUSIONS

1. EPA downregulates the expression of lipogenic genes (*FAS, SCD1* and *DGAT1-2)*, while upregulates genes involved in fatty acid oxidation (*CPT1A* and *ACOX1)*, which may prevent fat storage and promote fat burning in cultured human subcutaneous adipocytes from overweight/obese subjects.

2. EPA-treated adipocytes exhibit increased mitochondrial content along with an up-regulation of genes (*NRF-1, TFAM* and *COX IV)* and activation of master regulators (SIRT1, PGC1-α and AMPK) involved in the control of mitochondrial biogenesis. In parallel, EPA induces the expression of beige-related genes including *PRDM16, UCP1, CIDEA, TBX1* and *CD137.* These data suggest that EPA promotes a remodelling of adipocyte increasing mitochondrial biogenesis and beige-like markers in human subcutaneous adipocytes from overweight subjects.

3. MaR1 prevents TNF-α-induced glycerol release in 3T3-L1 adipocytes probably by preventing the decrease in both the lipid droplet-coating protein perilipin and the levels of the ATGL inhibitor G0S2, induced by the cytokine. Moreover, MaR1 reverses the cytokine-induced inhibition of phosphoHSL at Ser-565/total HSL ratio, while prevents the increased ratio of phosphoHSL at Ser-660/total HSL and phosphorylation of ERK1/2. These data suggest the ability of MaR1 to counteract the alterations induced by TNF-α on some of the main lipases and lipid droplet proteins controlling lipolysis.

4. MaR1 also counteracts the cytokine-induced decrease of p62 protein, an important autophagy indicator, and also prevents the induction of LC3II/LC3I, a key autophagosome formation marker, in TNF-α-treated 3T3-L1 adipocytes. These suggest that MaR1 can ameliorate the alterations induced by pro-inflammatory cytokines on autophagy in murine adipocytes.

5. *In vivo* treatment with MaR1 (intraperitoneal and oral gavage) reduces circulating levels of transaminases and liver triglycerides content in both *ob/ob* and DIO mice. Liver of MaR1-treated mice exhibits reduced protein content (FAS) or activation (by phosphorylation of ACC) of lipogenic enzymes, along with increased LC3II and reduced p62 protein levels. In DIO mice treated with MaR1 by oral gavage, these actions are accompanied by increased number of autophagic vacuoles, and upregulation of genes involved in autophagy (*Atg5* and *Atg7*) and fatty acid oxidation (*Cpt1a* and *Acox1*). These observations suggest that MaR1 reduces liver steatosis by decreasing lipogenic enzymes, while inducing fatty acid oxidation and hepatic lipophagy.

6. MaR1 also promotes AMPK phosphorylation in liver of DIO mice as well as after *in vitro* treatment of primary cultured hepatocytes. Interestingly, the stimulatory effects of MaR1 on fatty acid oxidation genes (*Cpt1a* and *Acox1*) and autophagy related genes (*Atg5* and *Atg7*) are inhibited by incubation of primary hepatocytes with the AMPK inhibitor Compound C. These facts suggest that the beneficial effects of MaR1 on liver fat accumulation could be in part mediated through AMPK activation.

7. Chronic treatment (10 days) with MaR1 (oral gavage) has profound effects on brown adipose tissue of DIO mice, characterized by increased UCP1 levels and upregulation of other thermogenic-related genes such as *Prdm16*, *Pgc1a* and *Dio2,* which was also accompanied by upregulation of glucose transporters (*Glut1-4*) and fatty acid oxidation-related genes (*Acox1*). Moreover, microPET studies with ^{18}F-FDG demonstrated that acute treatment with MaR1 potentiates cold-induced BAT activation in mice.

8. In cultured brown adipocytes, incubation with MaR1 also increases UCP1 protein content and upregulates thermogenic and mitochondrial biogenesis genes (*Tfam, Nrf1, Pgc1a, Ucp1, Gpr120* and *Fgf21*), as well as the oxygen consumption rate. These effects are accompanied with increased glucose uptake and upregulation of *Glut4* mRNA, and with a stimulation of both fatty acid uptake and oxidation and increased expression of *Cpt1a* and *Acox1*. Taken together, all these results strongly suggest the ability of MaR1 to activate brown adipocytes.

9. Chronic treatment (10 days) with MaR1 (oral gavage) induces beige adipocyte markers (*Ucp1*, *Pgc1a*, *Tmem26* and *Tbx1*) in subcutaneous WAT of DIO mice. Similar outcomes were observed in human mesenchymal cells (hMSC)-derived adipocytes treated with MaR1 along the differentiation process, but not in already mature hMSC-adipocytes treated with MaR1 for 24 h. These results point out that MaR1 could stimulate browning of WAT by promoting recruitment of beige adipocytes rather than inducing a transdifferentiation from white-to-brite adipocytes. However, mature hMSC-derived adipocytes treated with MaR1 (24 h) exhibit increased fatty acid oxidation rate and mitochondrial biogenesis-related genes.

GENERAL CONCLUSION

The current data demonstrate the ability of EPA to induce a remodelling of adipocyte metabolism characterized by the inhibition of fat storage genes and promotion of fatty acid oxidation genes, as well as by the increase in mitochondrial biogenesis and beige-like markers in human subcutaneous adipocytes from overweight subjects.

The current study also reveals that treatment with MaR1, a DHA-derived lipid mediator, could be helpful to reduce the alterations induced by pro-inflammatory cytokines on adipocyte lipid metabolism, particularly on TNF-α-induced lipolysis and autophagy.

On the other hand, MaR1 has been identified as a molecule able to ameliorate obesity-related liver steatosis in mice by decreasing lipogenic enzymes, while inducing

fatty acid oxidation genes and autophagy markers, which could be related to AMPK activation.

Finally, other relevant finding of this study is the characterization of MaR1 as an inducer of BAT activity and browning of WAT, which may contribute to its beneficial metabolic effects in obesity. In summary, MaR1 might represent a promising therapeutic candidate to tackle obesity comorbidities such as insulin resistance, type 2 diabetes and non-alcoholic fatty liver disease.

1. El EPA reprime la expresión de genes lipogénicos (*FAS, SCD1* y *DGAT1-2*), mientras que induce la expresión de genes implicados en la oxidación de ácidos grasos (*CPT1A* y *ACOX1*), lo que sugiere su capacidad para inhibir rutas que controlan el almacenamiento de grasa y promover aquellas implicadas en su oxidación, en adipocitos subcutáneos derivados de sujetos con sobrepeso/obesidad.

2. Los adipocitos tratados con EPA exhiben mayor contenido mitocondrial junto con un aumento tanto en la expresión de genes (*NRF-1, TFAM* y *COX IV*) como en la activación de factores (SIRT1, PGC1-α y AMPK) reguladores de la biogénesis mitocondrial. En paralelo, el EPA induce también la expresión de genes relacionados con el pardeamiento, tales como *PRDM16, UCP1, CIDEA, TBX1* y *CD137.* Estos datos sugieren que el EPA induce una remodelación de los adipocitos, caracterizada por un incremento de la biogénesis mitocondrial y de marcadores de adipocitos beige en adipocitos subcutáneos procedentes de sujetos con sobrepeso/obesidad.

3. MaR1 inhibe la liberación de glicerol inducida por el TNF-α en adipocitos 3T3-L1, lo que parece estar relacionado con sus acciones preventivas sobre con la disminución de perilipina, una proteína de la membrana de la gota lipídica, y de los niveles G0S2, inhibidor de ATGL, observados en los adipocitos tratados con dicha citoquina. Además, MaR1 revierte la disminución inducida por la citoquina del cociente fosfoHSLSer-565/HSL total, mientras que previene el aumento del cociente de fosfoHSLSer-660/HSL total y la fosforilación de ERK1/2 inducida por TNF-α. Estos datos sugieren la capacidad de MaR1 para contrarrestar las alteraciones inducidas por el TNF-α sobre algunas de las principales lipasas y proteínas de las gotas lipídicas que controlan la lipólisis.

4. MaR1, a su vez, contrarresta la disminución inducida por TNF-α en la proteína p62, un marcador de autofagia, y previene el aumento del cociente LC3II/LC3I, un marcador de formación de autofagosomas, observado en adipocitos 3T3-L1 tratados con TNF-α, lo que sugiere que MaR1 puede ser útil para mejorar las alteraciones inducidas por citoquinas pro-inflamatorias en la autofagia en adipocitos.

5. El tratamiento *in vivo* con MaR1 (intraperitoneal e intragástrico) reduce los niveles circulantes de las transaminasas y el contenido en triglicéridos hepáticos en modelos de obesidad genética (*ob/ob*) e inducida por la dieta (DIO). El hígado de los ratones tratados con MaR1 presenta un menor contenido (FAS) y menor activación (por fosforilación de ACC) de enzimas lipogénicas, junto con un aumento de LC3II y niveles reducidos de la proteína p62. En ratones DIO tratados con MaR1 por vía intragástrica, estas acciones están acompañadas por un aumento del número de vacuolas autofágicas, y el incremento de la expresión de genes implicados en la autofagia (*Atg 5* y *Atg7*), y en la oxidación de ácidos grasos (*Cpt1a* y *Acox1*). Estas observaciones sugieren que MaR1 reduce la esteatosis hepática al disminuir las enzimas lipogénicas, al mismo tiempo que induce la oxidación de ácidos grasos y la lipofagia hepática.

6. MaR1 también promueve la fosforilación de AMPK tanto en el hígado de ratones DIO como tras el tratamiento *in vitro* de cultivos de hepatocitos primarios. Además, los efectos estimuladores de MaR1 sobre genes implicados en la oxidación de ácidos grasos (*Cpt1a* y *Acox1*) y genes relacionados con la autofagia (*Atg5-7*) se bloquean en presencia del inhibidor de AMPK (Compound C) en los hepatocitos primarios. Estos hechos sugieren que los efectos beneficiosos de MaR1 sobre la acumulación de grasa hepática podrían deberse en parte a la activación de AMPK.

7. El tratamiento crónico (10 días) con MaR1 (vía intragástrica) tiene efectos muy relevantes en el tejido adiposo marrón de los ratones DIO, caracterizados principalmente por un aumento de los niveles UCP1 y de la expresión de otros genes relacionados con la termogénesis como *Prdm16, Pgc1a* y *Dio2,* los cuales se acompañan por una mayor expresión de los transportadores de glucosa *(Glut1 y Glut4)* y de genes relacionados con la oxidación de ácidos grasos *(Acox1).* Además, los estudios en microPET con ^{18}F-FDG demuestran que el tratamiento agudo con MaR1 potencia la activación del TAP inducida por el frío en ratones.

8. En cultivos de adipocitos marrones, la incubación con MaR1 también aumenta el contenido de proteína UCP1 y la expresión de genes involucrados en la biogénesis mitocondrial y la termogénesis *(Tfam, Nrf1, Pgc1a, Ucp1, Gpr120* y *Fgf21*), así como el consumo de oxígeno. Estos efectos estuvieron acompañados por un aumento de la captación de glucosa y en la expresión de *Glut4,* así como por una estimulación tanto de la captación de ácidos grasos, como de la oxidación de los mismos, asociada a un aumento de la expresión de *Cpt1a* y *Acox1.* En conjunto, todos estos hechos ponen de manifiesto la capacidad de MaR1 de incrementar la actividad de los adipocitos pardos.

9. El tratamiento crónico (10 días) con MaR1 (vía intragástrica) induce la expresión de marcadores de adipocitos beige *(Ucp1, Pgc1a, Tmem26* y *Tbx1)* en TAB subcutáneo de ratones DIO. Se observaron resultados similares en los adipocitos derivados de las células mesenquimales humanas (hMSC) tratados con MaR1 a lo largo del proceso de diferenciación, pero no cuando los adipocitos hMSC ya maduros se trataron con MaR1 durante 24 h. Estos resultados sugieren que MaR1 podría estimular el pardeamiento del TAB promoviendo el reclutamiento/formación de adipocitos beige desde precursores, en lugar de inducir una transdiferenciación de adipocitos blancos a beige. Sin embargo, los adipocitos maduros derivados de hMSC tratados con MaR1 (24 h) exhiben una mayor capacidad de oxidación de ácidos grasos y expresión de genes relacionados con la biogénesis mitocondrial.

CONCLUSIÓN GENERAL

Los resultados del presente trabajo demuestran la capacidad del EPA para inducir una remodelación del metabolismo de los adipocitos caracterizada por la inhibición genes implicados en el almacenamiento de grasas y la promoción de genes relacionados con la oxidación de ácidos grasos, así como por el incremento de marcadores de biogénesis mitocondrial y de adipocitos beige en adipocitos subcutáneos humanos procedentes de sujetos con sobrepeso/obesidad.

El presente estudio también revela que el tratamiento con MaR1, un mediador lipídico derivado del DHA, podría ser útil para reducir las alteraciones inducidas por citoquinas pro-inflamatorias sobre el metabolismo lipídico en adipocitos, particularmente sobre la lipólisis y la autofagia inducidas por TNF-α.

Por otro lado, la MaR1 se ha identificado como un agente capaz de mejorar la esteatosis hepática relacionada con la obesidad en ratones al disminuir enzimas lipogénicas, al tiempo que induce genes de oxidación de ácidos grasos y marcadores de autofagia, lo que podría estar relacionado con la activación de AMPK por MaR1.

Por último, otro hallazgo relevante de este estudio es la caracterización de MaR1 como un agente inductor de la actividad del TAP y del pardeamiento de TAB, lo que podría también contribuir a sus efectos metabólicos beneficiosos en la obesidad. En resumen, MaR1 podría ser un candidato terapéutico prometedor para combatir la obesidad y sus comorbilidades asociadas como la resistencia a la insulina, la diabetes tipo 2 y la enfermedad de hígado graso no alcohólico.

VII. THESIS SUMMARY

The present study aimed to determine the potential ability of EPA to remodel metabolism and phenotype of human subcutaneous adipocytes, as well as to characterize the ability of MaR1 to counteract inflammation-induced adipocyte disorders and to elucidate MaR1 actions on brown/beige adipose tissue and liver function in animal models of obesity and in culture cells.

This research demonstrated the ability of the n-3 PUFA EPA to increase mitochondrial content, and to activate master regulators of mitochondrial biogenesis and to promote the expression of genes that typify beige adipocytes in cultured fully differentiated human subcutaneous adipocytes from overweight subjects. Moreover, EPA up-regulated genes involved in fatty acid oxidation while down-regulated lipogenic genes. These data suggest that EPA promotes a remodelling of adipocyte metabolism which could be in part responsible for EPA beneficial effects in obesity.

Moreover, this research revealed for the first time that MaR1 was able to inhibit TNF-α-induced lipolysis. This effect seems to be associated to MaR1 ability to prevent the reduction of lipid droplet-coating protein perilipin and ATGL-inhibitor G0S2 protein expression induced by the cytokine. MaR1 also reversed the decrease on total hormone sensitive lipase (total HSL), and the ratio of phosphoHSL at Ser-565/total HSL, while preventing the increased ratio of phosphoHSL at Ser-660/total HSL as well as the phosphorylation of ERK1/2 induced by TNF-α. Moreover, MaR1 counteracted the cytokine-induced decrease of p62 protein content, a key autophagy indicator, and also prevented the induction of LC3II/LC3I ratio, an important autophagosome formation marker. These data point out that MaR1 might ameliorate TNF-α-induced alterations on lipolysis and autophagy in adipocytes, which could also contribute to the beneficial actions of MaR1 on adipose tissue inflammation and insulin sensitivity.

Furthermore, the current study also demonstrated the beneficial effects of MaR1 in reversing obesity-related liver steatosis in two different models of obesity (*ob/ob* and diet-induced obese (DIO) mice) and characterized the mechanisms involved. Interestingly, in *ob/ob* mice, MaR1 (2-10 µg/kg i.p., 20 days) reduced liver triglycerides content, FAS and SCD-1, while increased ACC phosphorylation and LC3II protein expression, in parallel with a drop in p62 levels. Similar effects on hepatic TG, ACC phosphorylation, p62 and LC3II were observed in DIO mice after MaR1 i.p. injection (2 µg/kg i.p., 10 days). Interestingly, oral gavage of MaR1 (50 µg/kg, 10 days) also

decreased serum transaminases, reduced liver weight and TG content. MaR1-treated mice exhibited reduced hepatic lipogenic enzymes content (FAS) or activation (by phosphorylation of ACC), accompanied by upregulation of genes involved in fatty acid oxidation (*Cpt1α* and *Acox1*) and autophagy (*Atg 5* and *Atg7*), along with increased number of autophagic vacuoles and reduced p62 protein levels. MaR1 also induced AMPK phosphorylation in DIO mice and in primary hepatocytes, and preincubation of hepatocytes with the AMPK inhibitor Compound C reversed MaR1 effects on *Cpt1α, Acox1, Atg5* and *Atg7* expression, suggesting the implication of AMPK in MaR1 actions.

The present study also reported that MaR1 treatment by oral gavage (50 μg/kg, 10 days) to DIO mice increased brown adipose tissue (BAT) UCP1 levels and upregulated other thermogenic-related genes such as *Pgc-1α, Prdm16* and *Dio2,* along with an increase in the mRNA levels of glucose transporters and fatty acid oxidation-related genes. In this line, in cultured brown adipocytes MaR1 also promoted glucose uptake and fatty acid utilization, in parallel with the upregulation of thermogenic genes and oxygen consumption rate. Interestingly, microPET studies with ^{18}F-FDG revealed that acute treatment with MaR1 potentiates cold-induced BAT activation in mice. Furthermore, MaR1 induced beige adipocyte markers (*Ucp1*, *Pgc-1α*, *Tmem26* and *Tbx1*) in subcutaneous white adipose tissue (WAT) of DIO mice as well as in human mesenchymal cells (hMSC)-derived adipocytes treated with MaR1 along the differentiation process. The fact that this effect was not observed when MaR1 treatment was tested on mature hMSC-derived adipocytes, point toward that MaR1 exerts its browning effect via recruiting brite adipocytes and not by promoting transdifferentiation from mature white to beige adipocytes. Nevertheless, mature white adipocytes treated acutely with MaR1 exhibited higher fatty acid oxidation rate. These data reveal MaR1 as a novel agent able to promote BAT activation and WAT browning, which could also contribute to its insulin-sensitizing properties in obesity.

In summary, the outcomes of the current project regarding the metabolic actions of MaR1 have uncover that MaR1 might constitutes a novel therapeutic candidate to tackle obesity comorbidities such as insulin resistance, type 2 diabetes and non-alcoholic fatty liver disease.