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MODULATION OF THE WNT/β-CATENIN PATHWAY IN OBESITY – A ROLE FOR BILE ACID METABOLIC IMPROVEMENT

Inês Catarina Marouvo Simões

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professor Doutor Carlos Palmeira (Universidade de Coimbra) e da Professora Doutora Anabela Rolo (Universidade de Aveiro)

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Abstract

Obesity is a pandemic public health problem, associated with a multitude of health problems such as cardiovascular disease, type 2 diabetes mellitus and other metabolic disorders. Considering the limited efficacy of current therapies to counteract the metabolic changes of obesity, new effective therapeutic strategies to increase energy expenditure and weight loss are under investigation. Bile acids and its anti-obesogenic effects have been recently associated with an increased number of multilocular UCP-1-positive fat cells in white adipose tissue depots. These cells, also known as brite or beige cells, induce a conversion of white adipocytes with typical fatty acid storage towards an oxidative phenotype, thereby stimulating energy metabolic fluxes and improving whole-body metabolism. However, the mechanism underlying bile acid metabolic effects are still unclear. In fact, it remains to be established whether they are the result of a direct action on the adipose tissue or a consequence of actions in other key metabolic organs such as liver. Due to the critical role of the Wnt/ β-catenin signaling pathway in adipogenesis and its protective effect against obesity and associated metabolic alterations, we proposed to address: if the bile acid chenodeoxycholic acid (CDCA) is able to decrease fat accumulation in 3T3-L1 adipocyte cultures; if these effects are dependent on UCP-1 increased mitochondrial oxidative capacity and the implication of the Wnt/ β-catenin signaling pathway. CDCA exposure during 96 h induced a concentration-dependent decrease in triglycerides accumulation in adipocytes, being this effect blocked when the Wnt/ β-catenin pathway was inhibited. The reduction on lipid accumulation caused by CDCA was associated with an increase in UCP-1 content, and consequent mitochondrial uncoupling. In the presence of XAV939, a blocker of the Wnt/ β-catenin pathway, CDCA was not able to decrease triglyceride accumulation neither to induce an increase in UCP-1 content. Despite UCP-1 induction, CDCA treatment did not increase the content in electron transport chain proteins neither reactive oxygen species generation; mitochondrial membrane potential was increased in cells incubated with 50 μM CDCA.

Importantly, our results showed for the first time that CDCA decreases lipid accumulation *in vitro,* an effect associated with increased UCP-1 content and involving the Wnt/β-catenin pathway. This favours the argument that CDCA action as general metabolic integrator is probably dependent on a direct effect on adipose tissue.

Keywords: adipocytes; bile acids; energy expenditure; canonical Wnt pathway.

Resumo

A obesidade é uma pandemia deste século, sendo um problema de saúde pública que está associado a outras doenças tais como doenças cardiovasculares, diabetes mellitus tipo 2 e outras doenças metabólicas. Considerando a limitada eficácia das atuais terapias no tratamento das alterações metabólicas associadas à obesidade, novas estratégias terapêuticas efetivas direcionadas para o aumento do gasto energético e perda de peso têm sido alvo de investigação. Os ácidos biliares e os seus efeitos anti-obesogénicos têm sido recentemente associados ao aumento de adipócitos multiloculares com expressão de UCP-1 em depósitos de tecido adiposo. Estas células, também conhecidas por células “brite” ou “beige”, induzem a conversão de adipócitos do tecido adiposo branco tipicamente com função de armazenar ácidos gordos em células com um fenótipo mais oxidativo. Esta conversão induz a estimulação dos fluxos metabólicos energéticos, conduzindo a uma melhoria do metabolismo de todo o organismo. No entanto, o mecanismo subjacente à ação dos ácidos biliares na indução destes efeitos não é ainda conhecido. Na realidade, está ainda por esclarecer se os efeitos dos ácidos biliares se devem a um efeito directo no tecido adiposo ou se por outro lado, são consequência da acção dos ácidos biliares em orgãos chave no metabolismo, tal como o fígado. Dado o papel que a via de sinalização Wnt/ β-catenina tem na adipogénese, assim como os seus efeitos protectores contra a obesidade e alterações metabólicas associadas, propomo-nos a perceber: se o ácido biliar, ácido quenodeoxicólico (CDCA), induz uma diminuição da acumulação de triglicerídeos em culturas de adipócitos 3T3-L1; se os efeitos do CDCA são dependentes de um aumento da capacidade oxidativa mitocondrial associada à UCP-1 e qual a implicação da via da sinalização Wnt/ β-catenina nestes efeitos. A exposição ao CDCA durante 96 horas induziu uma diminuição na acumulação de triglicerídeos nos adipócitos, sendo este efeito dependente da concentração de CDCA e bloqueado quando a via Wnt/ β-catenina foi inibida. A redução na acumulação de triglicerídeos induzida pelo CDCA esteve associada com um aumento do conteúdo em UCP-1, e um consequente desacoplamento mitocondrial. Na presença do XAV939, um inhibidor da via Wnt/ β-catenina, o CDCA não induziu uma diminuição da acumulação de triglicerídeos, nem um aumento do conteúdo em UCP-1. A exposição de 3T3-L1 a CDCA não alterou o conteúdo em proteínas da cadeia transportadora nem a produção de ROS; o potencial de membrana mitocondrial foi mais elevado em células incubadas com 50 µM CDCA.

Os nossos resultados mostraram pela primeira vez que o CDCA diminui a acumulação lipídica *in vitro*, estando este efeito associado a um aumento do conteúdo em UCP-1 e dependente da via da Wnt/ β-catenina. Assim, este estudo suporta o potencial efeito do CDCA como integrador metabólico, provavelmente através de um efeito direto no tecido adiposo.

Palavras chave: adipócitos; ácidos biliares; gasto energético; via Wnt/ β-catenina.

List of Abbreviations

^{18}F-FDG, ^{18}F-fluorodeoxyglucose
$\Delta\psi_m$, Mitochondrial membrane potential
AMPK, Adenosine monophosphate-activated protein kinase
AP-1, Activating protein-1
AR, Adrenergic receptor(s)
ATP, Adenosine-5'-triphosphate
BA, Bile acid(s)
BAT, Brown adipose tissue
BCA, Bicinchoninic acid
BMI, Body mass index
BMP, Bone morphogenetic protein(s)
BSA, Bovine serum albumin
C/EBP, CCAAT/enhancer binding protein
CA, Cholic acid
cAMP, cyclic adenosine monophosphate
CAR, Constitutive androstane receptor
CDCA, Chenodeoxycholic acid
cDNA, complementary DNA
CPT-1, Carnitine palmitoyltransferase-1
CRE, cAMP - response element
CYP7A1, Cholesterol 7 alpha-hydroxylase
D2, Type 2 iodothyronine deiodinase
DEX, Dexamethasone
DMEM, Dulbecco's modified Eagle medium
DMSO, Dimethyl sulfoxide
DNA, Deoxyribonucleic acid
ETC, Electron transport chain
EtHD-1 Ethidium homodimer-1
FABP, Fatty acid binding protein
FADH, Flavin adenine dinucleotide
FAT/CD36, Fatty acid translocase/ cluster of differentiation 36
FBS, Foetal bovine serum
FCCP, Carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone
FDA, Food and drugs administration

FFA, Free fatty acid(s)

FXR, Farnesoid X receptor

GLUT4, Glucose transporter type 4

H_2DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate

HIF-1α, Hypoxia-inducible factor-1α

HSL, Hormone sensitive lipase

IBMX, Isobutylmethylxanthine

IKK, IκB kinase

IL-6, Interleukin-6

IRS-1, Insulin receptor substrate-1

JNK, c-Jun NH_2-terminal kinase

LDH, Lactate dehydrogenase

LPL, Lipoprotein lipase

LRP5/6, Low-density lipoprotein receptor-related protein -5 or -6

MAPK, Mitogen-activated protein kinase

MCP-1, Monocyte-chemoattractant protein-1

mtDNA, Mitochondrial DNA

Myf5, Myogenic factor 5

NAD^+/NADH, Nicotinamide adenine dinucleotide, oxidized and reduced forms

NADPH, Nicotinamide adenine dinucleotide phosphate

NCS, New-born calf serum

NF, Nuclear factor

NP, Natriuretic peptide(s)

O_2, Molecular oxygen

OXPHOS, Oxidative phosphorylation

PAI-1, Plasminogen activated inhibitor-1

PBS, Phosphate buffered saline

PGC-1α, Peroxisome proliferator-activated receptor γ coactivator-1α

PKA, Protein kinase A

PPAR, Peroxisome proliferator-activated receptor

PRDM16, PRD1-BF-1-RIZ1 homologous domain-containing protein-16

PVDF, Polyvinylidene difluoride

PXR, Pregnane X receptor

RBP4, Retinol binding protein 4

ROS, Reactive oxygen specie(s)

SDS, Sodium dodecyl sulphate

SREBP, Sterol regulatory element-binding protein

T3, 3,5,3’-tri-iodothyronine

T4, Thyroxin

TBS, Tris-buffered saline

TBS-T, Tris-buffered saline-tween-20

TCF, T cell-specific factor

TG, Triglyceride(s)

TGR5, G protein coupled receptor

TMPD, Tetramethyl-p-phenylenediamine

TMRM, Tetramethylrhodamine methyl ester

TNF-α, Tumor necrosis factor-α

TZD, Thiazolidinedione

UCP-1, Uncoupling protein-1

VDR, Vitamin D receptor

VLDL, Very low-density lipoprotein

WAT, White adipose tissue

Wnt, Wingless-type mouse mammary tumor virus integration site family

CHAPTER 1

INTRODUCTION

Diets rich in caloric content associated with decreased physical activity have contributed to the high incidence of obesity. Data from the World Health Organization shows that this disorder is increasing at alarming rates, specifically, the worldwide obesity prevalence has doubled since 2008. This is due to the high occurrence of new cases of overweight and obesity not only in high-income countries but also in middle-income countries (World Health Organization, 2014). In Europe, it is estimated that over 50% of men and women were overweight, and approximately 23% of women and 20% of men were obese in 2008. Moreover, overweight is considered as the most common childhood disorder in the European region. Data estimates that over 60% of children who are overweight before puberty will be overweight in early adulthood (World Health Organization, 2011). As such, this disorder is considered to be one of the major public health problems in the world but not *per se*. This is related to the development of obesity-associated complications such as insulin resistance, high blood pressure and dyslipidemia. Together, these conditions constitute the metabolic syndrome, a known condition associated with an increased risk for type 2 diabetes, cardiovascular diseases and increasing mortality (Wilson et al., 2005).

Mitochondrial abnormalities have been reported in overweight and obese subjects (Goodpaster et al., 2001). The excessive nutrient supply increases lipid uptake into adipocytes and leads to an increase in mitochondrial activity with the generation of reactive oxygen species (ROS). The ROS accumulation mediates oxidative damage and compromises the mitochondrial function, number, morphology, and dynamics (Kusminski & Scherer, 2010). These alterations in mitochondria dysregulate the intracellular dynamic of the adipocyte and induces a markedly increase in the expression of genes coding for inflammation proteins (Bastard et al., 2006). Thus, a release of pro-inflammatory cytokines also contributes to an impairment of the insulin signaling pathway. The consequent release of fatty acids could trigger the development of insulin resistance in peripheral organs (Curtis et al., 2010). The excessive lipid accumulation promotes mitochondrial oxidative damage and a low oxidative capacity in skeletal muscle cells. Consequently, the generation of ROS and the accumulation of incompletely oxidized lipid intermediates interfere with the insulin signal transduction, and also contribute to the development of insulin resistance in liver and skeletal muscle and lipotoxicity in pancreatic β-cells (Meex et al., 2010).

Based on obesity and its related co-morbidities, intense research has been focused on therapies directed at improving mitochondrial function.

1.1 Adipose Tissue

1.1.1 White adipose tissue

1.1.1.1 Physiology

White adipose tissue (WAT) constitutes the majority of adipose tissue in the body and is considered the main organ responsible for energy storage (Trayhurn & Beattie, 2001). The tissue is located primarily in subcutaneous and in visceral depots, with other various distributed sites throughout the body. Depending on its location, the heterogeneity and the metabolic function of each depot are variable (Bjørndal et al., 2011).

WAT is composed by vascular-stromal fraction and mostly by mature adipocytes, but also fibroblasts, adipocyte precursors, endothelial cells and macrophages (Cristancho & Lazar, 2011). Adipocytes are unilocular (i.e., typically containing a single fat dropet) cells with a rounded appearance. The cells have few mitochondria and the larger lipid droplet occupies the most part of the cell volume with the nucleus and other cellular structures at the cell's periphery (Church et al., 2012). The lipid droplet is a specialized structure for triglyceride (TG) storage that can increase in size and in volume. It is constituted by a phospholipid bilayer that surrounds the hydrophobic core, which contains neutral lipids and sterol esters. The lipid droplet is coated by proteins specialized in the TGs stabilization namely caveolin and perilipin. Moreover, lipid droplets have specific machinery to the production and release of its lipid content (Walther & Farese Jr, 2009).

1.1.1.2 Function

1.1.1.2.1 Classical role

The primary role of WAT is the storage of TGs. During postprandial periods, glucose and lipids are converted into TGs via lipogenesis and subsequently, stored in the lipid droplet. The storage of large quantities of energy in the form of lipids occurs by the expansion of existing adipocytes (hypertrophy) and by the formation of new adipocytes from existing pre-adipocytes (hyperplasia). Conversely, in periods of food deprivation, the lipolysis of TGs is stimulated, occurring the release of glycerol and free fatty acids (FFAs) into circulation to maintain energetic homeostasis (Hajer et al.,

2008). It is noteworthy that mitochondria play a pivotal role in *de novo* lipogenesis, re-esterification of fatty acids and lipolysis. During *de novo* lipogenesis, mitochondria generate the key intermediates for the synthesis of TGs. More specifically, pyruvate is converted into acetyl-CoA in the matrix, while glycerol-3-phosphate is obtained via glyceroneogenesis. Furthermore, mitochondria are also responsible for lipid β-oxidation during lipolysis (Nye et al., 2008).

Besides the role of WAT in the storage of TGs, this tissue is also involved in other physiological processes such as coagulation, blood pressure and inflammation (Hajer et al., 2008).

1.1.1.2.2 Endocrine function

All the functions that have been already mentioned are, to some extent, regulated by a larger number of adipokines secreted by adipocytes. These hormone-like molecules have a paracrine or autocrine effect, depending on its site of action (Church et al., 2012).

Leptin is one of the hormones secreted by adipocytes. Its plasma levels are correlated with the amount of total body fat and are increased during fed status and decreased during fasting situations. This hormone is involved in the regulation of food intake and fat mass (Trayhurn & Beattie, 2001). Leptin production is stimulated by insulin, resulting in the suppression of food intake and the stimulation of energy expenditure. Leptin's actions are mediated by leptin receptors, which are mainly located in the central nervous system where the metabolic signals induce the expression of specific neuropeptides/neurotransmitters (Ahima & Flier, 2000). The integration of these signals between hypothalamus and peripheral tissues is mediated by the activity of hypothalamic adenosine monophosphate-activated protein kinase (AMPK), a well-studied and established metabolic sensor (Towler & Hardie, 2007). While hypothalamic AMPK is responsible for reducing food uptake, its activation in peripheral tissues is associated with energy production processes during periods of starvation (Long & Zierath, 2006). Therefore, leptin mediates the induction of lipid oxidation in the liver, lipolysis in adipose tissue and lipid oxidation and glucose uptake in skeletal muscle (Rasouli & Kern, 2008). When plasma leptin concentrations fail to suppress feeding or mediate weight loss, this leads to the occurrence of an obese state with leptin contributing to the production of pro-inflammatory cytokines (Lancha et al., 2012). In addition, leptin has a role in systemic effects namely in the modulation of T-cell immune response (Ahima & Flier, 2000).

Another important protein exclusively produced by adipocytes is adiponectin. This protein is highly expressed in lean organisms, so its levels are inversely related to the body fat mass. Adiponectin plays a role in insulin sensitization (Matsuda & Shimomura 2013). The mechanism involves the binding of adiponectin to specific transmembrane receptors located in skeletal muscle and liver. Hormone binding triggers AMPK activation which reduces insulin receptor substrate-1 (IRS-1) inhibitory serine phosphorylation and activates the expression of peroxisome proliferator-activated receptor (PPAR)-α target genes (Wang et al. 2007). Adiponectin inhibits hepatic glucose uptake, increases fatty acid oxidation in muscle and liver, enhances glucose uptake in muscle and stimulates energy expenditure (Galic et al., 2010). In addition to these actions, this hormone has anti-atherosclerotic functions based on its anti-inflammatory effects (Okamoto et al., 2002).

Moreover, WAT secretes other types of hormones such as resistin, retinol binding protein 4 (RBP4) and visfatin (Rasouli & Kern, 2008). However, the importance of these proteins in the development of insulin resistance phenotype is still less clear and poorly understood than those of leptin and adiponectin in humans.

Besides adipokine production, WAT also secretes cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte-chemoattractant protein-1 (MCP-1) and plasminogen-activated inhibitor (PAI-1) (Galic et al., 2010). These cytokines are highly secreted by the non-adipose cells when an increase in adipose tissue mass occurs. Thus, alterations in adipokines secretion and an increment in cytokines release are mainly associated with the chronic inflammation and insulin resistance phenotypes, typically observed in obesity (Fain, 2010).

1.1.2 Brown adipose tissue

1.1.2.1 Physiology

Brown adipose tissue (BAT) is referred as an energy dissipating tissue and its distribution is inversely correlated to the age and body mass index (BMI) (van Marken Lichtenbelt et al., 2009; Lowell & Flier, 1997).

Brown adipocytes are derived from multipotent mesenchymal stem cells and are distributed in different locations (Cypess & Kahn, 2010). The discrete location predominates in cervical-supraclavicular, perirenal/adrenal and paravertebral regions. These brown fat pads are closer to the major blood vessels and their cells derive from muscle progenitor cells - Myf5 (myogenic factor 5) expressing positive cells (Timmons

et al., 2007). On other hand, the already proven existence of brown adipocytes diffused in WAT depots have a different origin, since these cells derive from Myf5 negative cells (Seale et al., 2009; Ravussin & Galgani, 2011).

The induction of brown adipogenesis triggers a cascade of events that ultimately leads to lipid accumulation, mitochondrial biogenesis and the increase in expression and content of the hallmark protein of this tissue, the mitochondrial native uncoupling protein-1 (UCP-1), also known as thermogenin (Ravussin & Galgani, 2011). The key regulator of brown adipogenesis is the zinc-finger containing transcription co-factor PRD1-BF-1-RIZ1 homologous domain-containing protein-16 (PRDM16), which interacts with PPAR-γ and CCAAT/enhancer binding protein (C/EBP)-β (Lee et al., 2013). When fully mature, BAT cells express UCP-1, β_3-adrenergic receptors (ARs) and other BAT proteins such as type 2 iodothyronine deiodinase (D2) and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) (Anghel et al., 2007). These proteins play a critical role in the activation of thermogenesis, enhancing mitochondrial biogenesis and oxidative metabolic pathways in the cells.

BAT is physiologically and functionally different of WAT. It has a rich capillary network and is innervated by a high density of noradrenergic fibers. Brown adipocytes are small multilocular cells (small lipid droplets) with a polygonal shape, and a high mitochondrial content (Lee & Cowan, 2013). BAT mitochondria (Figure 1.1) are rich in laminar cristae and contain a unique protein, UCP-1, in the inner mitochondrial membrane. When activated, this protein dissipates the proton gradient of the intermembrane space to the mitochondrial matrix with high mitochondrial substrate oxidation mainly from fatty acids (Bouillaud et al., 1992). This process is a chemical energy dissipating process that uncouples electron transport through respiratory chain from ATP production, resulting in heat production - process known as thermogenesis.

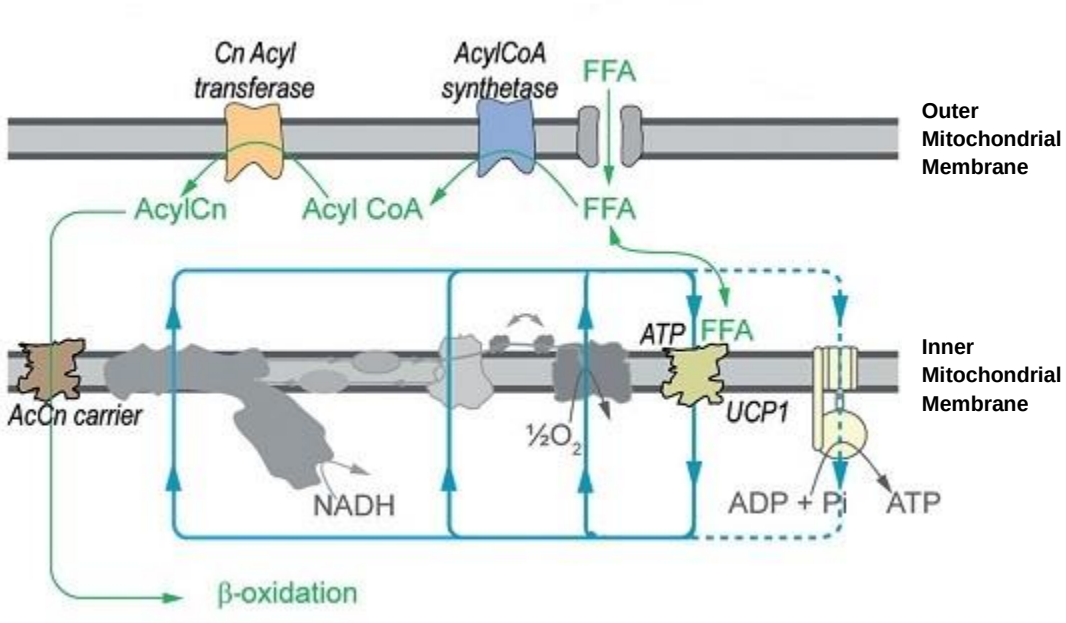

Figure 1.1 - Schematic representation of BAT mitochondrion. Free fatty acids (FFAs) are transferred into the mitochondria via the general activation of carnitine shuttle system. FFAs can bind to UCP-1, activating its condutance. In another way, acylcarnitine molecules are then oxidized in the mitochondria through β-oxidation, with the released acetyl CoA moities being oxidized in the citric acid cycle, generating reduced molecules of nicotinamide adenine dinucleotide (NADH). These molecules donate electrons to Complex I of the mitochondrial respiratory chain. The blue arrows illustrate the protonic (H^+) flow between the matrix and the inter-membrane space, as consequence of the electron transport to the final acceptor, molecular oxygen (O_2). Protons can reenter the mitochondrial matrix through ATP synthase, generating adenosine-5'-triphosphate (ATP) or, in another way, protons can reenter through uncoupled protein-1 (UCP-1). ADP, Adenosine diphosphate; P1, Inorganic phosphate (Adapted from Nicholls, D. G., 2013).

1.1.2.2 The renaissance of BAT in humans

Until a few years ago, it was believed that BAT was only present in larger mammals such as ourselves during childhood, and the amount of this tissue decreased during the ageing process, until it was virtually inexistent in adulthood (Lean, 1989). The small amounts of BAT in adults were considered absent or irrelevant to biological processes. However, using positron emission tomography in combination with computed tomography, Virtanen and collaborators demonstrated the existence of an highly active BAT in adult humans (Virtanen et al., 2009). During cold exposure, it was shown an increase in glucose uptake in some distinct areas, which coincided with an

increase in gene expression profile characteristic of BAT, namely the expression of the *UCP-1* gene. Furthermore, histological analyses confirmed the presence of multilocular cells filled with lipid droplets.

The existence of BAT in healthy adult individuals has supported the resurgence of global interest in the study of this tissue (Virtanen et al., 2009). The existence of activatable BAT in adult humans suggested that this capacity could have tremendous metabolic significance on body weight control.

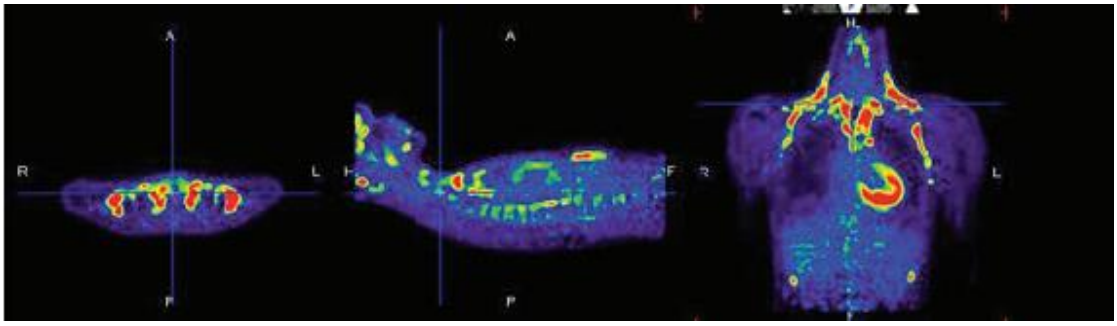

Figure 1.2 - Anatomical location of active BAT in humans measured by positron emission tomography in combination with computed tomography. From left to right, are represented a transversal slice of clavicles, a sagittal slice of the spine and a coronal slice of the thorax. The active BAT are represented by red and green areas (Adapted from Ravussin & Galgani, 2011).

1.1.2.3 Function

1.1.2.3.1 Thermogenic process

Considering that BAT is an energy efficient tissue for heat production in small mammals, it plays a role in body temperature regulation during periods of hibernation or torpor. This is a process of interest in hibernating animals and in newborns because of their necessity to maintain body temperature autonomically (Enerbäck, 2010).

The exposure to temperatures below thermoneutrality induces thermogenesis by two mechanisms, the shivering of skeletal muscle and the adaptative non-shivering in BAT (Saito, 2013).

The non-shivering thermogenesis of BAT implies sympathetic nervous system stimulation (Bartness, 2010). This process is also regulated by the thyroid hormone, being the process dependent on the activity and expression of D2 (Silva, 2006). This enzyme is responsible for the conversion of thyroxin (T4) into 3,5,3'-tri-iodothyronine (T3) which triggers the UCP-1 transcription (Ribeiro et al., 2000). Otherwise, during sympathetic stimulation, the activation of noradrenergic fibers present in BAT leads to

the release of norepinephrine. This cathecolamine binds to β_3-ARs expressed in brown adipocytes, and activates adenylate cyclase. The cyclic adenosine monophosphate (cAMP) activates protein kinase A (PKA) which in turn phosphorylates and activates the p38 mitogen-activated protein kinase (MAPK) pathway. The increase in the p38 MAPK activation enhances the action of transcriptional factors that regulates thermogenic gene expression. This regulation includes transcriptional induction of the genes encoding for UCP-1, components of the enzymatic machinery responsible for oxidative phosphorylation and cellular machinery responsible for the uptake of lipids and glucose (Cannon & Nedergaard, 2004). The action of PKA also includes the phosphorylation and activation of perilipin and hormone sensitive lipase (HSL). Activation of HSL induces its translocation to lipid droplets and consequently the hydrolysis of TGs into FFAs and glycerol (Vosselman et al., 2013).

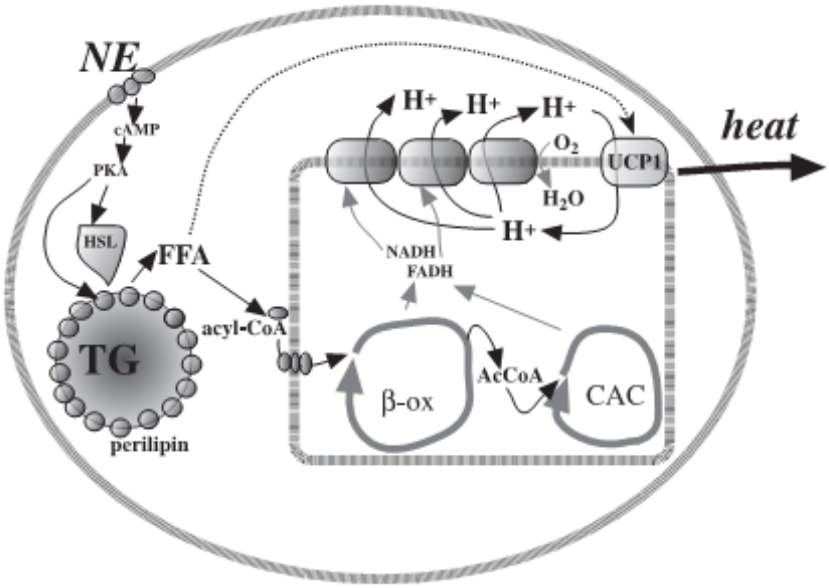

Figure 1.3 - Schematic representation of regulation of the thermogenic process through sympathetic nervous system stimulation. Norepinephrine (NE) activates protein kinase A (PKA), leading to the hydrolysis of triglycerides (TGs) into free fatty acids (FFAs) and glycerol. Despite its function as uncoupling protein-1 (UCP-1) activators, these molecules are oxidized in the mitochondria, resulting in the formation of reducing equivalents namely nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). The process results in the release of energy by heat production; cAMP, cyclic adenosine monophosphate; CAC, Citric acid cycle; H^+, Hydrogen ion; H_2O, Molecular water; HSL, Hormone sensitive lipase; O_2, Molecular oxygen (From Cannon & Nedergaard, 2004).

The released FFAs not only serve as activators of UCP-1 but are also the main substrate for thermogenesis (Vosselman et al., 2013).

The high availability of substrates and the numerous UCP-1-rich mitochondria of BAT contributes to the consumption of large amounts of fat and are associated with an intense thermogenic function (Cannon & Nedergaard, 2004).

1.1.2.3.2 Regulation of glucose and lipid metabolism

Several studies have pointed a role for BAT as a chemical energy dissipation tissue that promotes weight loss, controls body fat deposition and protects mice from diet-induced obesity, diabetes and insulin resistance (Ghorbani et al. 1997; Hamann et al. 1996). In fact, evidence showed a role for BAT in energy homeostasis since mice lacking BAT became severely obese (Hamann et al., 1996). Transgenic mice with markedly reduced BAT and UCP-1 expression also had impaired metabolic parameters as increased total body lipid content, hyperglycemia and hyperinsulinemia (Hamann et al., 1995). In accordance, it has also been shown that mice resistant to obesity and diabetes had higher amounts of BAT and UCP-1 (Kopecky et al., 1995).

In line with recent findings, BAT is involved in the regulation of plasma TG metabolism. The activation of BAT induces the clearance of triglyceride-rich-lipoproteins and fatty acids in blood of mice (Bartelt et al., 2011). A short-term cold exposure induces the channeling of lipid excess levels into BAT due to an increase in the expression of lipoprotein lipase (LPL) and fatty acid translocase/ cluster of differentiation 36 (FAT/CD36) that stimulate lipolysis and the uptake of lipids into brown adipocytes, respectively (Bartelt et al., 2011). BAT also plays a role in glucose metabolism. Cold induced an increase in glucose uptake in human brown adipocytes, being this process dependent on the expression of glucose transporter type 4 (GLUT4) (Orava et al., 2011).

Therefore, the activation of BAT and the enhancement of energy expenditure due to sympathetically activation have glucose and lipid-lowering effects. Considering all the metabolic effects of BAT, a role for this tissue in combating obesity and related disorders in rodents has been proved (Kopecky et al., 1995).

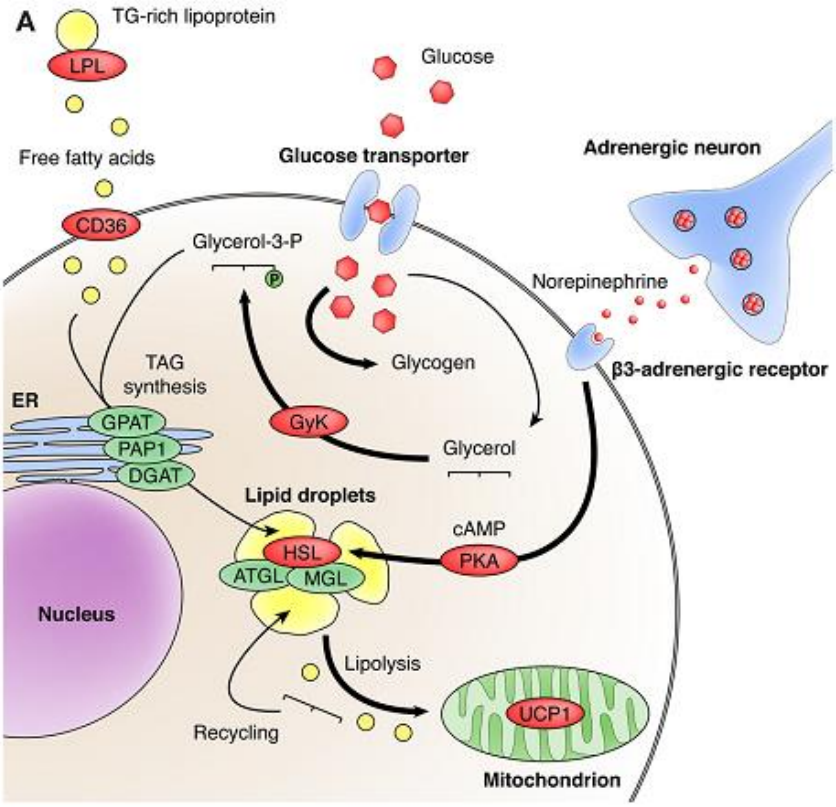

Figure 1.4 - Schematic representation of lipid and glucose metabolism in BAT induced by cold exposure. The cold exposure induced the clearance of serum lipids by increasing lipoprotein lipase (LPL) activity and free fatty acids (FFAs) uptake through fatty acid translocase/ cluster of Differentiation 36 (FAT/CD36). Otherwise, the sympathetic innervation also induces glucose uptake. The increase in glucose content contributes not only to the production of adenosine triphosphate (ATP) by anaerobic pathways but also to the glyceroneogenesis, contributing to the triglyceride (TG) synthesis. All this leads to increased lypolysis, leading to FFAs oxidation and energy expenditure in the mitochondria. ATGL, Adipose triglyceride lipase; DGAT, Diacylglycerol acyltransferase; GPAT, Glycerol 3-phosphate acyltransferase; GyK, Glycerol kinase; HSL, Hormone sensitive lipase; MGL, Monoacylglycerol lipase; PAP1, Lipid phosphate phosphatase 1; PKA, Protein kinase A; TAG, Triacylglycerol; UCP-1, Uncoupling protein-1 (Adapted from Festuccia et al., 2011).

Despite the study of the significance of BAT in humans is far from complete, it is demonstrated the inverse relationship between obesity and BAT. A detailed analysis showed that the prevalence and the activity of cold-activated BAT is inversely correlated with adiposity and also dependent of other parameters such as BMI, age and sex (Cypess et al., 2009). Moreover, it was demonstrated that a defective activation of BAT contributes to age-related accumulation of body fat (Yoneshiro et al., 2011). These findings suggest a role of BAT in the regulation of energy balance and

body fat content, protecting against body fat accumulation in humans as it has been demonstrated in mice.

1.1.2.4 BAT activation

BAT as a non-shivering thermogenic tissue has a role in energy balance in mice and humans. The metabolically active role of BAT is revealed by several studies in which BAT is a stimulator of energy expenditure and a glucose/fatty acids regulator (Bartelt et al., 2011; Kopecky et al., 1995). Then, the renewed attention in BAT and the advances in its function understanding reinforce the importance of this tissue as a potential tool with therapeutic benefits in the control of energy balance in humans. These recent observations have increased the search for strategies to activate and recruit BAT.

The capacity of BAT to be activated in humans is demonstrated firstly by cold-induced metabolically active BAT in healthy humans (Saito et al., 2009). Secondly, evidence showed that patients with pheochromocytoma, a cathecolamine-secreting tumor, had a high rate of ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake in BAT with an increase in metabolic rate and weight loss (Fukuchi et al., 2004). Indeed, cathecolamines stimulate BAT thermogenesis because when the tumor is resected, the ^{18}F-FDG uptake levels disappeared and the metabolic rate decreased (Cannon & Nedergaard, 2004). Thus, BAT is a flexible tissue with capacity to dissipate energy and can be activated in the presence of different stimuli. Cold exposure is the most widely known strategy to induce BAT activation. Adding to the effects of the acute response, a prolonged exposure to cold induces a chronic response characterized by higher UCP-1 levels, mitochondrial biogenesis and hyperplasia of brown adipocytes (Lowell & Flier, 1997). Another pathway is the activation of the adrenergic systems. However, treatment with β-agonists has not been successful (Arch, 2002). β_3-specific agonists are used to increase energy expenditure but somehow, these agonists do not reach the biological levels to activate the β_3-ARs. In addition, the overstimulation of these receptors has been linked to vascular events as heart attack and stroke (Cypess et al., 2012). Recent studies have focused on novel activators such as irisin, a protein secreted during exercise, and cardiac natriuretic peptides (NPs), a class of molecules produced in the heart (Boström et al., 2012; Whittle & Vidal-Puig, 2012). This data pointed to the action of both molecules at inducing energy expenditure, UCP-1 expression and stimulating brown fat-like cells development.

Although, the activation of BAT, via either environmental or pharmacologic, could be used to increase energy expenditure, many questions remain regarding about the efficacy, safety and durability of these treatments.

1.2 Obesity

1.2.1 Progression of adipose tissue dysfunction in obesity

The increase in adiposity in genetic or diet-induced obesity models has been linked to the insulin resistance phenotype (Guilherme et al., 2008). This cause-and-effect relationship pointed to an impairment of the secretory function of WAT as a cause of the metabolic alterations observed in peripheral tissues, specifically on skeletal muscle and liver (Langin, 2010).

Different factors contribute to the WAT dysfunction state (Cinti, 2012). Due to high caloric intake, the high-energy excess accumulation in adipocytes leads to adipocyte hypertrophy. As such, in an obese condition, the enlargement of adipocytes can *per se* promote an insufficient vascularization, compromising the oxygen supply of the cells (Cinti, 2012). Thus, in obesity, hypoxia is considered an upstream event from oxidative stress, endoplasmic reticulum stress, adipokine dysregulation, adipocyte death and inflammation (Wood et al., 2009; Fischer-Posovszky et al., 2011). This initial event (Figure 1.5) includes the recruitment of the transcription factor, hypoxic inducible factor-1α (HIF-1α). The molecular mechanisms induced by this factor in the presence of ROS, free fatty acids and pro-inflammatory cytokines are mediated through the activation of several intracellular signaling pathways that implicate nuclear factor (NF)-κB, IκB kinase (IKK), activating protein-1 (AP-1) and c-Jun NH_2-terminal kinase (JNK) (Cai et al., 2006; Guilherme et al., 2008). These transcription factors lead to alterations in gene expression pattern of adipocyte specific genes and also other genes related to inflammation and cellular dysfunction in adipose tissue during obesity (Guilherme et al., 2008). All these pathways could interact with insulin signaling via serine/threonine inhibitory phosphorylation of insulin receptor substrate-1 (IRS-1) (Bastard et al., 2006).

Obesity is associated with a pro-oxidant environment, being oxidative stress one of the major causes of mitochondrial dysfunction in adipocytes. The mitochondrion constitutes a major source of ROS that are considered as byproducts of aerobic respiration. During this process, as a result of imperfectly coupled electron transport associated with oxidative phosphorylation, the leakage of electrons principally from

complex I and complex III of the electron respiratory chain leads to reduction of a small proportion of oxygen molecules to superoxide anion ($O_2^{\cdot-}$) (Wood et al., 2009). This radical can react with other molecules and form secondary products such as peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$) (Green et al., 2004). Oxidative stress depends on the ratio between the production of these radicals and its removal by several cell defense mechanisms. These mechanisms include several antioxidant enzyme systems that are able to scavenge ROS (Li et al., 2013). However, its effectiveness is limited and when the levels of ROS increase, these radicals are able to induce mitochondrial oxidative damage of lipids, proteins and DNA (Curtis et al., 2014). Therefore, mitochondrial ROS can cause a decrease in mitochondrial ATP synthesis, dysregulate cellular calcium homeostasis, and induce the mitochondrial permeability transition pore. This leads to a compromised mitochondrial function and to the induction of cellular stress responses that ultimately causes apoptosis (James & Murphy, 2002).

In obesity, elevated levels of fatty acids may provide an additional source of excess oxidative phosphorylation substrates, inducing ROS production (Kahn & Flier, 2000). Therefore, elevated ROS appear to upregulate the expression of NADPH oxidase and decreased the activity of antioxidant enzymes which establish a vicious cycle that augments oxidative stress in WAT and blood (Furukawa et al., 2004). The increased ROS generation is linked to mitochondrial damage, causing reduced mitochondrial DNA (mtDNA) content, reduced electron transport chain (ETC) enzymatic activity as well as alterations in mitochondrial network (Curtis et al., 2014). As such, ROS production compromised all important mitochondrial functions in adipocytes, namely oxidative phosphorylation, β-oxidation and mitochondrial biogenesis (Choo et al., 2006). Moreover, oxidative stress is linked to a dysregulation of adipocytokines which includes an increase in the expression of IL-6, PAI-1, MCP-1 and a decrease in the expression of PPAR-γ and adiponectin (Furukawa et al., 2004). The increased expression of MCP-1, a potent chemoattractant for monocytes and macrophages, causes the infiltration of these cells and the inflammation of the adipose tissue (Furukawa et al., 2004).

Despite the effects on macrophage activity and in WAT metabolism, the alterations on cytokines production also cause alterations in other organs by systemic action (Furukawa et al., 2004).

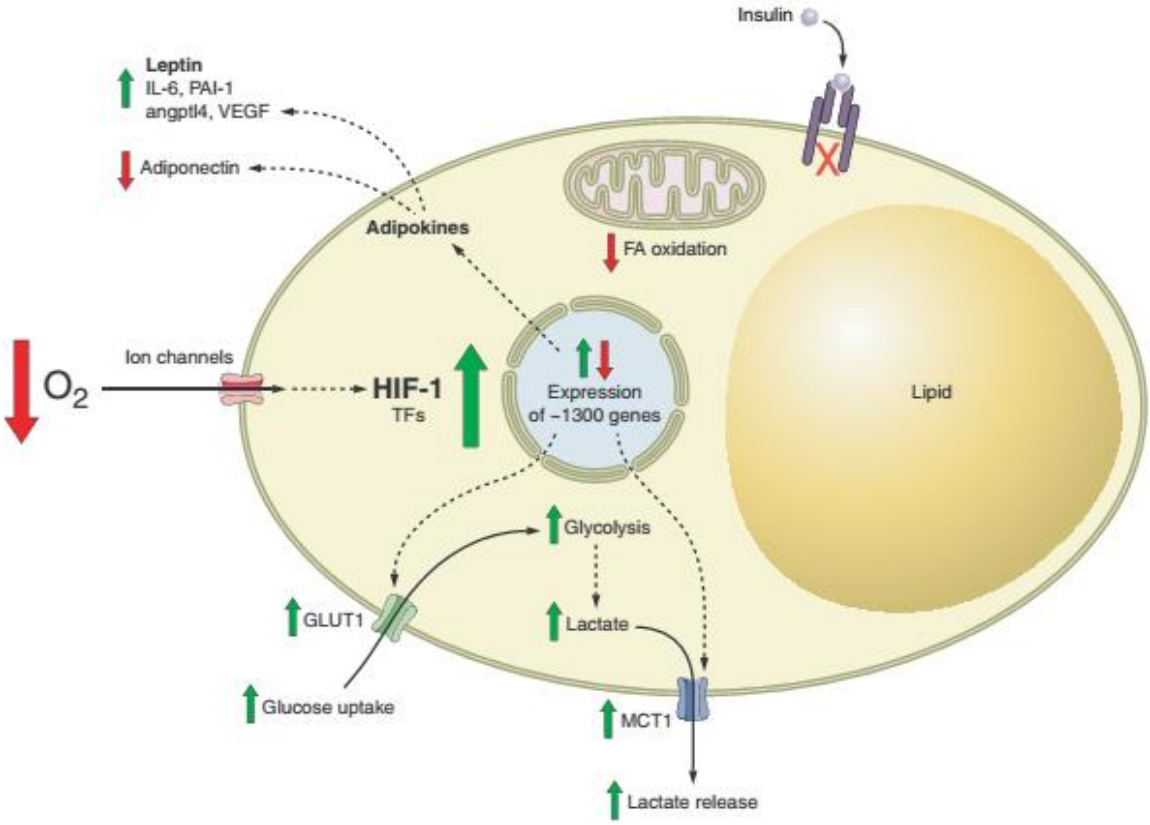

Figure 1.5 - Schematic representation of the events that leads to the adipose tissue dysfunction in obesity. The low levels of molecular oxygen (O_2) induce the production of transcriptional factors (TFs) that causes alterations in gene expression pattern. This includes alterations in the mitochondrial function, in the secretion of adipokines and in glucose metabolism. These effects are beyond the induction of a pro-inflammatory state as well as in the development of white adipose tissue (WAT) insulin resistance. Angptl4, Angiopoietin-like 4; FA, Fatty acid; GLUT1, Glucose transporter-1; HIF-1, Hypoxia-inducible factor 1; IL-6, Interleukin 6; MCT1, Monocarboxylate transporter 1; PAI-1, Plasminogen activator inhibitor-1; VEGF, Vascular endothelial growth factor (Adapted from Trayhurn, 2013).

1.2.2 Adipocyte dysfunction linking obesity to insulin resistance

Fischer-Posovszky and coworkers showed that adipocyte death (apoptosis or necrosis) is the initial event that leads to the macrophage recruitment and consequently, the inflammation process (Fischer-Posovszky et al., 2011; Cinti et al., 2005). The mobilization and infiltration of macrophages seems to be dependent on an increased production of ROS that increase the expression of MCP-1 (Xu et al., 2003). During this process, a switch of M2 macrophages towards M1 activated macrophages is induced (Lumeng et al. 2007). The M1 macrophages form crown-like structures

around dead adipocytes before the removal process by phagocytosis. Because of the size of adipocytes and the time required for its removal, a chronic low-grade inflammation is induced (Cinti et al., 2005). Interestingly, the degree of infiltration of macrophages is higher in visceral fat than in subcutaneous fat in an obese state. These observations are based on the capacity of visceral adipocytes to reach more rapidly its critical size based on its smaller size (Murano et al., 2008). Hence, visceral fat accumulation is associated with an increased cardiovascular risk.

The adipose-specific inflammation seems to be an important step in the insulin resistance phenotype and obesity related complications (Xu et al., 2003). During obesity, the local increase in oxidative stress in accumulated fat causes the dysregulated production of adipocytokines. Adipocytes and macrophages secrete large amounts of TNF-α, IL-6, leptin, resistin and other pro-inflammatory cytokines while serum adiponectin levels are diminished in obesity (Hotta et al., 2000). Despite the action of other factors, TNF-α is considered the major factor responsible for the development of insulin resistance in WAT. It seems to interact with insulin cascade signaling *via* serine/threonine inhibitory phosphorylation of IRS-1, contributing to the insulin resistant phenotype (Hotamisligil & Spiegelman, 1994). In addition, TNF-α and alterations in adipokines secretion also contributes to reduce the ability of adipocytes to accumulate TGs, increasing the levels of circulating FFAs (Maassen et al., 2007).

Therefore, the high levels of circulating FFAs can be taken up and accumulate in skeletal muscle (Figure 1.6) and liver, contributing to general insulin resistance. The intracellular lipid accumulation has been linked to an increased fatty acid flux that leads to an accumulation of lipid intermediates (diacylglycerol, long-chain acyl CoAs and ceramides) in the cytosol (Szendroedi et al., 2012). These intermediates can interfere with the insulin-signaling cascade and muscle glycogen synthesis, promoting a decrease in the glucose uptake (DeFronzo & Tripathy, 2009). Moreover, the accumulation of FFAs induces deleterious effects on skeletal muscle mitochondrial function with a disruption in mitochondrial oxidative phosphorylation (Kelley et al., 2002).

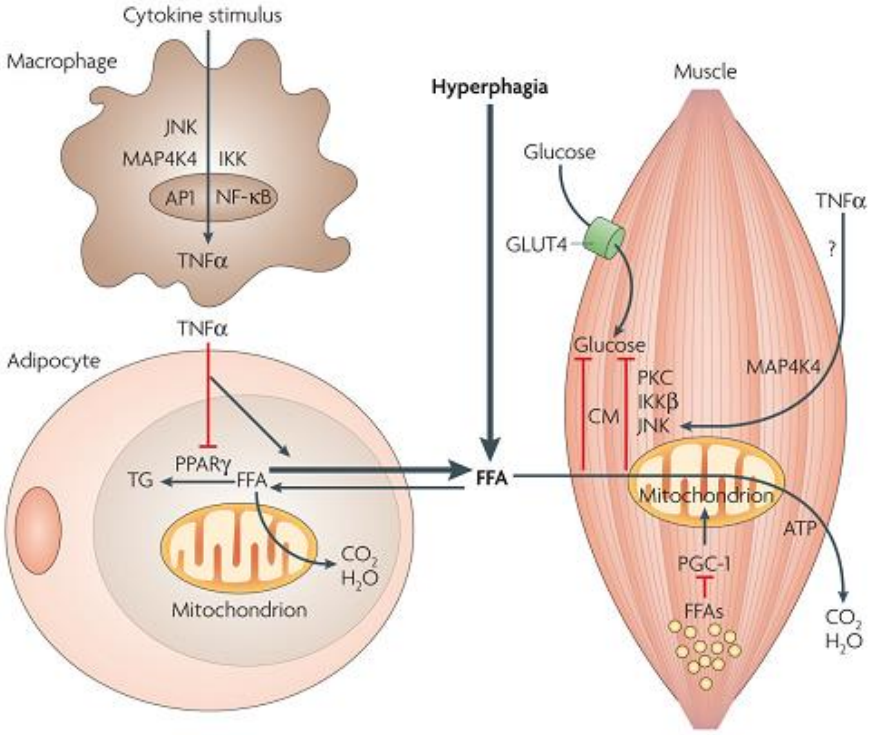

Figure 1.6. Chronic inflammation in adipose tissue triggers insulin resistance in skeletal muscle. The recruitment of macrophages also contributes to alterations in cytokine expression and secretion. Specifically, macrophages secrete tumor necrosis factor-α (TNF-α) which impairs triglyceride (TG) storage and increases lypolysis. The circulating free fatty acids (FFAs) can be taken up and accumulate in skeletal muscle cells. The accumulation of lipid intermediates can interfere with several pathways, inhibiting glucose uptake and imparing the expression of genes involved in mitochondrial function. AP1, Activator protein-1; ATP, Adenosine triphosphate ; CM, Ceramide; CO_2, Carbon dioxide; GLUT4, Glucose transporter type 4; H_2O, Water; IKK, IκB kinase; JNK, Jun N-terminal kinase; MAP4K4, Mitogen-activated protein kinase kinase kinase kinase-4; NF-κB, Nuclear factor-κB ; PKC, Protein kinase C; PGC-1, PPARγ co-activator-1; PPAR-γ, peroxisome proliferatoractivated receptor-γ; (From Guilherme et al. 2008).

1.3 Therapeutical strategies

1.3.1 Classical therapies

The development of different type of approaches, whether behavioral or pharmacologic, is required to counteract obesity and related disorders. The current obesity therapies are mainly focused on regulating energy intake and energy expenditure (Clapham & Arch, 2007).

The behavioral modifications are based in lifestyle modifications, which includes diet and physical activity. Although these type of alterations are potentially effective in weight loss, they are difficult to implement in obese people and sometimes not successful as desired (Godpaster et al., 2011).

On the other hand, pharmacologic approaches have showed limited efficacy besides adverse side effects. There are only three drugs (sibutramine, phentermine, and orlistat) approved by the food and drugs administration (FDA) for weight loss based on the reduction of energy intake or in the reduction of intestinal absorption. However, none of them have adequate long-term clinical efficacy (Cypess & Kahn, 2010). As such, other approaches have been developed to restore the correct equilibrium between energy intake and energy expenditure. Drug treatments based on thiazolidinedione (TZD) drug class, as pioglitazone and rosiglitazone, improve insulin sensitivity by several mechanisms. They promote adipocyte differentiation and enhance mitochondrial biogenesis, increasing lipid storage in WAT and fatty acid oxidation capacity. Although the significant effects on decreasing insulin resistance, these agents have associated some side effects (Willson et al., 2001). The bariatric surgery is an effective approach implemented in recent years but it is only suitable for severely obese people with co-morbidities. Although the metabolic improvement verified in those obese people, bariatric surgery is an invasive method with possible surgery complications (Sjöström et al., 2012).

The described approaches have associated risks and are not useful for all the obesogenic population. Thus, novel therapeutic tools based on targeting energy expenditure are being researched. Recently, these therapeutic tools are based on BAT characteristics to reduce adiposity in WAT (Zafrir, 2013).

1.3.2 BAT recruitment

The research community has described BAT and its targeting as an efficient approach in the treatment of dyslipidemia, obesity, and type 2 diabetes (Cypess & Kahn, 2010). These effects are beyond the metabolic effects of BAT by elevating whole-body energy expenditure and by regulating glucose and fatty acids levels in circulation. However, the activation of BAT is not an efficient weight loss strategy in overweight or obese humans (van Marken Lichtenbelt et al., 2009).

Instead of therapeutical strategies targeting the activation of BAT, novel approaches in order to stimulate and increase BAT mass in the body are under development. This is based on several studies that show that, in mice, the prolonged

cold exposure stimulation, treatment with β_3-AR agonists or PPAR-γ agonists protect mice from diet-induced obesity by inducing an increase in BAT mass and UCP-1 levels. (Petrovic et al., 2010; Kopecky et al., 1995). Moreover, the transplantation of BAT caused an improvement of the metabolic profile in mice, being these effects BAT mass dependent (Stanford et al., 2013). Thus, this increase in BAT mass improved glucose tolerance, increased insulin sensitivity and lean mass, and decreased leptin, triglycerides and cholesterol levels.

Several works have shown an increase in multilocular UCP-1-positive fat cells in anatomical sites corresponding to WAT (Barbatelli et al., 2010), referred as brite cells. One of the potential mechanisms to explain the origin of these cells is the direct conversion of mature white adipocytes into brown-like adipocytes through a transdifferentiation process that is called “browning” of WAT (Himms-Hagen et al., 2000). On other hand, it is suggested that brite cells could be derived from “masked” brown adipocytes in WAT that can be unmasked upon prolonged stimulation by the presence of a physiological or pharmacological stimuli that elevates the intracellular cAMP levels (Cousin et al., 1992). Although other processes to explain this phenomenon are still debated, the transdifferentiation theory is considered the main process responsible for white to brown adipocyte conversion. In this process, white adipocytes have the capacity to directly be transformed into mature cells with morphological and functional similarities of brown adipocytes (Cinti, 2012; Barbatelli et al., 2010). This is supported by data demonstrating that the appearance of this type of cells coincides with a decline in the number of white adipocytes simultaneously with an increase in the number of brown-like adipocytes at fat depots (Himms-Hagen et al., 2000; Frontini et al., 2013).

1.3.2.1 Brite cells

In the presence of a thermogenic stimulus, the “browning” of WAT induces the appearance of an intermediary phenotype in white cells, the UCP-1-positive brown-like adipocytes in WAT depots (Petrovic et al., 2010). These cells are called inducible, beige, or “brite” (from the combination of the words brown and white) cells and despite they are distinct from “classical” brown adipocytes, they are not considered as a distinct cell type on their own (Townsend & Tseng, 2012).

Brite cells have a developmental origin, characteristics and gene expression profile distinct from WAT and “classical” BAT. The brown-like adipocytes that arise in classical white adipose depots did not express the myogenic lineage marker Myf5, the

early marker of myogenesis (Barbatelli et al., 2010). This shows that brite cells have a different cell lineage from classical brown adipocytes which express Myf5, a common feature shared with skeletal muscle precursor cells. Thus, brite cells are probably directly induced from mature WAT, which the cell lineage is characterized as Myf5-negative cells (Seale et al., 2009).

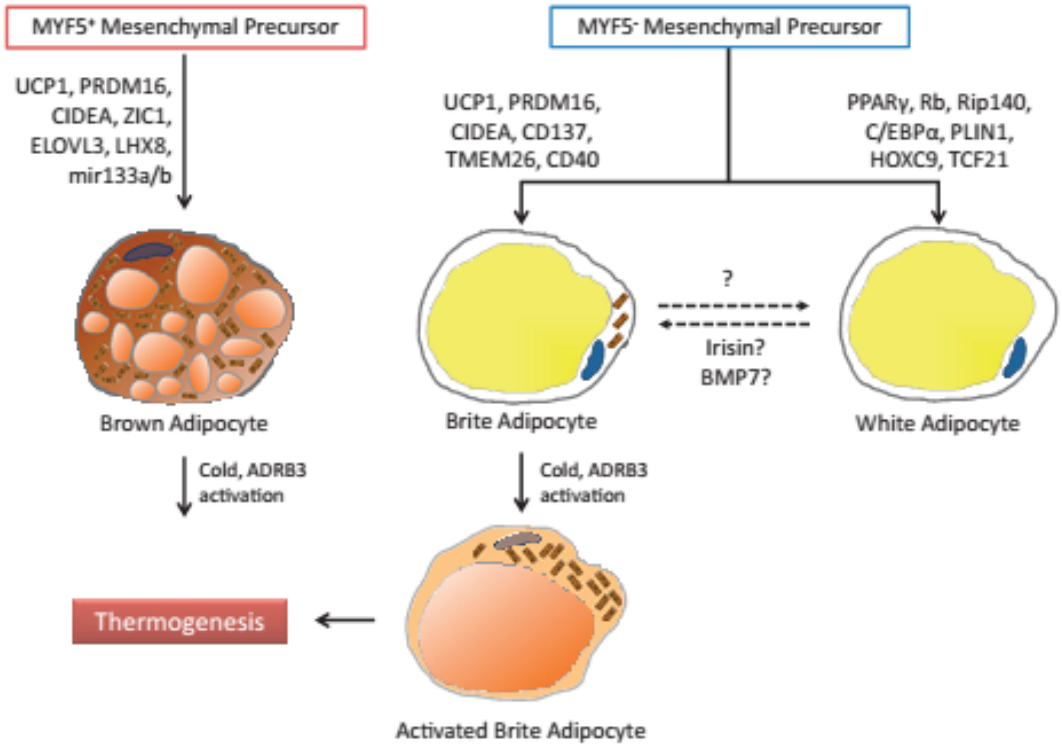

Figure 1.7 – Developmental origin of brite adipocytes. Myf5-negative mesenchymal precursors can be differentiated into brite cells or into white adipocytes, given the right stimuli. Furthermore, white adipocytes can undergo transdifferentiation into brite cells. The activation of these cells demonstrates that they are thermogenically competent cells, similar to brown adipocytes. ADRB3, β_3-adrenergic receptor; BMP7, Bone morphogenic protein 7 (From Peschechera & Eckel, 2013).

The majority of brown-like adipocytes in humans showed molecular and functional characteristics similar to brown adipocytes in BAT depots (Giralt & Villarroya, 2013). The brite cells are constituted by large cells with an irregular shape as well multiple and heterogeneous lipid droplets (Lehr et al., 2009). Moreover, these cells have high expression levels and proteic content of UCP-1 and mitochondrial oxidative enzymes (Petrovic et al., 2010). Some data points to the fact that the transdifferentiation process induces the appearance of mixed features between white

and brown adipocytes phenotype (Barbatelli et al., 2010). Although some differences, brite cells are competent cells in thermogenesis and in energy expenditure at the same levels of classical brown adipocytes (Giralt & Villarroya, 2013).

In terms of gene profile, the expression of regulators as PGC-1α, C/EBPβ or PRDM16 during the transdifferentiation process, contributes to a similar pattern of gene expression in brite cells when compared to brown adipocytes (Pisani et al., 2011). The expression of UCP-1 can be considered a cell marker of the transdifferentiation process. In addition to the expression of genes implicated in thermogenesis, the expression of other genes related to mitochondrial biogenesis is upregulated after this process. Interestingly, brite cells are also characterized by the expression of certain WAT genes (Lo & Sun, 2013). Although the up-regulation of BAT specific markers, it is noteworthy that brite cells store energy surplus and have a whiter adipocyte phenotype in the absence of a thermogenic stimulus. This capacity seems to provide evidence that the conversion of white adipocytes into brite cells is a bidirectional interconversion process (Lee & Cowan, 2013).

1.3.2.1.1 Regulation of the "browning" process

The process of transdifferentiation can be induced by the stimuli of a wide spectrum of molecules. These molecules have been reported to induce the activation of BAT function *in vivo*, but they have also been reported to induce beige cell development. (Hondares et al., 2011; Karamitri et al., 2009; Pisani et al., 2011).

A number of studies showed that the expression of some transcription factors is sufficient to the development of beige cells in mice and in pheochromocytoma patients (Sharp et al., 2012; Frontini et al., 2013). Transcriptional factors involved in the process are critically required at different stages of the transdifferentiation process, contributing to the expression of characteristic BAT markers, especially at mitochondrial level (Hondares et al., 2011). During this process, the transcriptional factors act by binding to conserved cAMP-response element (CRE) sites, promoting the transcription of CRE-containing genes which are required for full brown-like adipocyte formation (Karamitri et al., 2009). The main regulators of "browning" are PRDM16, PPAR-γ and PGC-1α (Lo & Sun, 2013).

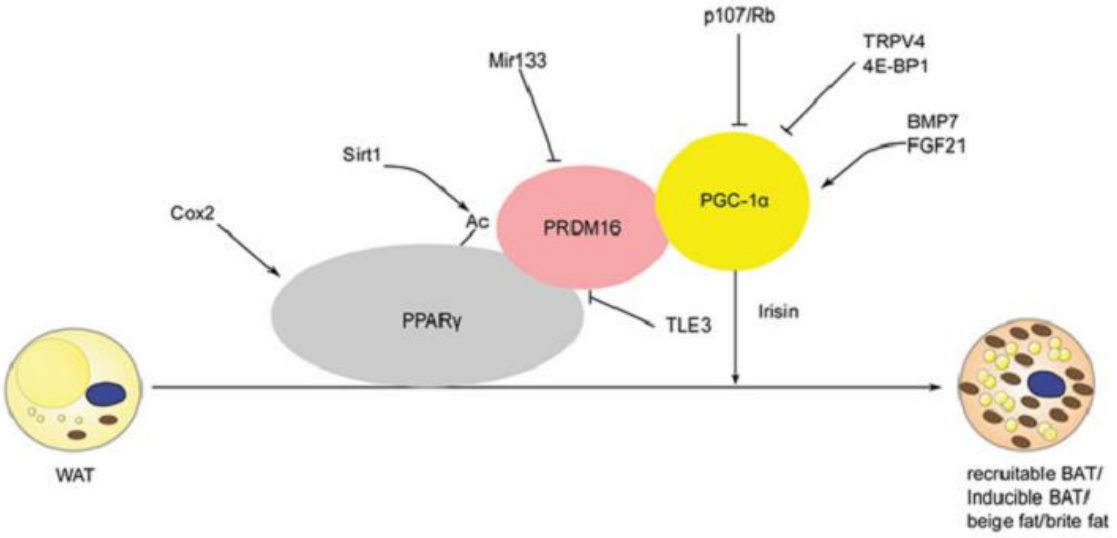

Figure 1.8 - The main "browning" transcriptional regulators involved in the transdifferentiation process. Peroxisome proliferator-activated receptor-γ (PPAR-γ), PR domain containing 16 (PRDM16) and PPAR-γ co-activator 1-α (PGC-1α) are involved in the induction of BAT genes and also in the repression of WAT genes. BAT, Brown adipose tissue; WAT, White adipose tissue (Adapted from Lo & Sun, 2013).

PPAR-γ is one of the transcriptional factors involved in the regulation of the transdifferentiation program. A full activation of this factor is required to enhance UCP-1, PGC-1α and other genes expression related to mitochondrial biogenesis (Nedergaard et al., 2005). PPAR-γ is not only involved in the induction of brown fat genes but also in the repression of white fat genes (Vernochet et al. 2009). In addition, the transcription cofactor PGC-1α also plays a role in the development of brite cells. Several studies showed that mice deficient in PGC-1α have impaired UCP-1 and mitochondrial genes expression while the ectopic expression of this factor induced an increase in mitochondrial and thermogenic genes expression (Puigserver et al., 1998; Tiraby et al., 2003; Uldry et al., 2006). Hence, PGC-1α is critical for the expression of genes involved in the regulation of mitochondrial biogenesis, oxidative metabolism and thermogenesis (Puigserver et al., 1998).

The development of brown adipocytes is also dependent of other transcriptional regulator - PRDM16. This factor seems to stimulate the appearance of brown-like adipocytes by association with other co-regulators as PPAR-α, PPAR-γ, PGC-1α and also C/EBPβ (Sharp et al., 2012). These interactions provoke the expression of thermogenic genes and others brown fat genes while causing the repression of white fat specific genes through association with co-repressors (Kajimura et al., 2008). As

such, PRDM16 seems to be the critical transcriptional factor for the development of brite cells and it is also a metabolic integrator on the regulation of thermogenesis in differentiated brown adipocytes (Hondares et al., 2011).

In another way, PPAR-α is also mentioned like an important transcription factor that plays a role in BAT development. This transcription factor is involved in the cellular lipid uptake and in mitochondrial and peroxisomal β-oxidation due to the interaction between PPAR-α and PRDM16 (Mandard et al., 2004; Seale et al., 2009). Furthermore, PPAR-α induces PGC-1α gene transcription which contributes to the induction of the thermogenic function (Barbera et al., 2001). Thus, while PPAR-γ seems to be important in the induction of thermogenic genes mainly in the early steps of BAT differentiation, the PPAR-α seems to be more important in the terminal steps. As such, PPAR-α induces fatty acid oxidation and consequently, it is a critical factor in fully differentiated brown adipocytes (Hondares et al., 2011).

All the factors described above are considered as brown-like adipocyte development key markers in the acquisition of a complete BAT-like phenotype. Hence, the development of brite cells leads to an increase in UCP-1, fatty acid oxidation enzymes and ETC proteins that contributes to a higher uncoupling and respiratory capacity (Tiraby et al., 2003). The energy dissipation that is associated with a higher metabolic rate in mitochondria contributes to a decrease in fat mass and to a possible amelioration of insulin sensitivity.

1.3.2.1.2 Mitochondrial Biogenesis

Mitochondrial biogenesis is a program that results in increased mitochondrial mass. This process involves the synthesis, import of protein and lipids and the replication of the mitochondrial genome. Mitochondrial biogenesis requires the integration of multiple transcriptional regulatory pathways controlling the expression of mitochondrial genome and nuclear encoded oxidative phosphorylation genes (Hock & Krali, 2009). This coordination is regulated by PGC-1α, a transcriptional co-activator of the expression of other transcription factors, namely, the nuclear respiratory factor (NRF)-1. NRF-1 is responsible for the activation of the expression of nuclear genes coding for electron respiratory chain (ETC) enzymes, for example β-ATP synthase, cytochrome *c*, cytochrome *c* oxidase subunit IV (Puigserver & Spiegelman, 2003). In addition, NRF-1 also regulates the expression of mitochondrial transcription factor A (TFAM). Its translocation into mitochondria induces the transcription and replication of

the mitochondrial genome (Wu et al., 1999). The co-ordinate regulation of these systems contributes is essential to a balanced assembly and function of mitochondrion.

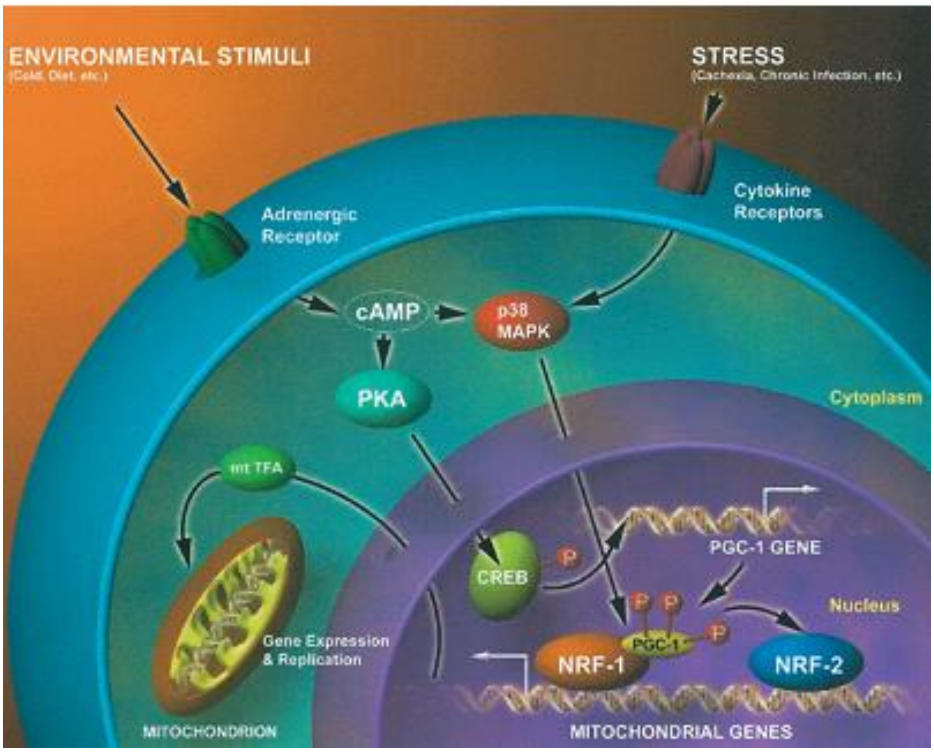

Figure 1.9 - The action of peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) in mitochondrial biogenesis. The expression of PGC-1α is induced by protein kinase A (PKA) and/ or p38 mitogen-activated protein kinase (MAPK) activation. This factor leads to the nuclear respiratory factor (NRF) -1/-2 expression, resulting in the expression of subunits of electron respiratory chain and in an increase of mitochondrial DNA replication and transcription. cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; mt TFA, Mitochondrial transcription factor A (From Puigserver & Spiegelman, 2003).

During the “browning process”, the combined action of PPAR-γ and PGC-1α induced mitochondrial biogenesis in white adipocytes (Lo & Sun, 2013). Thus, the expression of “browning” transcriptional regulators increase mitochondrial oxidative capacity, associated with increased expression of mitochondrial OXPHOS and fatty-acid oxidation enzymes (Hondares et al., 2011). In fact, the analysis of brite cells confirmed the existence of a high mitochondrial density, with mitochondria exhibiting an intermediate morphology between white and brown adipocytes (Frontini et al. 2013).

There is evidence that the mitochondrial dysfunction and consequently, the defective oxidative metabolism are involved in fat accumulation and in the development of insulin resistance during visceral obesity (Nisoli et al., 2007). Given that, it is

noteworthy that the induction of PGC-1α and mitochondrial biogenesis may be crucial for the restoration of mitochondrial function in adipocytes (Meex et al., 2010). The induction of UCP-1 in WAT and the stimulation of mitochondrial biogenesis have been shown to enhance mitochondrial content and activity and to stimulate oxidative capacity. In fact, it was showed an increase in the expression of cytochrome *c*, cytochrome *c* oxidase and the expression of mitochondrial fatty acid enzymes carnitine palmitoyltransferase-1 (CPT-1) and medium chain acyl-coenzyme A dehydrogenase (MCAD) under conditions of PGC-1α ectopic expression in adipocytes (Tiraby et al., 2003). Therefore, the high oxidative capacity and the energy expenditure in adipocytes enhance fat oxidation. It is noteworthy that the reduction in body weight is accompanied not only by an improvement in WAT function but also in the pathophysiology of several risk factors linked to obesity (Furukawa et al., 2004).

1.4 Wnt/ β-catenin signaling pathway

The wingless-type mouse mammary tumor virus integration site family (Wnt)/ β-catenin signaling is a pathway implicated in the differentiation and in the development of various cells types of numerous tissues. In particular, this pathway plays a role in white and brown adipogenesis (Bennett et al., 2002; Longo et al., 2004).

The Wnt/ β-catenin signaling pathway is regulated by Wnts and others secreted molecules. The Wnts are glycoproteins that bind to its receptor complex composed of Frizzled transmembrane receptors and low-density lipoprotein receptor-related protein -5 or -6 (LRP5/6) co-receptors which serves as docking site for axin (Clevers, 2006). This scaffold protein forms a cytoplasmic multi-protein complex which also comprises adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β). The stabilization of axin enhances the efficiency of GSK3β – mediated phosphorylation of β-catenin, the key mediator of the Wnt/ β-catenin pathway (Metcalfe & Bienz, 2011).

Although the central role that GSK3 plays in the canonical Wnt signaling, this kinase that consists of highly homologous α and β isoforms is involved in numerous cellular processes. Owing to its role in the regulation of the insulin signaling cascade, the modulation of GSK3 activity leads to alterations in insulin action. In fact, a high GSK3 activity is reported in insulin-resistant obese rodent models where the GSK3 activation causes the negative modulation of glycogen synthesis and glucose transporter activity (Henriksen & Dokken, 2006).

In the Wnt/ β-catenin signaling pathway, the absence of Wnt ligand inhibits the pathway. The glycogen synthase kinase 3β (GSK3β) is active and causes the

phosphorylation of β-catenin, which leads to its proteasome-mediated degradation. The phosphorylated β-catenin is targeted for ubiquitylation and subsequent proteasomal degradation. Hence, the inhibition of β-catenin translocation into the nucleus represses β-catenin-mediated transcriptional activation (Kang et al., 2005).

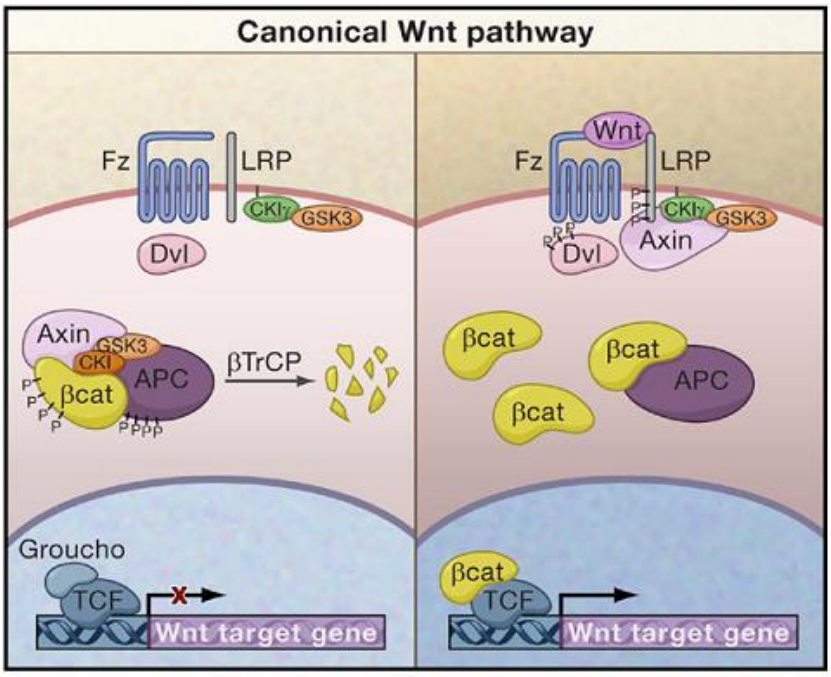

Figure 1.10 - The canonical Wnt signaling pathway. (Left panel) In the absence of Wnt ligands, β-catenin is recognized by the destruction complex and is phosphorylated by glycogen synthase kinase-3 (GSK3). Phosphorylated β-catenin is targeted for ubiquitination, being degraded at the proteosome level. In the nucleus, the transcription of Wnt target genes is inhibited. (Right panel) In the presence of Wnt ligands, β-catenin is stabilized, being translocated into the nucleus where by interaction with T cell-specific factor (TCF), promotes the transcription of Wnt target genes. β-TrCP, F box/WD repeat protein; APC, Adenomatous polyposis coli; CK1, Casein kinase I; Dvl, Disheveled proteins; Fz, Frizzled receptors; LRP, Low-density lipoprotein receptor-related protein (From Clevers, 2006).

In another way, the presence of Wnt ligands causes the activation of the Wnt/β-catenin signaling pathway. The binding of Wnt proteins to the Frizzled receptors and to LRP5/6 recruits Disheveled proteins to the plasma membrane (Figure 1.10). The Disheveled proteins interacts with axin which recruits its associated proteins, namely GSK3β (Metcalfe & Bienz, 2011). The inhibition of GSK3β induces the stabilization of β-catenin, causing the cytosolic accumulation of the hipophosphorylated β-catenin in the cytoplasm. After a while, β-catenin is translocated into the nucleus where it interacts with T cell-specific factors (TCFs), forming a co-activator complex that

regulates gene expression (Cristancho & Lazar, 2011; Prestwich & MacDougald, 2007). Recently, PPAR-γ is considered as an inhibitor of Wnt signaling pathway by interacting and stimulating the activity of GSK3β that in turn leads to proteasomal-dependent β-catenin degradation (Liu et al., 2006).

Although some questions remained to be addressed to understand the cascade of events that occurs after the induction of the master adipogenic transcription factors expression, it is known that the activation of the pathway do not appear to influence the transcription of C/EBPβ or C/EBPδ but inhibits the expression of PPAR-γ and C/EBPα (Kang et al., 2005). The activation of the pathway modulates the relative levels of cell type specific transcription factors involved in adipogenesis and brown adipogenesis while the inhibition of the pathway is required to induce these processes. It is noteworthy that the canonical Wnt signaling is regulated by several secreted inhibitors that influence the activation or the inhibition of the pathway (Chung et al., 2012). In fact, the overexpression of Wnt10b, an activator of the pathway, induces a reduction in adipogenesis and confers resistance to diet-induced obesity in mice (Wright et al., 2007). Alterations in Wnt signaling system have been associated with the development of obesity and type 2 diabetes (Christodoulides et al., 2009).

1.4.1 "Browning" process - Wnt/ β-catenin dependence

The activation of the canonical Wnt signaling pathway prevents the expression of critical transcription factors for the differentiation of brown adipocytes, such as C/EBPβ and PPAR-γ both *in vitro* and *in vivo* (Longo et al., 2004; Kang et al., 2005). Interestingly, this activation is also responsible for the suppression of UCP-1 expression in mature brown adipocytes which may be associated with the suppression of PGC-1α transcription (Kang et al., 2005).

The activation of the canonical pathway can be modulated at several levels by the expression of Wnt ligands or other molecules that interfere in the signaling cascade pathway (Ehrlund et al., 2013). For instance, the presence of endogenous activators of this pathway (*Wnt10a* and/or *Wnt10b*) or a specific GSK3β inhibitor, CHIR99021, promoted an impairment of BAT function (Longo et al., 2004). This included a reduction in the expression of brown adipocyte markers genes, namely PGC-1α. Moreover, the same study indicated that the activation of the Wnt pathway caused the suppression of mitochondrial and nuclear-encoded mitochondrial gene expression, some of which involved in fatty acid oxidation and also oxidative phosphorylation (OXPHOS) (Kang et al., 2005). Despite the absence of brown adipocyte phenotype, the cells presented

unilocular droplets and a continuous expression of white adipocytes markers in mice (Kang et al., 2005).

In line with previously findings that Wnt/ β-catenin signaling pathway regulates not only brown adipogenesis and brown adipocyte genes expression, but also the differentiation of brown into white adipocytes, it is suggested a role for this pathway in the "browning" process (Kang et al., 2005). Despite the scarce knowledge about the Wnt dependence, recent data supported this role by demonstrating the Wnt involvement in the plasticity of WAT (Kang et al., 2005). Hence, the modulation of this pathway might promote the expression of genes involved in the potentiation of beige cell development and the subsequent mitochondrial biogenesis (Ehrlund et al., 2013).

1.5 Bile acids

1.5.1 Synthesis and Function

Bile acids (BAs) are a family of steroid molecules that are produced in the liver, accumulated in the gallbladder and excreted into the small intestine (Mano et al. 2004). They are synthesized from cholesterol by the action of hepatic enzymes via classical pathway or an alternative pathway that seems to be less common (account only to 6% of BAs synthesis) (Mano et al., 2004; Schubring et al., 2012). The classical pathway consists of 7α-hydroxilation of cholesterol by cholesterol 7 alpha-hydroxylase (CYP7A1) and the subsequent reactions that include the modification of the ring structure of cholesterol, namely oxidation and shortening of the side chain, and the conjugation of the bile acid with an amino acid (Thomas et al. 2008). The resulting products are the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) which are converted into secondary bile acids by the action of intestinal flora (Fiorucci et al., 2009).

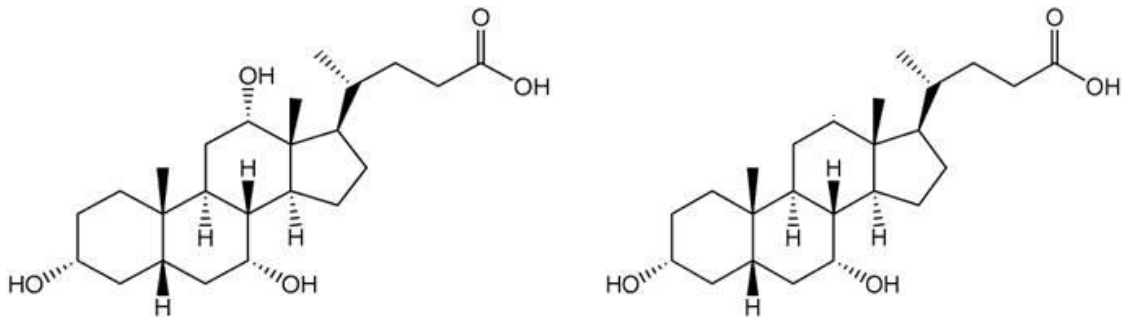

Figure 1.11 - Molecular structure of primary bila acids CA (left) and CDCA (right).

The conjugated BAs have different chemical properties and distinct biological activities. In the intestinal lumen, the amphipathic nature of BAs promotes digestion and the absorption of dietary lipids and vitamins. To maintain a functional pool of BAs, they are reabsorbed and transported back into the liver, being extensively recycled (Mano et al., 2004). Besides their function in dietary lipid absorption, in the regulation of their own synthesis and cholesterol homeostasis, BAs are responsible for the activation of several signaling pathways involved in lipid and glucose homeostasis (Trauner et al., 2010).

1.5.2 Bile acids and energy homeostasis

It is well established that BAs have a beneficial effect in the maintenance of metabolic homeostasis. Evidence shows that BAs are implicated in decreasing serum and hepatic levels of TGs in a model of obesity, insulin resistance and hypertriglyceridemia (Watanabe et al., 2004). These results are consistent with another study where the administration of CA prevented and reversed diet-induced weight gain with a decrease in adiposity (Watanabe et al., 2006). These effects are associated with an increase in the expression of several genes involved in the control of energy expenditure in BAT in mice (Watanabe et al., 2006). Recently, evidence showed that another bile acid, CDCA also reversed obesity and related disorders in mice. The CDCA administration induced an increase in UCP-1 content in BAT, being the beneficial effects of this steroid molecule justified at least in part by UCP-1 mediated thermogenesis (Teodoro et al., 2014). Moreover, in the same study, it was demonstrated that CDCA mediated the appearance of UCP-1 expressing cells in WAT (Teodoro et al., 2014).

The mechanism underlying these BAs effects has remained elusive. Some research has been focused on BAs as ligands of the farnesoid X receptor (FXR) and the G protein coupled receptor (TGR5). Therefore, BAs might be able to exert its function triggering the induction of the signaling cascade pathway activated by one of these receptors (Watanabe et al., 2004; Kawamata et al., 2003).

Based on the BAs beneficial effects in the whole body metabolism and considering its effects on elevating UCP-1 levels, BAs could contribute to a reduction in adiposity, acting as metabolic integrators in the reversion of dietary obesity and the stabilization of a lean phenotype (Watanabe et al., 2012). BAs seem to be a promising therapeutic approach for the clinical management of metabolic disorders.

1.5.2.1 Bile acids and FXR

FXR is a nuclear receptor that when activated by BAs protects against body weight gain and fat deposition in liver and skeletal muscle (Cipriani et al., 2010). These effects are attributed to an increase in the expression of genes involved in lipoprotein clearance while the genes involved in the biosynthesis of TGs are repressed (Claudel et al., 2005). In fact, the activation of FXR induces the apolipoprotein C2, a co-activator of LPL, which decreases serum levels of TGs and FFAs (Neuschwander-Tetri, 2012). In particular, in adipocytes, the activation of FXR induces an increase in adipose tissue storage by the expression of PPAR-γ, sterol regulatory element-binding protein (SREBP)-1c and fatty acid binding protein (FABP) (Rizzo et al., 2006). The activation of FXR in liver seems to induce the expression of PPAR-α and its target genes, increasing fatty acid oxidation and decreasing the capacity to secrete or store TGs (Teodoro et al., 2011). The role of FXR in lipid metabolism is supported by data showing that FXR deficient mice had increased serum and hepatic TGs, and cholesterol levels due to an increase in the production of VLDLs (Thomas et al., 2008; Neuschwander-Tetri, 2012). Moreover, the activation of FXR seems to have a key role in enhancing glucose uptake and promoting insulin sensitivity, counteracting insulin resistance in liver and skeletal muscle tissue in an obese animal model (Cipriani et al., 2010).

Despite the beneficial effects of BAs, recent studies provided controversial effects of synthetic FXR agonists when compared with BAs effects. In fact, the synthetic FXR agonist GW4064 exacerbates insulin resistance and glucose intolerance, inducing obesity and diabetes in high fat fed mice (Watanabe et al., 2011). In line with the above observations, evidence also showed an improvement of glucose

homeostasis in a FXR deficiency in obesity (Prawitt et al., 2011). These data suggest that BAs might exert its beneficial effects through a pathway independent of FXR.

1.5.2.2 Bile acids and TGR5

BAs are also ligands of TGR5. Diet supplementation with BAs in obese mice fed a high fat diet reversed diet-induced obesity, decreased WAT mass and improved glucose tolerance (Watanabe et al., 2006). The authors attribute these effects to the activation of TGR5.

The activation of this receptor leads to the activation of cAMP-PKA signaling pathway and the subsequent expression of *D2*, a thyroid hormone-activating enzyme. Thus, the conversion of T4 into T3 and the increasing levels of intracellular T3 induces the binding of transcription factors to specific sequences in the promoter of T3 responsive genes such as *UCP-1*, and *PGC-1α*, the master regulator of mitochondrial biogenesis (Watanabe et al., 2006; Casas et al., 2008; Hall et al., 2010). Thus, the signaling pathway TGR5-cAMP-D2 is reported as an interesting target in the regulation of energy expenditure in obese patients. This is in the line with recent findings where the administration of BAs induced a high consumption of O_2 simultaneously with an increase in mitochondrial activity and energy expenditure in brown adipocytes as well as in human skeletal myocytes (Watanabe et al., 2006; Blanchet et al., 2012).

Conversely, some reports have now questioned the role of TGR5 as a target for metabolic control. Interestingly, the exposure of BAs derivatives in mice lacking or overexpressing *D2* or *TGR5* has the same BAs effects (Sato et al., 2008; da-Silva et al., 2011). In addition, primary and secondary BAs appear activate TGR5 at high concentrations demonstrating a low affinity of BAs to this receptor (Kawamata et al., 2003). Hence, more research will be necessary to understand the mechanism through which BAs exert its beneficial metabolic effects.

1.6 Aims/ Objectives

Bile acids are signaling molecules implicated in the prevention and reversion of metabolic disorders. Its beneficial effects have been reported in a mice model of obesity, insulin resistance and hypertriglyceridemia (Watanabe et al., 2004). The authors attributed these effects in the whole body metabolism at least in part by UCP-1-mediated thermogenesis in BAT. Additionally, bile acids have also been shown to induce an increase in mitochondrial content and a higher expression of mitochondrial

biogenesis markers in BAT (Watanabe et al., 2006; Teodoro et al., 2014). Moreover, there is evidence that the natural bile acid CDCA induce the appearance of some UCP-1 positive adipocytes interspread in white adipose tissue. In line with these findings, BAs could induce UCP-1 expressing adipocytes, being the fatty acid storage phenotype of these cells converted towards a fat utilization phenotype. However, it remains to be established if the anti-obesity effect of CDCA is a direct effect in adipose tissue or a consequence of actions in other key metabolic organs. Moreover, although controversy remains, the canonical Wnt signaling pathway may be implicated in BAs effects.

In this work, we aimed to explore BAs effects in adipocytes cultures and their dependence on the Wnt/ β-catenin signaling pathway. With this in mind, CDCA-induced transdifferentiation was evaluated in the presence of an activator (CHIR99021) or an inhibitor (XAV939) of the Wnt/ β-catenin pathway.

Since mitochondria are a key organelle in the metabolism of the adipocytes and also in the development of adipocyte dysfunction during obesity, we addressed the effects of CDCA on adipocytes mitochondrial function.

Considering the growing interest in the development of therapeutic strategies to counteract the alterations that leads to obesity and adipocyte dysfunction, this study will help us to unravel BAs effects in adipocytes and the molecular mechanisms underlying such effects.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Except when the source of the compound is described, all compounds were purchased from Sigma-Aldrich (St Louis, MO). All other reagents were obtained with the highest grade of purity commercially available.

2.2 Cell line

3T3-L1 cell line (ATCC CL-173) was derived by clonal expansion from murine Swiss 3T3 cells. Due to its potential to differentiate from fibroblasts to adipocytes, 3T3-L1 cell line has been largely used as an *in vitro* model in adipocyte differentiation and in studies related to obesity and metabolic diseases (Green & Meuth 1974).

2.3 Cell culture

3T3-L1 cells were maintained in a pre-adipocyte expansion medium - Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM D-glucose, 30 mM sodium bicarbonate, 2 mM L-glutamine and 1 mM sodium pyruvate, with 1% antibiotic/antimycotic (Life Technologies, Carlsbad, CA) and 10% new-born calf serum (NCS) (Life Technologies). The cells were cultured in 75-cm^2 tissue culture flasks (Sarstedt, Nümbrecht, Germany) and maintained in a humidified atmosphere incubator with 5% CO_2 at 37 °C. The medium of the cells was changed every two days and when cells reached approximately 70-80% confluence, cells were detached with 0.05% trypsin (Life Technologies) and passaged with a typical 1:6 dilution. For assay preparation, cells were counted with an automated cell counter (TC10 Automated Cell Counter, Bio-Rad Laboratories, Hercules, CA) and seeded in 12-well plates (Orange Scientific, Braine-l'Alleud, Belgium) at a density of 50,000 cells per well.

Based on conventional protocols (Kovacic et al. 2011; Martini et al. 2012), the differentiation of the 3T3-L1 pre-adipocytes was not successfully achieved. The differentiation of the cells was too low and the cells detach easily from the plates which did not allow to proceed with the differentiation protocol neither cells treatment. In order to try to solve these problems, we tested some modifications in the protocol. In fact, we tested some protocols using the known commonly differentiative agents insulin,

dexamethasone (DEX), isobutylmethylxanthine (IBMX) and rosiglitazone. The protocols tested were summarized in Table 2.1.

Table 2.1. Protocols tested for the effective differentiation of 3T3-L1 pre-adipocytes.

	Protocol 1	**Protocol 2**	**Protocol 3**	**Protocol 4**	**Protocol 5**
Day 0-3	BMI	BMI	BMI	BMI	BMI
Day 3-5	DMII IBMX Insulin DEX Rosiglitazone	DMII IBMX DEX Rosiglitazone	DMII IBMX Insulin DEX	DMII Insulin	DMII Insulin Rosiglitazone
Day 5-7	DMII Insulin	DMII	DMII Insulin	DMII Insulin	DMII Insulin
Day 7- until differentiation	DMII	DMII	DMII	DMII	DMII

Abbreviations: BMI, Basal medium I: DMEM 25 mM glucose, 10% NCS and antibiotics; DMII, Differentiation medium II: DMEM 25 mM glucose, 10% foetal bovine serum (FBS) and antibiotics; DEX, Dexamethasone; IBMX, Isobutylmethylxanthine.

The differentiation of each tested protocol was followed during approximately 2 weeks, being the progression of adipogenesis examined microscopically. This examination was based on the number and size of multiple lipid particles in the cytoplasm of the cells along the protocol. After the morphologic analysis at microscope, we concluded that the further described protocol allows a complete differentiation of the fibroblasts into adipocytes.

This protocol was based on the experiences described by Zebisch and coworkers (Zebisch, K et al. 2012). Cells were maintained in 12-well plates until reaching absolute confluence. After 48 h, the medium was replaced by a differentiation medium containing DMEM 25 mM glucose with 10% foetal bovine serum (FBS) (Life Technologies) and supplemented with 0.5 mM IBMX, 0.25 µM DEX, 1 µg/mL insulin and 2 µM rosiglitazone (Cayman Chemical Company, Ann Arbor, MI). After 48 h, the medium was removed from each well and was replaced by DMEM 25 mM glucose only supplemented with 1 µg/mL insulin for two additional days. After that, the cells were

maintained in adipocyte maintenance medium containing DMEM 25 mM glucose with 10% FBS. The medium was refreshed every two days. 100% differentiated adipocytes were acquired approximately around day 7 of the differentiation process. The differentiation was confirmed by morphologic analysis (Figure 2.1).

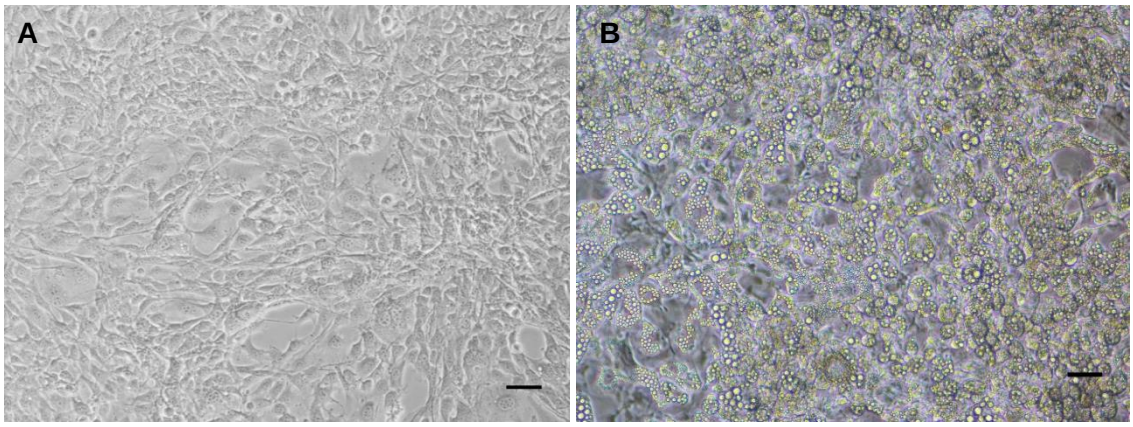

Figure 2.1. Microscopic appearance of 3T3-L1 fibroblasts and 3T3-L1 adipocytes. (A) 3T3-L1 fibroblasts at day 2 of the differentiation with no accumulation of lipids. (B) 3T3-L1 adipocytes at day 8 of the adipocyte differentiation protocol described previously. The cells became more spherical and nearly all of the cells contained lipid droplets in their cytoplasm. This differentiation state remained unchanged during the subsequent days. Scale bar, 20 µm.

Once the cells reached the 100% differentiated state, they were submitted to different treatment conditions:

- In a first task, the cells were submitted to different concentrations of CDCA (10 and 50 µM) during 96 h;
- In a second task, the cells were submitted to 10 µM CDCA and 3µM CHIR99021 treatment during 96 h;
- In a third task, the cells were submitted to 10 µM CDCA and 1 µM XAV939 treatment during 96 h.

In these experiments, control cells were maintained in the same conditions and received an equivalent amount of vehicle, ethanol (in the case of CDCA treatment) and DMSO (in the case of XAV939 or CHIR99021 treatment). Thereafter, the cells were subsequently used for experiments.

All the photometry and fluorescence measurements were made in a Victor^3 1420-050 series (Perkin-Elmer, Waltham, MA) plate reader.

2.4 Live-dead assay

Viability and the death of the cells were evaluated with LIVE/DEAD® Viability/Cytotoxicity Kit (Life Technologies) using the fluorescent marker ethidium homodimer-1 (EtHD-1). The fluorescent marker EtHD-1 indicates the loss of plasma membrane integrity. When the cell membrane is disrupted, EtHD-1 enters and intercalates in the DNA, producing a bright red fluorescence in dead cells (Palmeira et al. 2007). As such, the total of both viable and non-viable cells was detected using Hoechst 33342, a specific fluorescent molecule that interacts with chromatin. In that way, Hoechst 33342 provides a direct measurement of the total number of cells in a well, resulting in a bright blue fluorescence in all the nucleus of the cells (Richards et al. 1985). Non-viable cells were identified as those who exhibited simultaneously EtHD-1 and Hoechst 33342 staining.

After the treatment period, the cells were incubated in the presence of 4 µM EtHD-1 and 1 µL/mL Hoechst 33342 (Life Technologies) in a phosphate buffered saline (PBS) solution during 30 min at 37 °C in the dark, according to the manufacturer's instructions. The medium was changed for a new medium (PBS) to remove the excess of probe and then used for the viability analysis. Cells were observed and distinct randomly selected areas per well were recorded using a fluorescence microscope (Nikon eclipse TS100, Amsterdam, Netherlands) and a NIS elements D4.11.01 Nikon software. The amount of the cells present in recorded images was counted, with the data expressed according to the percentage of viable cells in the total number of cells per well. The count of the cells was made using ImageJ software.

2.5 LDH release

Cell viability and membrane integrity were evaluated by the amount of cytoplasmic enzyme lactate dehydrogenase (LDH) released into the medium of the cells. The assay is based on the conversion of pyruvate to L-lactate, which results on the conversion of NADH into NAD^+. The rate of conversion of NADH into NAD^+ is proportional to LDH activity (Moran & Schnellmann 1996). As such, LDH activity is determined comparing the activity of LDH in medium to total LDH activity in medium and cell lysates using a LDH assay kit purchased from Hospitex Diagnostics (Sesto Fiorentino, Italy).

After the respective treatment during the time indicated, the medium of each well was collected. In addition, the cells were also collected from the wells using RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% DOC, 0.1% SDS) containing a cocktail of protease inhibitors (Sigma-Aldrich). The cell lysates were obtained by passing the cells through a 26-gauge syringe. Then, the samples were collected and transferred to a clean 96-well plate where the enzymatic analysis was determined according to the assay kit at 37 °C. Firstly, 200 µL of medium assay reaction (4:1 mixture of R1 and R2 reagents), was added to each well and the basal absorbance was acquired. After that, 20 µL of each cell lysate sample was added into each well. The reaction was monitored at 340 nm and the LDH activity was calculated based on ΔA/min x 16030, according to the assay kit.

2.6 Sulforhodamine B assay

The sulforhodamine B assay is a colorimetric assay well suited to determining the cellular protein content. The dye sulforhodamine binds to basic aminoacids residues under acidic conditions, which provides a sensitive method of measuring the protein content in comparison with other assays. The determination was made as previously described by Skehan and co-workers with slight modifications (Skehan et al. 1990).

After the treatment period and the subsequent assays, it is necessary to normalize the values obtained according to the cellular protein content in each well. The cells were washed twice with PBS and fixed with p-formaldehyde (4% in PBS) during 30 minutes on a shaking table. Then, the cells were washed twice with PBS and once with distilled water. After that, the plates were air dried and then the sulforhodamine B solution (0.5% in 1% acetic acid) was added to each well, being the plates incubated at room temperature during 30 min in the dark on a shaking table. The plates were washed four times with 1% acetic acid to remove the unbound dye, and the plates were air dried once more. To solubilize the protein-bound dye, 10 mM Tris base solution (pH 10) was added to each well in an equal volume to the original culture medium. The plates were submitted to a gentle stirring on the shaking table during 5 min. The absorbance was measured at 540 nm.

2.7 Bicinchoninic acid assay

The bicinchoninic acid (BCA) assay is a well described assay used to determine protein concentration (Walker 1994). The reaction is based on the reduction of Cu^{2+} to Cu^{+} by proteins in the presence of alkaline conditions. The purple coloured product of this reaction is linear to the protein concentration of unknown samples.

BCA solution was prepared by mixing 50 parts of bicinchoninic acid solution with 1 part of copper (II) sulphate solution. After diluting the unknown samples, 25 µL of each sample was added to 200 µL of BCA solution in a 96-well plate. In parallel, a standard curve with known concentrations of bovine serum albumin (BSA) (0.1%) was prepared (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL). The plate was incubated during 30 min at 37 ºC in the dark. Then, the absorbance was measured at 540 nm. The protein concentrations were determined based on standard curve and considering the dilution factor of the sample.

2.8 Oil red O staining

The oil red O assay constitutes a method for quantifying lipid deposition. The lipid accumulation is proportional to the extent of differentiation of the cells and the assay specifically allows the quantification of triglycerides accumulation in the differentiated adipocytes (Ramírez-Zacarías et al. 1992).

After the treatment period, the cells were washed twice with PBS and fixed with p-formaldehyde (4% in PBS) during 30 min on a shaking table. Then, cells were washed twice with PBS and once with distilled water. After this, the cells were stained with oil red-O-dye (6:4, 0.6% oil red in isopropanol) during 1 h on a shaking table. After this, the cells were washed three times with distilled water to remove the excess of dye in each well. The incorporated dye in the cells was dissolved in 200 µL of isopropanol, and the absorbance measured at 490 nm. The values were normalized according to the protein content in each well which was measured with the sulforhodamine B assay, described in section 2.6. The values were expressed in comparison with controls set as 100%.

2.9 Measurement of mitochondrial membrane potential

The monitoring of the mitochondrial membrane potential ($\Delta\psi_m$) was determined based on the accumulation of tetramethylrhodamine methyl ester (TMRM) (Life

Technologies), in the mitochondrial matrix, in a direct proportion to their $\Delta\psi_m$. This cell permeant, cationic fluorescent probe allows the detection of small changes in $\Delta\psi_m$ which is indicative of mitochondrial function state (Distelmaier et al. 2008).

The monitoring of $\Delta\psi_m$ was done according to a modified procedure of Rolo and co-workers (Rolo et al. 2003). 3T3-L1 fibroblasts were seeded in 12-well plates, differentiated and after the treatment period, the medium of each well was removed and the cells were incubated with 6.6 µM TMRM (prepared in dimethyl sulfoxide (DMSO)) in 1 mL of medium without FBS at 37 °C, in the dark during 30 min. After this incubation period, the medium of the cells was removed, and a new medium without FBS was added to remove the excess probe. The fluorescence was measured using excitation and emission wavelengths of 485 and 590 nm, respectively. Initially, the basal fluorescence was recorded and then, $\Delta\psi_m$ was estimated based on the complete depolarization caused by the incubation of the cells with 1 mM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP). The values were normalized according to the protein content in each well, measured with the sulforhodamine B assay, described in section 2.6.

2.10 Measurement of reactive oxygen species generation

The evaluation of ROS generation was determined fluorometrically, using the 2',7'-dichlorodihydrofluorescin diacetate (H_2DCF-DA) probe. This probe easily diffuses through the cellular membrane and can be hydrolysed by intracellular esterases, becoming cell trapped. The resulting product can then be oxidized to a fluorescent product, 2',7'-dichlorofluorescin, in the presence of ROS (Gomes et al. 2005).

The determination of ROS formation was performed according to a method with slight modifications (Zhou et al. 2001). 3T3-L1 fibroblasts were seeded in 12-well plates, differentiated and after the treatment period, the medium of each well was removed and the cells were incubated with 50 µM H_2DCF-DA (prepared in DMSO) in 1 mL of medium without FBS in the incubator at 37 °C in the dark during 30 min. After this incubation period, the medium of the cells were removed, and 1 mL of new medium without FBS was added. The fluorescence was measured using excitation and emission wavelengths of 485 and 538 nm, respectively. To calculate the amount of ROS generation, the basal measurement of ROS was acquired, followed by the recorded fluorescence when 5 µL of 0.5 mM antimycin A was added. The addition of antimycin A blocks complex III of the mitochondria chain, provoking a maximal ROS

generation. The results were normalized accordingly to the protein content in each well, measured with a sulforhodamine B assay, described in section 2.6.

2.11 Measurement of cytochrome *c* oxidase activity

In order to study the mitochondrial function, it is important to evaluate the function of mitochondrial proteins, namely cytochrome *c* oxidase.

The activity of this protein was evaluated in a closed chamber of an oxygen sensitive electrode (Hansatech Instruments Ltd, Norfolk, UK). The monitoring of oxygen concentration was polarographically measured using a Clark electrode as previously described (Brautigan et al. 1978). The reactions were performed at 25 °C in 0.5 mL of PBS medium. This medium was supplemented with 2 µM rotenone, 10 µM cytochrome *c* and 150 µg of protein samples. After recording the basal cytochrome *c* oxidase activity, 5 mM ascorbate and 0.25 mM tetramethyl-p-phenylenediamine (TMPD) were added to the reaction medium. The reaction was initiated and the cytochrome *c* oxidase activity was recorded. The activity is expressed in nAtoms O/ min/ mg protein.

2.12 Western blot analysis

The Western blot is a technique that enables the quantification of specific proteins in a cell or tissue sample.

Total cellular protein extracts were prepared from differentiated cells after the treatment period. The cells were washed three times with PBS and then, the cells were collected from the wells using RIPA buffer containing a cocktail of protease inhibitors. The cell lysates were obtained by passing the cells through a syringe (BD Plastipak, Franklin Lakes, NJ) and then, cells lysates were centrifuged at 10,000 xg for 10 min at 4 °C. The resulting supernatant was collected and used for the subsequent protocol. The protein quantification was determined through BCA assay described in section 2.7. Then, protein samples were prepared using Laemmli sample buffer (Bio-Rad) with 5% β-mercaptoethanol. For western blot analysis, 50 µg of protein was loaded per lane and electrophoresed on a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel in buffer 25 mM Tris-HCl containing 192 mM glycine and 0.1% SDS pH 8.3 at 120 mV.

After electrophoresis, protein samples were transferred to polyvinylidene difluoride (PVDF) membranes using 25 mM Tris-HCl containing 192 mM glycine and

20% methanol during 90 min at 100 mV. Then, membranes were blocked in 5% non-fat milk in tris-buffered saline-tween-20 (TBS-T: 20 mM Tris (pH 7.6), 150 mM NaCl and 0.1% Tween-20) for 2 h with agitation. After that, the membranes were washed three times with tris-buffered saline (TBS). Membranes were incubated with primary antibodies (Table 1) diluted in wash buffer (Life Technologies) with 5% (v/v) of blocking buffer (Life Technologies), overnight at 4 °C. Membranes were washed three times with TBS-T and incubated with biotin-conjugated secondary antibodies, anti-mouse or anti-rabbit, (Life Technologies) with agitation for 1 h. After that, membranes were washed with TBS three times and incubated with Qdot-625 streptavidin conjugate (Life Technologies). To confirm the equal amount of protein per lane, it was used a loading control for the total protein content in the samples.

Membranes were imaged using a Bio-Rad Gel Doc EZ Imager and an Image Lab 4.1 software. The densitometric analysis was performed using ImageJ software.

Table 2.2. List of primary antibodies used for Western Blotting analysis.

Antibody	Specie	Dilution	Supplier
COX-I	Mouse	1:100	MitoSciences (Eugene, OR)
COX-IV	Mouse	1:1000	MitoSciences
UCP-1	Rabbit	1:500	Abcam (Cambridge, MA)
Cleaved caspase-3	Rabbit	1:500	Cell Signaling (Danvers, MA)

2.13 Real-time polymerase chain reaction

To determine the expression pattern of some genes, mRNA expression was analysed using semi-quantitative real-time polymerase chain reaction (RT-PCR).

Ribonucleic acid (RNA) was extracted from the cells using the PureLink RNA mini kit (Life Technologies), with the extraction performed according to manufacturer's instructions. Briefly, cells were lysed by adding lysis buffer with 2-mercaptoethanol to each well. Each cell lysate was manually homogenised manually by passing the lysate through a syringe. 70% ethanol was added and each sample was mixed and transferred to a spin cartridge. In the spin cartridge, RNA bound to the membrane and after it was washed, purified RNA was eluted into a recovery tube.

RNA quantification was determined on a Qubit® 2.0 Fluorometer (Life Technologies). The assay was prepared by adding 10 µL of each sample to 190 µL of Qubit working solution prepared by diluting Qubit reagent (Life Technologies) in Qubit buffer (1:200) (Life technologies). Then, tubes were incubated at room temperature for 2 min. Assay tubes were inserted in the Qubit fluorescence system and stock concentration of RNA samples were determined. After that, cDNA (complementary DNA) was synthesized with the Bio-Rad iScript cDNA synthesis kit using 500 ng of RNA. Each sample reaction was performed with iScript reaction mix and iScript reverse transcriptase in a Minicycler thermal cycler (Bio-Rad). The expression of genes of interest was evaluated by the real time semi-quantitative PCR on a MiniOpticon Real time PCR system (Bio-Rad). Each sample reaction was performed using iQ SYBR Green Supermix (Bio-Rad) and respective forward and reverse primers. The expression of each gene of interest was normalised considering the expression of the housekeeping gene 18S ribosomal (18S rRNA).

The primers used and its sequences are described in Table 2.

Table 2.3. List of primers and its respective nucleotide sequences.

Gene		Primer Sequence
18S	Forward	GCCCGAGCCGCCTGGAATAC
	Reverse	CCGGCGGGTCATGGGAATAAC
COX-III	Forward	TCATCGTCTCGGAAGTATTTTT
	Reverse	ATTAGTAGGGCTTGATTTATGTGG
COX-IV	Forward	AGAAGGCGCTGAAGGAGAAGGA
	Reverse	CCAGCATGCCGAGGGAGTGA
CPT-1α	Forward	GCAGCTCGCACATTACAAGGACAT
	Reverse	AGCCCCCGCCACAGGACACATAGT
FAS	Forward	GGCTGCCTCCGTGGACCTTATC
	Reverse	GTCTAGCCCTCCCGTACACTCACTCGT
GLUT4	Forward	ACCGGCTGGGCTGATGTGTCT
	Reverse	GCCGACTCGAAGATGCTGGTTGAATAG
PEPCK	Forward	GGCAGCATGGGGTGTTTGTAGGA
	Reverse	TTTGCCGAAGTTGTAGCCGAAGAAG
PGC-1α	Forward	CCCAAAGGATGCGCTCTCGTT
	Reverse	TGCGGTGTCTGTAGTGGCTTGATT
PPAR-α	Forward	TGCGCAGCTCGTACAGGTCATCAA
	Reverse	CCCCCATTTCGGTAGCAGGTAGTCTTA
PPAR-γ	Forward	GGCGAGGGCGATCTTGACAGG
	Reverse	GGGCTTCCGCAGGTTTTTGAGG
UCP-1	Forward	CACGGGGACCTACAATGCTTACAG
	Reverse	GGCCGTCGGTCCTTCCTT
TFAM	Forward	AGGAGCTGAAGGCATGCGGTGAAG
	Reverse	GTCCAGTGCGTGCGGGTGAAC

2.14 Statistical analysis

Data were statistically analysed using GraphPad Prism 5.03 (GraphPad Software, Inc.,La Jolla, CA) and are presented as mean ± standard errors of mean (SEM). Statistical significance between groups was performed using one-way ANOVA followed by a Bonferroni post-hoc test for 3 or more groups comparison. In the analysis, a value of $P < 0.05$ was considered statistically significant.

CHAPTER 3

RESULTS

3.1 CDCA effect in 3T3-L1 adipocytes

3.1.1 Cell viability

Bile acids have been described as amphipatic compounds which might interfere with cell membranes causing alterations in cell viability in isolated and cultured hepatocytes, as well as in a human breast carcinoma cell line (Im et al., 2001; Perez & Briz, 2009). Taking this into account, cell viability was evaluated in 3T3-L1 adipocytes incubated with CDCA to determine the potential cytotoxic effects of this molecule in this cell line.

Firstly, cell viability was assessed based on the capacity of EtHD-1 to enter into cells that have lost membrane integrity. The results showed that CDCA caused no alterations on cell viability when compared to control, at both concentrations (Figure 3.1).

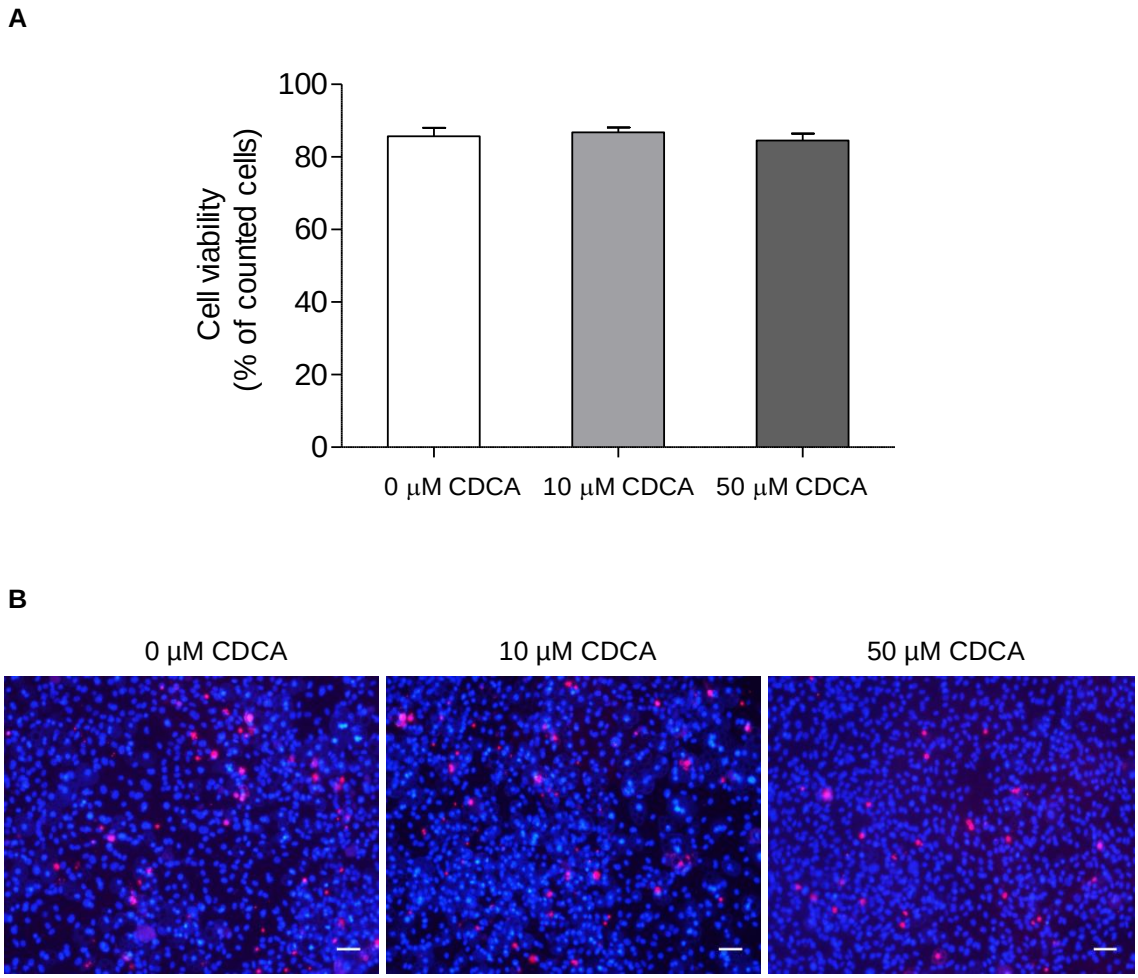

Figure 3.1 - 3T3-L1 adipocytes viability after CDCA treatment. (A) 3T3-L1 adipocytes were cultured and treated with different concentrations of CDCA (10 μM and 50 μM) for 96h. The viability of the cells was assessed by incubating the cells with EtHD-1/ Hoechst 33342 fluorescent markers according to Live-dead assay described in Methods (Chapter 2). Data are expressed in terms of percentage of viable cells (number of viable cells in the total number of counted cells per area selected) and represent the mean ± SEM of different experiments (n=3). No statistically significant differences were found ($P<0.05$). (B) Representative fluorescence images for each condition of CDCA treatment showing dead cells with EtHD-1 (red) and nuclear staining with Hoechst 33342 (blue) in 3T3-L1 adipocytes. Scale bar, 20 μm.

The data is consistent with the results observed for cell viability determined by LDH release (Figure 3.2). Any of the CDCA concentrations tested induced significant LDH release.

Both results indicate that CDCA does not induce necrotic cell death in 3T3-L1 adipocytes incubated with 10 μM or 50 μM for 96h.

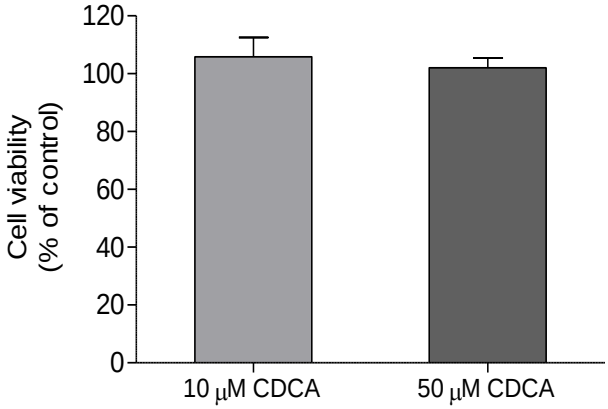

Figure 3.2 - 3T3-L1 adipocytes viability after CDCA treatment. 3T3-L1 adipocytes were cultured and treated with different concentrations of CDCA (10 μM and 50 μM) for 96h. The viability of the cells was determined by estimating the release of LDH into the media, according to the LDH assay kit described in Methods (Chapter 2). The cell viability was expressed as the percentage of LDH no released into the medium of total LDH when compared to control, considered as 100%. Data represent the mean ± SEM of different experiments (n=4). No statistically significant differences were found ($P<0.05$).

In addition and in the line with the observations that CDCA did not induce necrosis, it was evaluated if CDCA could interfere with any intracellular mechanism implicated in programmed cell death – apoptosis. Caspase 3 has been referred as an effector caspase of the cell's entry point into the apoptotic pathway which can be activated by two distinct pathways – the extrinsic and the intrinsic pathway (Boatright & Salvesen, 2003). The cleaved form of this protein is considered as a hallmark of the activated apoptotic pathway (Porter & Jänicke, 1999). To further dissect the possible role of CDCA in apoptosis, the content of cleaved-caspase 3 in 3T3-L1 adipocytes was evaluated.

As shown in Figure 3.3, no differences were detected in cleaved-caspase 3 content in cells treated with 10 μM and 50 μM CDCA when compared to control. Thus, the data clearly indicates that there is no evidence of significant necrosis or apoptosis in 3T3-L1 adipocytes treated with the tested CDCA concentrations.

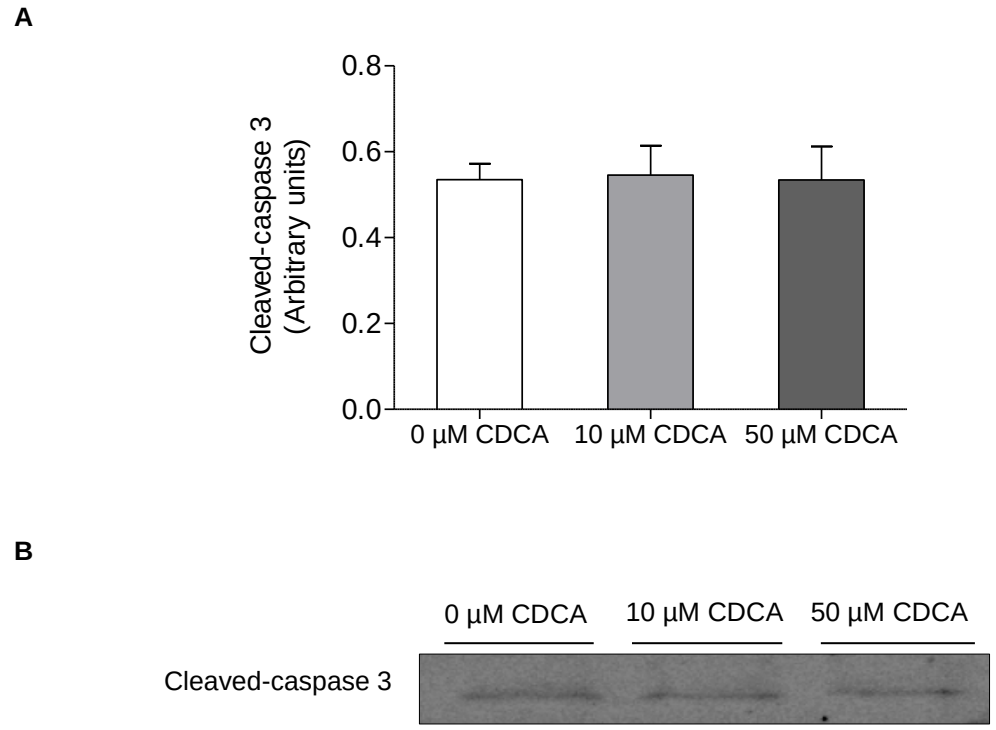

Figure 3.3 – Cleaved-caspase 3 content in 3T3-L1 adipocytes after CDCA treatment. (A) 3T3-L1 adipocytes were cultured and treated with different concentrations of CDCA (10 µM and 50 µM) for 96h. Cleaved-caspase 3 content were assessed in cell lysates by Western blotting analysis and normalized with a loading control. Data are expressed as arbitrary units and represent the mean ± SEM of different experiments (n=5). No statistically significant differences were found ($P<0.05$). (B) Image of Western blotting is representative of protein content in each condition.

3.1.2 Triglycerides accumulation

Bile acids have been reported to improve several parameters of TG metabolism in mouse models of hypertriglyceridemia (Watanabe et al., 2004). Considering the potential therapeutic applications of bile acids in the modulation of metabolic disorders, it is important to dissect the role of CDCA on the metabolism of these cells. Since adipocytes store energy in the form of TGs, TGs content was determined in 3T3-L1 adipocytes.

CDCA decreased the lipid accumulation in 3T3-L1 adipocytes after 96h of exposure (Figure 3.4). This effect was statistically significant for both concentrations used (10 µM and 50 µM CDCA) when compared to control. However, the effect was

more pronounced in cells treated with 50 µM CDCA. This data suggested that CDCA reduce lipid accumulation in a CDCA dose-dependent manner.

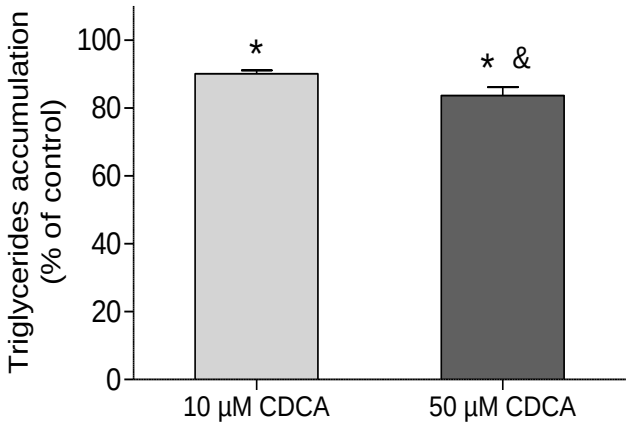

Figure 3.4 - Triglycerides accumulation in 3T3-L1 adipocytes after CDCA treatment. 3T3-L1 adipocytes were cultured and treated with different concentrations of CDCA (10 µM and 50 µM) for 96h. TG accumulation was assessed using oil red O staining. Data are expressed as percentage of control, considered as 100%, and represent the mean ± SEM of different experiments (n=5). * indicates statistically significant different versus control (P<0.05); & indicates statistically significant different versus 10 µM CDCA (P<0.05).

3.1.3 Mitochondrial function

3.1.3.1 Mitochondrial membrane potential

The mitochondria in adipose tissue are involved in the regulation of lipid metabolism through the regulation of lipogenesis and lipolysis. Moreover, abnormal mitochondrial function has been linked to lipid accumulation in adipocytes. Therefore, the decrease in lipid content of adipocytes suggests a high oxidative capacity due to changes in the mitochondrial function (Medina-Gómez, 2012).

Mitochondria have an electrochemical potential across their inner membrane and alterations in this potential reflect the state of mitochondrial function. In this work, it was determined the effect of CDCA in mitochondrial membrane potential in 3T3-L1 adipocytes. The results showed that 10 µM CDCA did not induced any alterations in mitochondrial membrane potential in CDCA treated cells when compared to control (Figure 3.5). However, 50 µM CDCA induced a statistically significant increase in mitochondrial membrane potential when compared to 10 µM CDCA-treated cells.

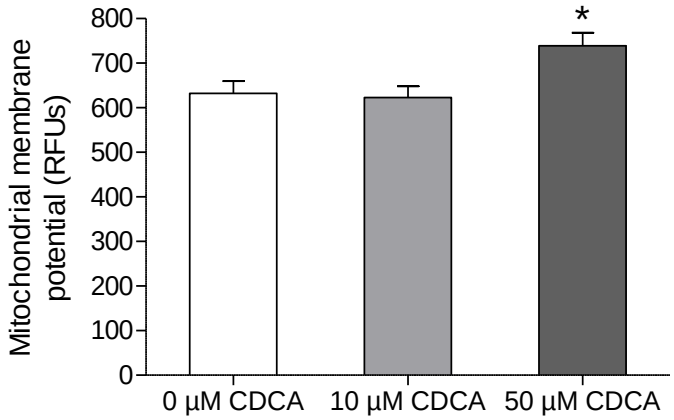

Figure 3.5 - Mitochondrial membrane potential ($\Delta\psi_m$) in 3T3-L1 adipocytes after CDCA treatment. 3T3-L1 adipocytes were cultured and treated with different concentrations of CDCA (10 µM and 50 µM) for 96h. The mitochondrial membrane potential, expressed as relative fluorescence units (RFUs), was assessed fluorometrically using the TMRM probe as described in Chapter 2. Data represent the mean ± SEM of different experiments (n=5). * indicates statistically significant different versus 10 µM CDCA ($P<0.05$).

3.1.3.2 Generation of reactive oxygen species

Mitochondria are considered as an important source of ROS. An enhancement of ROS production has been linked to alterations in cell signaling, increased mutations in mtDNA and an abnormal mitochondrial function (Bonnard et al., 2008).

In order to determine if CDCA treatment alters ROS generation, ROS levels were assessed in 3T3-L1 adipocytes using the fluorescent probe H_2DCF-DA.

The results showed no differences for the basal generation of ROS in 3T3-L1 adipocytes exposed to 10 µM and 50 µM CDCA when compared to control (Figure 3.6). Also, in the presence of antymicin, an inhibitor of complex III of mitochondria, no alterations were reported in the mitochondrial capacity to generate ROS. Thus, there is no evidence that CDCA affected the generation of ROS.

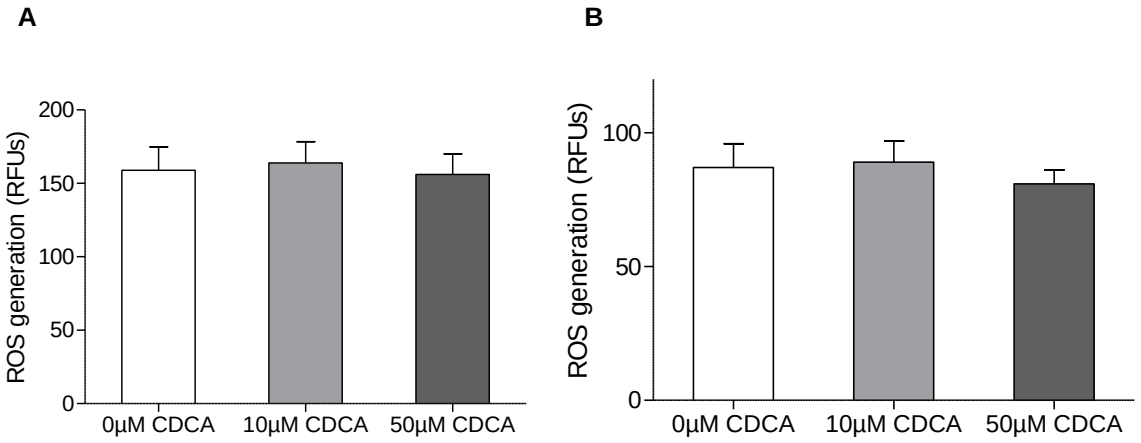

Figure 3.6 – Reactive oxygen species (ROS) generation in 3T3-L1 adipocytes after CDCA treatment. 3T3-L1 adipocytes were cultured and treated with different concentrations of CDCA (10 µM and 50 µM) for 96h. The ROS generation, expressed as relative fluorescence units (RFUs), was assessed fluorometrically using the H_2DCF-DA probe. After the basal fluorescent measurement (A), antimycin was added to block complex III of electron transport chain to induce maximal ROS generation (B). Data represent the mean ± SEM of different experiments (n=7). No statistically significant differences were found (P<0.05).

3.1.3.3 Cytochrome *c* oxidase activity

In order to better characterize the mitochondrial function of 3T3-L1 adipocytes submitted to CDCA treatments, the enzymatic activity of cytochrome *c* oxidase (COX) was also evaluated. This complex is part of the electron respiratory chain and its activity is an indicator of mitochondrial oxidative phosphorylation competence and undoubtedly of mitochondrial function (Li et al., 2006).

The results showed no significant differences in mitochondrial activity in 3T3-L1 adipocytes when compared to control (Figure 3.7).

This data was in agreement with the previous results shown in mitochondrial membrane potential (for 10 µM CDCA condition) and ROS production (for all conditions tested). Furthermore, the increase in mitochondrial membrane potential in cells treated with 50 µM CDCA was not associated with increased COX activity.

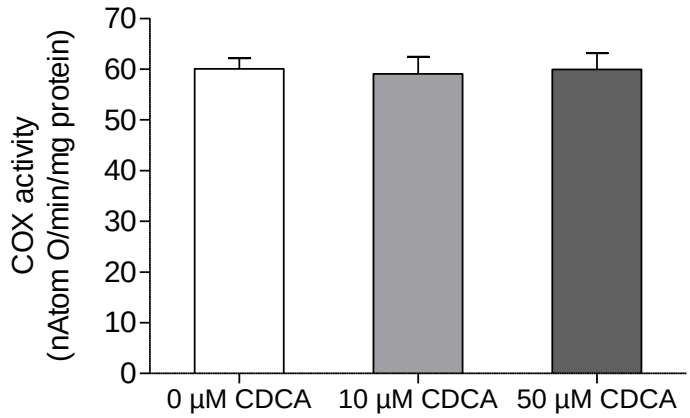

Figure 3.7 - Cytochrome c oxidase (COX) activity in 3T3-L1 adipocytes after CDCA treatment. 3T3-L1 adipocytes were cultured and treated with different concentrations of CDCA (10 µM and 50 µM) for 96h. The COX activity was measured using a Clark-type oxygen electrode. Data are expressed in nAtoms O/ min/ mg protein and represent the mean ± SEM of different experiments (n=5). No statistically significant differences were found (P<0.05).

3.1.3.4 Mitochondrial proteins content

Since CDCA has described to induce an increase in markers of mitochondrial biogenesis in BAT in mice (Teodoro et al., 2014), it was hypothesized whether CDCA effects in 3T3-L1 adipocytes could be explained by an increase in mitochondrial protein content.

Thus, the content in cytochrome *c* oxidase subunit I (COX-I), a mitochondrial-encoded protein and in cytochrome *c* oxidase subunit IV (COX-IV), a nuclear-encoded protein were evaluated in 3T3-L1 adipocytes.

This data showed no statistical differences in the content of COX-I and COX-IV (Figure 3.8), which was correlated with the previous results obtained for COX activity.

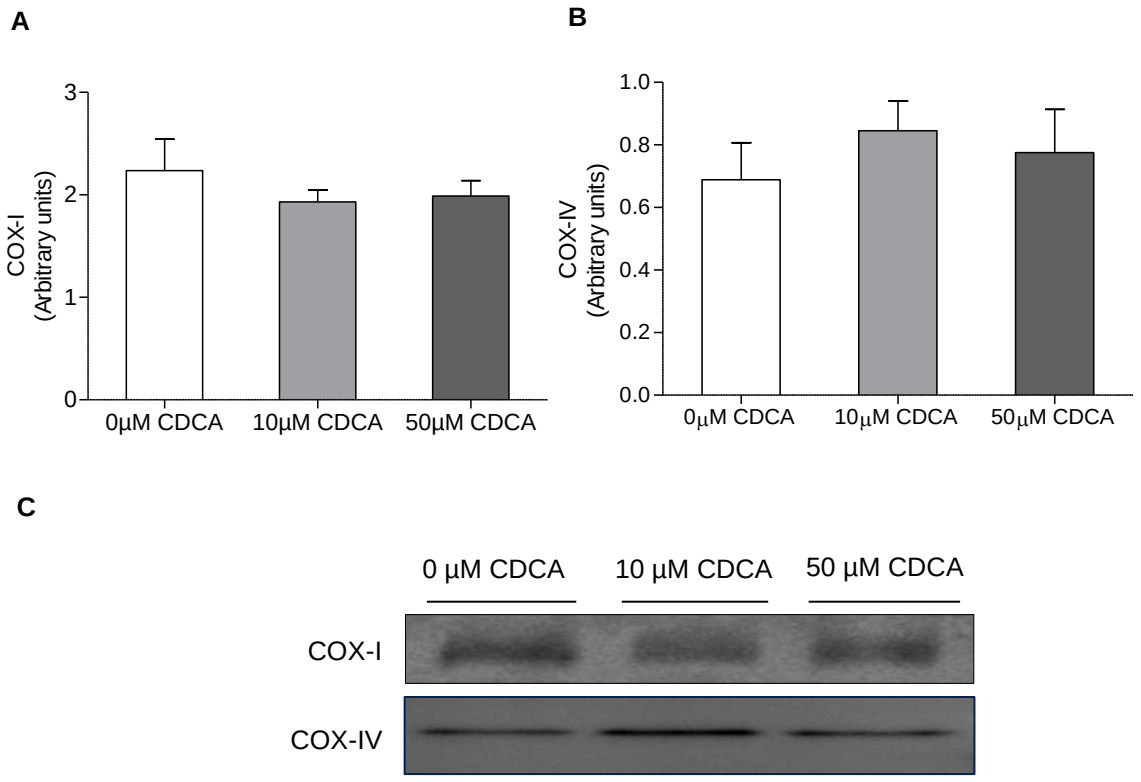

Figure 3.8 - Cytochrome c oxidase subunit I (COX-I) and cytochrome c oxidase subunit IV (COX-IV) content in 3T3-L1 adipocytes after CDCA treatment. 3T3-L1 adipocytes were cultured and treated with different concentrations of CDCA (10 μM and 50 μM) for 96h. Content in mitochondrial-encoded protein COX-I (A) and nuclear-encoded protein COX-IV (B) were assessed in cell lysates by Western blotting and normalized with a loading control. Data are expressed as arbitrary units and represent the mean ± SEM of different experiments (n=4). No statistically significant differences were found ($P<0.05$). (C) Images of Western blotting are representative bands of each protein content in each condition.

3.1.3.5 UCP-1 content

Although CDCA decreased TG content in 3T3-L1 adipocytes, it did not increase mitochondrial oxidative capacity, as shown by no alterations in neither COX activity nor protein content.

According to the literature, the transdifferentiation of white adipocytes into brite cells leads to the appearance of UCP-1-expressing adipocytes in WAT depots. These cells are known to function as competent brown adipocytes, dissipating energy mainly through the oxidation of stored lipids (Giralt & Villarroya, 2013). Moreover, BAs have been described to prevent and reverse fat accumulation by increasing energy expenditure in BAT (Watanabe et al., 2006).

Taking this into account, it was hypothesized that CDCA decreased lipid accumulation by UCP-1-mediated mitochondrial uncoupling in 3T3-L1 adipocytes.

Our data showed an increase in UCP-1 content (Figure 3.9) after exposure of 3T3-L1 adipocytes to 10 µM and 50 µM of CDCA for 96h. Interestingly, the increase in UCP-1 content was only statistically significant when cells were treated with 10 µM CDCA. An increase in UCP-1 content and, consequently, an increase in energy expenditure were in agreement with the reduction of lipid accumulation in adipocytes treated with 10 µM CDCA. Indeed, the presence of UCP-1 provoked an increase for energy demand that was provided by higher fat oxidation of metabolites as TG stored in the cells. However, the decrease in lipid accumulation for 50 µM CDCA was not consistent with UCP-1 content. This data possibly indicates that the CDCA effect was not only induced by UCP-1 dependent thermogenesis but also depends on another mechanism not yet determined.

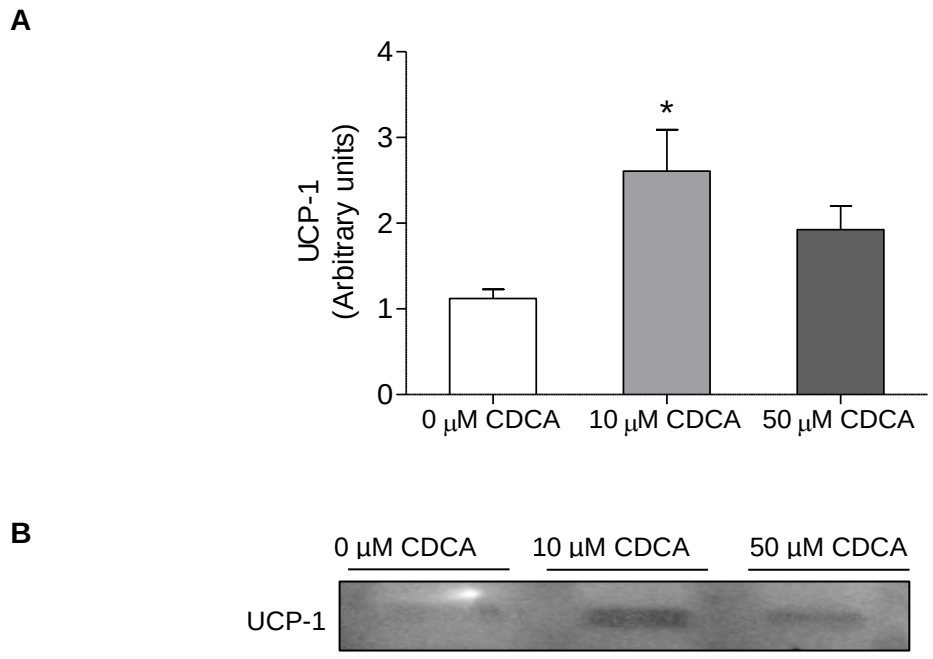

Figure 3.9 - Uncoupling protein-1 (UCP-1) content in 3T3-L1 adipocytes after CDCA treatment. (A) 3T3-L1 adipocytes were cultured and treated with different concentrations of CDCA (10 µM and 50 µM) for 96h. UCP-1 content was assessed in cell lysates by Western blotting normalized with a loading control. Data are expressed as arbitrary units and represent the mean ± SEM of different experiments (n=4). * indicates statistically significant different versus control ($P<0.05$); (B) Image of Western blotting is representative of protein content in each condition.

3.1.4 mRNA relative expression

In this study, the relative expression of key genes potentially implicated in the effects of CDCA was also analysed. This analysis was performed using semi-quantitative real time – PCR in 3T3-L1 adipocytes.

The analysis of genes expression related to adipocyte metabolism revealed that the expression of genes involved in fatty acid oxidation (*PPAR-α*) as well as in the pathway of mitochondrial fatty acid oxidation (*CPT1α*) were not changed after treatment with CDCA (Figure 3.10). In addition, CDCA had no effect on the expression of genes involved in lipogenesis (fatty acid synthase (*FAS*) and phosphoenolpyruvate carboxykinase (*PEPCK*)) and on the expression of glucose transporter *GLUT4*.

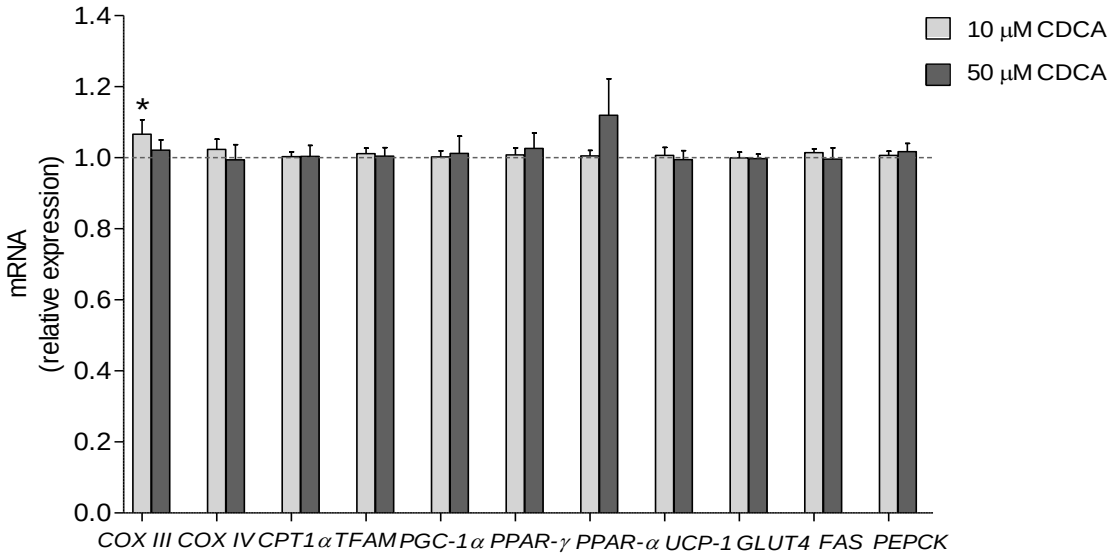

Figure 3.10 – Relative gene expression analysis in 3T3-L1 adipocytes after CDCA treatment. 3T3-L1 adipocytes were cultured and treated with different concentrations of CDCA (10 μM and 50 μM) for 96h. The semi-quantitative Real Time-PCR analysis was performed as described in methods in chapter 2. The relative gene expression of *COX III*, *COX IV*, *CPT1α*, *TFAM*, *PGC-1α*, *PPAR-γ*, *PPAR-α*, *UCP-1*, *GLUT4*, *FAS* and *PEPCK* was determined based on standard curves and normalised using 18S RNA as a housekeeping gene (0 μM CDCA = 1, dashed line). Data represent the mean ± SEM of different experiments (n=4). * indicates statistically significant different versus control (P<0.05).

Furthermore, CDCA did not lead to alterations in the transcription of the master regulator of mitochondrial biogenesis PGC-1α, neither in its target gene *TFAM*. These observations were in agreement with the no significant differences in the expression of

the gene *PPAR-γ* gene which is implicated in the induction of UCP-1 and mitochondrial biogenesis (Pisani et al., 2011).

Although CDCA did not induce any alterations in the majority of the genes analysed, it was found a statistically significant increase in COX III expression in cells treated with 10 µM CDCA when compared to control. Nevertheless, no alterations were observed for the expression of nuclear encoded gene *COX-IV*.

Consistent with the low expression for PGC-1α and PPAR-γ, the effect of CDCA did not induce an increase in *UCP-1* gene expression in 3T3-L1 adipocytes.

The gene expression studies confirm that CDCA treatment during 96h did not induce any significant effects at the expression levels of the majority of the genes analysed. Despite the absence of alterations at the thermogenic level, the changes in COX III expression reinforces the hypothesis that CDCA might play a role at the mitochondria transcriptional level.

3.2 CDCA and Wnt/ β-catenin signaling pathway

3.2.1 Triglycerides accumulation

In order to study how the activation or the inhibition of Wnt/ β-catenin signaling pathway might modulate CDCA effects, TG accumulation was analysed in 3T3-L1 adipocytes exposed simultaneously to CDCA and CHIR99021 or XAV939.

The simultaneously treatment with CDCA and XAV939, a Wnt/ β-catenin inhibitor, caused a significant increase in TG accumulation when compared to the cells treated only with 10 µM CDCA (Figure 3.11). These alterations indicated that CDCA effects were blocked by the presence of XAV939.

Interestingly, CHIR99021 (a Wnt/ β-catenin activator) effects were opposite to XAV939 effects. The results showed that the treatment of the cells only with CHIR99021 significantly decreased the triglycerides accumulation when compared to control. Treatment of 3T3-L1 adipocytes simultaneously with CHIR99021 and CDCA maintained the reduction on lipid accumulation showed only in the presence of the activator.

These findings showed that CDCA effects declined when the Wnt/β-catenin signaling pathway is inhibited. Opposingly, CDCA effects were maintained in the presence of the activator of the pathway. Together, we can conclude that CDCA effects are dependent of the Wnt/β-catenin signaling pathway.

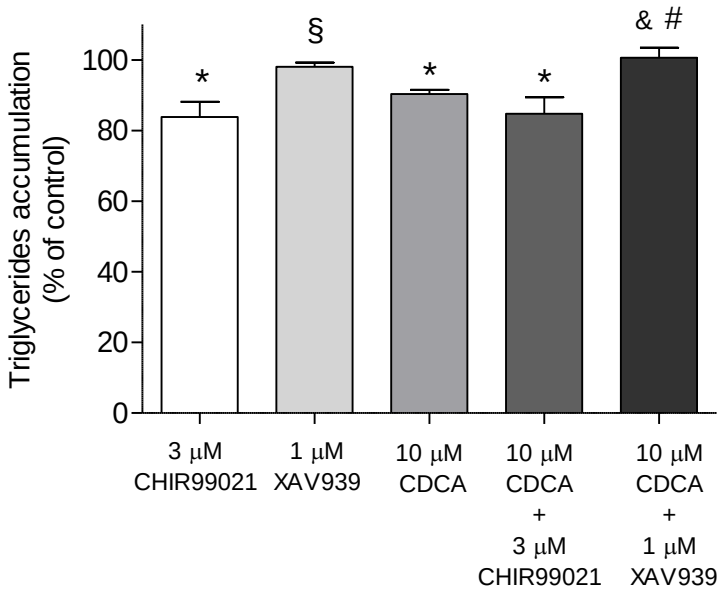

Figure 3.11 - Triglycerides accumulation in 3T3-L1 adipocytes after CDCA treatment. 3T3-L1 adipocytes were cultured and treated with 3 μM CHIR99021, 1 μM XAV939, 10 μM CDCA and simultaneously with 10 μM CDCA and 3 μM CHIR99021 or 1 μM XAV939 for 96h. The triglycerides accumulation was assessed using oil red O staining. Data are expressed as percentage of control, considered as 100%, and represent the mean ± SEM of different experiments (n=7). * indicates statistically significant different versus control (P<0.05); § indicates statistically significant different versus 3 μM CHIR99021 (P<0.05); & indicates statistically significant different versus 10 μM CDCA (P<0.05); # indicates statistically significant different versus 10 μM CDCA + 3 μM CHIR99021 (P<0.05).

3.2.2 Mitochondrial function

As CDCA effects has been found to be modulate through the Wnt/β-catenin signaling pathway in 3T3-L1 adipocytes, we next sought to determine if mitochondrial protein content was altered when XAV939 blocked CDCA effects. XAV939 did not induce any statistical differences in the content of COX-I and COX-IV (Figure 3.12).

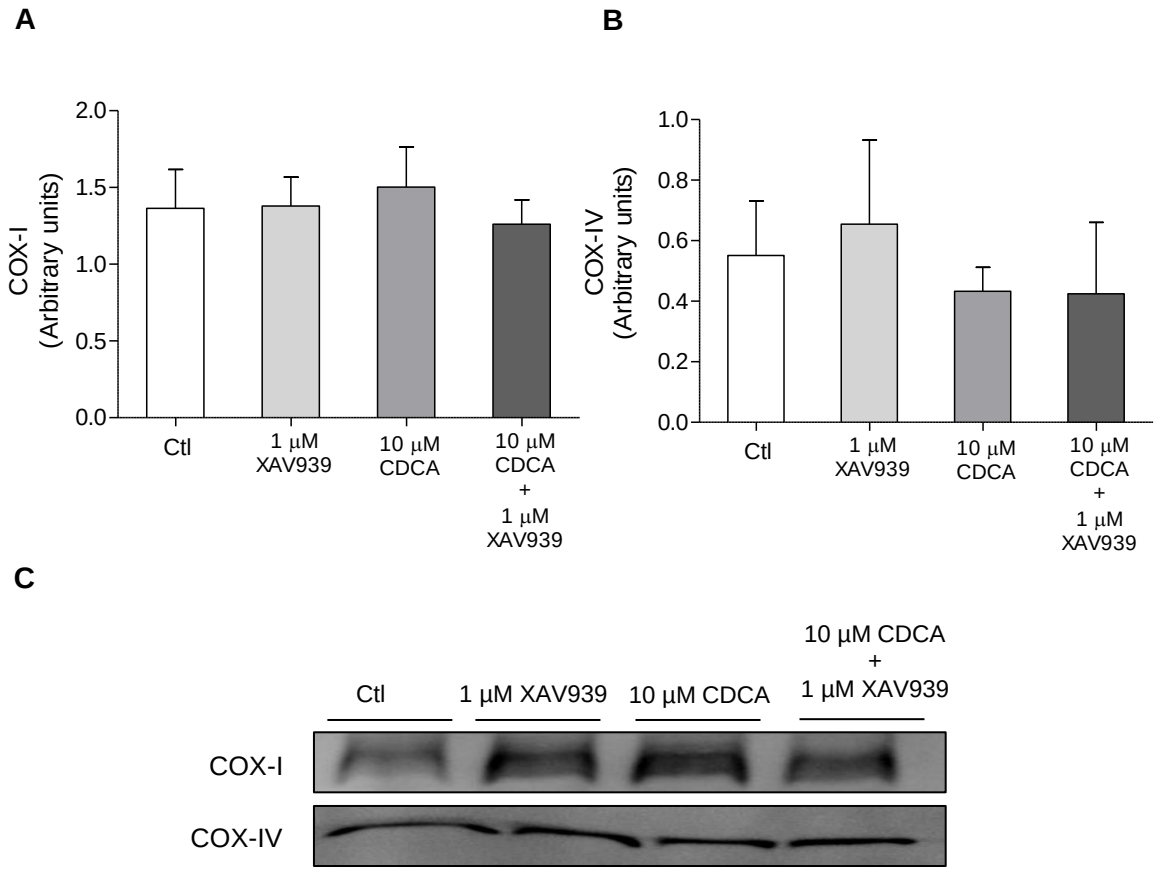

Figure 3.12 - Cytochrome *c* oxidase subunit I (COX-I) and cytochrome *c* oxidase subunit IV (COX-IV) content in 3T3-L1 adipocytes after CDCA and XAV939 treatment. 3T3-L1 adipocytes were cultured and treated with 10 μM CDCA or/and 1 μM XAV939 for 96h. The protein levels of mitochondrial-encoded protein COX-I (A) and nuclear-encoded protein COX-IV (B) were assessed in cell lysates by Western blotting and the protein content is normalized to a loading control. Data are expressed as arbitrary units and represent the mean ± SEM of different experiments (n=4). No statistically significant differences were found ($P<0.05$). (C) Images of Western blotting are representative bands of protein content in each condition.

3.2.2.1 UCP-1 content

CDCA showed to induce a decrease in TG accumulation which is blocked in the presence of XAV939. Since CDCA effects seemed to be justified in part by an increase in UCP-1 content, it is important to understand if XAV939 blocked the decrease in lipid accumulation through inducing alterations at the UCP-1 level.

Therefore, UCP-1 content in 3T3-L1 adipocytes exposed to both 10 μM CDCA and 1 μM XAV939 treatment was evaluated. Despite no statistically differences were

detected in the UCP-1 content, a slight decrease in the UCP-1 content were detected in 3T3-L1 adipocytes submitted to CDCA and XAV939 treatment when compared to adipocytes treated only in the presence of CDCA (Figure 3.13). This slight decrease might suggest that XAV939 blocks CDCA effects by a decrease in UCP-1 content. However, the effects at UCP-1 level not be sufficient to increase lipid accumulation as shown in Figure 3.11.

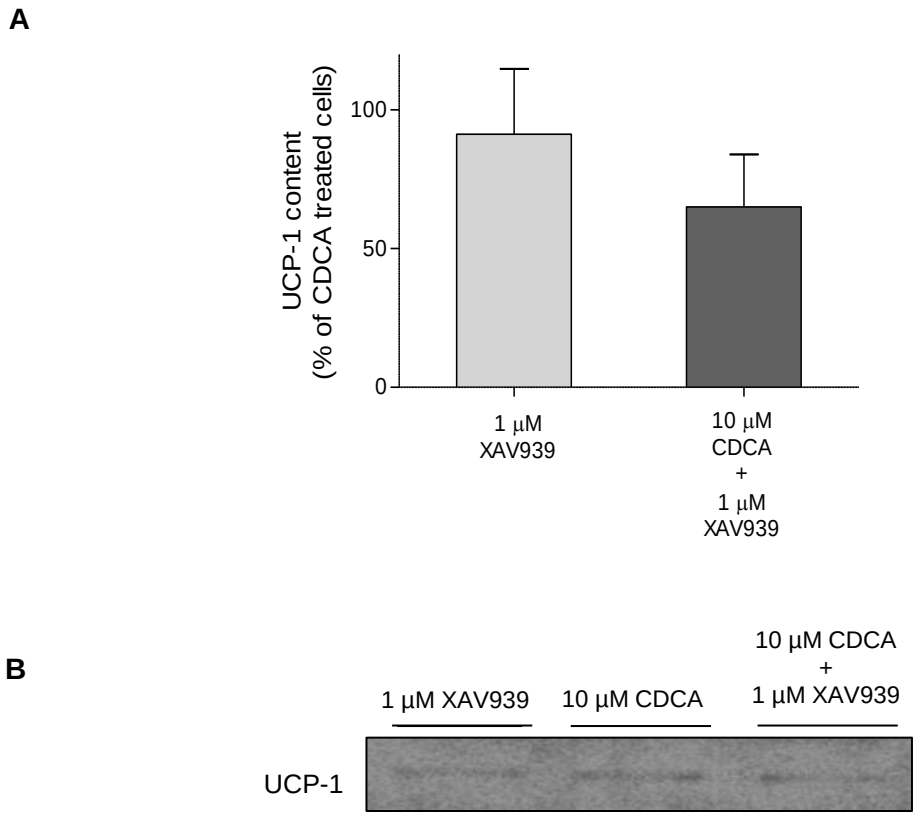

Figure 3.13 - Uncoupling protein-1 (UCP-1) content in 3T3-L1 adipocytes after CDCA and XAV939 treatment. (A) 3T3-L1 adipocytes were cultured and treated with 10 μM CDCA and/ or 1 μM XAV939 for 96h. The protein levels of UCP-1 were assessed in cell lysates by Western blotting and normalized with a loading control. Data are expressed as percentage of CDCA treated cells, considered as 100%, and represent the mean ± SEM of different experiments (n=4). No statistically significant differences were found ($P<0.05$); (B) Image of Western blotting is representative of protein content in each condition.

CHAPTER 4

DISCUSSION

Obesity is rapidly becoming one of the major health problems affecting the world's population. Due to increased positive energy balance, this disorder is characterized by an excessive accumulation of fat in adipocytes. In these conditions, fat accumulation and the generation of ROS are considered as the initial events that trigger the dyrsregulation of a large number of endocrine and inflammatory pathways that have a negative impact on insulin signaling, and ultimately, cause insulin resistance, impairment of triglyceride storage and an increase in lipolysis in WAT (Eckel et al., 2005; Guilherme et al., 2008; Médina-Gomez, 2012). Concomitantly, the increase of fatty acids in circulation can affect insulin-stimulated glucose metabolism in skeletal muscle and also impairs other actions of insulin in other organs, including liver, heart and the vasculature (Kim et al., 2008). Insulin resistance is a key factor in the progression of type 2 diabetes mellitus, a metabolic disorder characterized by hyperglycemia and insufficiency of secretion or receptor insensitivity to endogenous insulin. The complications of diabetes include cardiovascular diseases and other complications that affect kidney and peripheral nerves (Qatanani & Lazar, 2007). However, the mechanisms underlying the development of these pathologies are yet to be fully understood.

Collectively, there is strong evidence to support the idea that excessive fat accumulation is a key factor underlying adipose tissue dysfunction. Considering that a loss of 5-10% of the WAT mass is sufficient to improve dyslipidemia, hypertension and insulin resistance, the reduction of accumulated fat levels constitutes an interesting target for the development of new therapies (Furukawa et al., 2004; Jones et al., 2007). Given that, a potential therapeutic approach to prevent and reverse these initial events in the progression of obesity is based on the targeting of WAT, and in particular, mitochondria of adipocytes.

In this context, CDCA emerge as a potential pharmacological compound to the management of obesity and metabolic syndrome by inducing weight loss, improve glucose tolerance and insulin sensitivity in animal models (Watanabe et al., 2006). However, it is important to dissect whether the role of CDCA in preventing and limitating fat accumulation (Teodoro et al., 2014) is a direct or an indirect effect of CDCA in WAT.

This study demonstrates for the first time that the natural bile acid CDCA induced the appearance of UCP-1 expressing adipocytes in cultures of 3T3-L1 adipocytes. The induction of the transdifferentiation of these cells leads to a decrease on lipid accumulation, which is correlated with previous results that demonstrate BAs beneficial effects in the improvement of the metabolism *in vivo* (Watanabe et al., 2004,

2006, 2012). This suggests that CDCA may improve metabolism by a direct effect on the white adipose tissue. We furthermore show that CDCA effects were at least, mediated through the Wnt/ β-catenin signaling pathway. In fact, the decrease in TG accumulation induced by CDCA was not altered by CHIR99021, an activator of the pathway but it was blocked by XAV939, an inhibitor of the pathway.

Although BAs are essential for solubilization and transport of dietary lipids, these molecules exert multiple biological functions as signaling agents by binding to membrane and nuclear receptors, endowing them with an endocrine function (Sharma et al., 2011). In this study, we showed that CDCA induced a decrease on lipid accumulation in 3T3-L1 adipocytes after 96h of treatment. Although the exposure to excessive BAs is reported to be cytotoxic, in our study, CDCA did not alter cell viability of 3T3-L1 adipocytes. The concentrations used in this study were lower that the cytotoxic concentrations reported in several experiments and other cell lines (Im et al., 2001; Rolo et al. 2003). In these studies, BAs are showed to inhibit cell proliferation in cultured HepG2 and human cervical carcinoma cells while other studies showed that BAs can induce necrosis, and at a lower concentration, can induce apoptosis through intrinsic and extrinsic pathways (Im et al., 2001; Rolo et al. 2003) Thus, our results are in agreement with reported experiments which showed that CDCA did not induce any effects in cell viability (Im et al., 2001).

The observed CDCA effect are in accordance with published data showing that administration of bile acids such as CA prevented and reversed fat accumulation in high-fat fed animals through a decrease in WAT mass (Watanabe et al., 2006, 2011). CA has been shown to induce a thermogenic gene response that involves an increase in fat oxidation capacity resulting in bile acid-induced energy expenditure (Watanabe et al., 2006). Similarly, CDCA has been also described to induce UCP-1 mediated thermogenesis whereby a stimulation of energy expenditure caused a reduction in adiposity (Teodoro et al., 2014). It is known that TG accumulation depends on the net difference between the synthesis and breakdown of TG in adipocytes. Mitochondria play a pivotal role in the control of fatty acid β-oxidation (Medina-Gómez, 2012). Several studies have reported that increased energy expenditure is associated with an induction of UCP-1 positive-like cells in WAT (Watanabe et al., 2006, 2012), being the stimulation of UCP-1 mediated thermogenesis an effective strategy to energy expenditure and for the stabilization of a lean phenotype (Barbatelli et al., 2010; Teodoro et al., 2014). The recruitment of this type of cells also known as brite or beige cells causes an increase in UCP-1 content and mitochondrial biogenesis, promoting

high mitochondrial activity, including fatty acid oxidation and electron transport chain activity (Smorlesi et al., 2012).

Importantly, our study demonstrated CDCA-induced an increase in UCP-1 content in 3T3-L1 adipocytes. Previous work has shown that UCP-1 may be ectopically induced in white adipocytes (Barbatelli et al., 2010; Böstrom et al., 2012; Petrovic et al., 2010), and this expression reduced lipid accumulation in 3T3-L1 adipocytes (Senocak et al., 2008; Si et al., 2007). Thus, the higher UCP-1 content induced by CDCA is related with reduced triglycerides accumulation in white adipocytes that contributes to energy expenditure both *in vitro* and *in vivo* (Kopecky et al., 1995; Orci et al., 2004; Tiraby et al., 2003). These results are consistent with the energy dissipative function of UCP-1 that is known to uncouple oxidative phosphorylation, promoting the dissipation of cellular energy as heat (Rousset et al., 2004).

The inhibition of the Wnt/ β-catenin signaling pathway blocked the CDCA-decreased TG accumulation. In line with these findings, the inhibition of the pathway by XAV939 also induced a slight decrease in the UCP-1 content. In fact, the reduction in UCP-1 and its dissipatory energy function are in agreement with the observed increase in TG accumulation.

The Wnt/ β-signaling pathway has been established as an important regulator of adipogenesis. PPAR-γ and C/EBPα are the key adipogenic factors of this process, thereby inducing the expression of the downstream target genes of the adipogenic process (Christodoulides et al., 2009). Wnt signaling pathway is regulated by endogenous Wnt inhibitors. The activation of SFRP5, an inhibitor of Wnt pathway, increased susceptibility to obesity in high fat fed mice (Mori et al., 2012). As such, recent studies have assigned particular importance to the activated canonical Wnt pathway, because of its role in inhibiting adipogenesis. The exposure to kirenol showed to downregulate the key adipogenic factors, preventing lipid accumulation by modulating components of Wnt/ β-catenin pathway in 3T3-L1 adipocytes (Kim et al., 2014). Wnt10b also prevent adipogenesis, thereby inhibiting WAT development *in vivo* (Wright et al., 2007). The activation of the pathway induced direct effects on visceral adiposity, and improves glucose and lipid metabolism in the body overall (Choi et al., 2013). The downregulation of expression of genes involved in adipogenesis causes a reduction in lipid accumulation and visceral adiposity, which exerts beneficial effects against obesity (Shen et al., 2014). Therefore, CDCA and its capacity in regulating obesity and metabolic disorders may be dependent on the modulation by this pathway.

The impact of the Wnt/ β-catenin signaling pathway on UCP-1 expression is intriguing. In brown adipocytes, Wnt/ β-catenin activation by CHIR99021 has been

shown to decrease PGC-1α-mediated UCP-1 expression while limiting lipid accumulation (Kang et al., 2005). In contrast, Wnt inhibitory factors of the frizzled-related family proteins have been shown stimulate to mitochondrial biogenesis and function in both adipose tissue and cultured adipocytes (Mori et al., 2012).

It should be noted that when Wnt/ β-catenin signaling is activated by inhibition of GSK3β, free β-catenin molecules are stabilized in the cytosol, that can modulate the relative levels of the cell type specific transcription factors, supressing them (Prestwich & MacDougald, 2007). In fact, the activation of the canonical Wnt signaling does not appear to influence C/EBPβ or C/EBPδ but appears to repress the expression of the adipogenic transcription factors PPAR-γ and C/EBPα (Kang et al., 2005). Since C/EBPα induces adipogenesis through the action of PPAR-γ, when β-catenin interacts negatively with this transcription factor, this results in the repression of adipogenesis (Prestwich & MacDougald, 2007). Although β-catenin may interfere with transcriptional activation of UCP-1 as shown in brown adipocytes (Kang et al., 2005), one explanation for these results can be based on a complete inhibition of PPAR-γ expression. Thus, loss of PPAR-γ function impairs the TG storage capacity of the adipocytes, thereby inducing a decrease in TG accumulation. This hypothesis provides an explanation not only for the CDCA and CHIR99021 effects but also for the CHIR99021 effects *per se* in the absence of CDCA.

Accordingly, XAV939 mediates tankyrase inhibition, leading to the stabilization of axin and consequently, to the formation of the β-catenin destruction complex composed by GSK3β, axin and APC (Huang et al., 2009). In this case, the degradation of β-catenin prevents its interaction with the adipogenic transcription factor PPAR-γ, preventing the decrease in TG accumulation induced by CDCA. Given that, we can demonstrate that canonical Wnt signaling might play a role in the control of TG accumulation by a PPAR-γ mechanism. As such, induction of UCP-1 by CDCA may involve other mechanisms that have not been clarified yet.

CDCA might regulate body fat mass by inducing a metabolic coordination between UCP-1 and other enzymes of oxidative metabolism (Watanabe et al., 2012). In fact, mice fed on a high fat diet containing CA display increase oxygen consumption, which was correlated with an increase in the number of lamellar cristae (Watanabe et al., 2006). However, our data did not support an up-regulation of mitochondrial oxidative phosphorylation activity as previously others studies demonstrated (Watanabe et al., 2006, 2012). In this study, CDCA did not alter mitochondrial activity neither mitochondrial protein content. Although consistent, our data are not in accordance with above observations using CA and CDCA in mice (Watanabe et al.,

2006, 2012). Considering the UCP-1 function in dissipating the flux of protons from the intermembrane space into the mitochondrial matrix, it would be expected that UCP-1 should decrease the mitochondrial membrane potential as well as the ROS generation (Baumruk et al., 1999; Fink et al., 2005; Rousset et al., 2004; Tejerina et al., 2009). Under hyperglycemic conditions, ROS generation has shown to increase in parallel with the accumulation of lipids during the differentiation of 3T3-L1 pre-adipocytes into adipocytes (Furukawa et al., 2004). In these conditions, alterations in mitochondrial membrane potential and ROS accumulation leads to deleterious effects at mitochondrial level, which has been linked to the disruption of the intracellular dynamic of the adipocytes (Curtis et al., 2014). In this study, the lowest concentration of CDCA induced no alterations in mitochondrial membrane potential while the highest concentration of CDCA increased the mitochondrial membrane potential. On the other hand, CDCA was unable to reduce ROS generation in 3T3-L1 adipocytes. Indeed, these observations are correlated with the absence of alterations observed in the others mitochondrial parameters analysed. These differences can be due to the fact that the exposure of the 3T3-L1 adipocytes to a high glucose media did not induce alterations in mitochondrial membrane potential neither ROS generation that allow the detection of changes in the parameters when UCP-1 is induced. Moreover, it should be considered that UCPs form a small family of proteins that comprises UCP-1 but also UCP-2 and UCP-3. Despite UCP-1 is a well-characterized protein responsible for uncoupling respiration, UCP-2 and UCP-3 appear to be more involved in restricting the production of ROS in cells (Rousset et al., 2004). In this regard, the induction of UCP-1 on adipocytes might not induce alterations in ROS generation maybe due to the fact that the physiological function of the protein depends on the tissue where it is expressed. In fact, UCP-1 can reduce mitochondrial ROS emission when it is expressed in muscle. In contrast, UCP-1 cannot control ROS formation in BAT (Adjeitey et al., 2013). Considering the equal metabolic signatures between BAT and UCP-1 expressing adipocytes, it is suggested that when expressed in UCP-1 expressing adipocytes, UCP-1 also not regulate mitochondrial ROS emission.

BAs seem to regulate energy expenditure by inducing the expression of genes involved in BAT thermogenesis (Watanabe et al., 2006). During this process, UCP-1-positive like cells, once stimulated, can enrich not only the expression of UCP-1 and other thermogenic genes but also the expression of mitochondrial genes (Wu et al., 2012). In line with recent findings, the induction of thermogenic activity involves the combined action of several regulators (Seale et al., 2009). Besides the role of PGC-1α in the regulation of adaptative thermogenesis, this transcriptional co-activator is known

to be one of the main regulators of cellular energy metabolism, mitochondrial biogenesis and fatty acid β-oxidation (Puigserver et al., 2003). The administration of bile acids such as CA or CDCA was reported to induce an increase in the expression of *PGC-1α* and *UCP-1* genes in WAT (Teodoro et al. 2014; Watanabe et al., 2011). In addition, CDCA treated mice exhibited increased expression of mitochondrial biogenesis markers (Teodoro et al., 2014). However, in our study, CDCA did not induce alterations in the UCP-1 and PGC-1α expression levels while induced an increase in COX-III expression. This pattern of expression seems to be not in accordance with the previous reported findings. However, the discrepancies between the content and the expression of COX-III are possibly justified by a post-transcriptional event that act, maybe at the protein synthesis level. On other hand, the UCP-1 gene expression levels and the protein content of this protein can be justified by the cellular machinery which could promote an increase in protein stability and a downregulation in gene expression as a metabolic adaptation to the energy state (Senocak et al., 2008).

In order to determine the molecular basis for the reduced TG accumulation, we investigated the profile changes in the gene expression of enzymes implicated in major metabolic processes in adipocytes. The CDCA treatment did not induce alterations in the transcript levels of lipogenic enzymes, including FAS and PEPCK. In addition, our data indicated a slight increase in the expression of PPAR-α. PPAR-α plays an important role in the regulation of genes involved in lipid catabolism and thermogenic gene expression through the induction of UCP-1 (Hondares et al., 2011). Therefore, CDCA may interfere with cellular energy status, by the mechanistic link between UCP-1 and AMPK. Previous studies reported that the UCP-1 expressing adipocytes undergo metabolic adaptations to compensate the inefficiency of oxidative phosphorylation to generate ATP (Senocak et al., 2008; Si et al., 2009). In fact, it was showed that mitochondrial uncoupling in adipocytes leads to a decrease in intracellular ATP levels in vitro (Klaus et al., 2012; Si et al., 2009). In this respect, the lower ATP yield can stimulate the AMPK pathway which has been shown to stimulate glucose transport and increase glycolysis (Kemp et al., 2003). Thus, AMPK activation induces glycolytic ATP generation while causes a down-regulation of energy consuming processes in adipocytes. In accordance, the reduction in TG accumulation would be caused by a reduction of lipid synthesis rather than an up-regulation of fatty acid oxidation (Si et al., 2009). Findings suggest that the maintenance of constant ATP levels depends on anaerobic processes which increase ATP generation by lactate production (Si et al., 2009). In fact, prior studies reported that UCP-1 expression induces an increase in glucose oxidation (Si et al., 2007; Tejerina et al., 2009). Although 3T3-L1 adipocytes

were cultured with an abundant supply of glucose in the media, our data showed no alterations in *GLUT4* expression (Figure 3.10) (Si et al., 2007). Despite the apparent contradiction about lipogenesis and glucose oxidation routes between our study and prior studies, it should be noted that the metabolic cellular adjustments depend on the time by which adipocytes are exposed to the uncoupling agent (Senocak et al., 2008). In fact, various cell types, when treated with uncoupling agents, respond by primarily up-regulating glucose consumption and anaerobic ATP synthesis and, only after, by down-regulating oxidative phosphorylation (Cabrero et al., 1999; Rossmeisl et al., 2000). In this regard, it is suggested that the primary adaptation of 3T3-L1 adipocytes in the response to mitochondrial uncoupling is an increase in glucose oxidation. Thus, this hypothesis warrants further investigation to clarify the mechanisms for reduced lipid accumulation. These additional studies should involve the quantification of metabolic fluxes using isotopic tracers. This analysis would be also important to confirm the results obtained for the gene expression analysis.

BAs are signaling molecules which act as ligands of TGR5 and members of the nuclear hormone receptor superfamily that comprises not only FXR but also the constitutive androstane receptor (CAR), the pregnane X receptor (PXR) and the vitamin D receptor (VDR) (Schaap et al., 2014). Although the biological relevance of all receptors, the two best characterized bile acid- activated receptors that are targeted for bile acid action are the TGR5 and the FXR. Therefore, the activation of bile acid-TGR5-cAMP-D2 signaling pathway is one promising pathway supporting bile acids effect. TGR5 is a metabotropic receptor of bile acid that is known to be expressed in adipose tissue (Kawamata et al., 2003). As such, CDCA might induce the activation of the cAMP-dependent thyroid hormone-activating enzyme D2 (Tiwari A. & Maiti P., 2009). During this process, T4 derived from foetal bovine serum in media is converted into the active form of thyroid hormone (Watanabe et al., 2006). Since T3 is implicated in the induction of thermogenesis in mammals, this hormone may be able to induce thermogenesis in 3T3-L1 cells in a same manner. However, in our study, the lack of evidence of higher mitochondrial function remains contradictory. Therefore, to address such issues, it would be interesting to study the oxygen consumption on CDCA-treated cells. Moreover, CDCA might induce its effects through the induction of FXR. FXR regulates the expression of genes involved in lipoprotein clearance, reducing glucose, TG and cholesterol plasma levels (Claudel et al. 2005). Despite its lower content in adipose tissue, the activation of FXR leads to the induction of adipose tissue capacity storage by altering adipokines secretion and the expression of PPAR-Ɣ, SREBP1c and FABP. Moreover, the activation of FXR in liver seems to induce the expression of

PPAR-α and its target genes, increasing fatty acid oxidation and decreasing the capacity to secrete or store TGs (Teodoro et al. 2011). Although the activation of FXR has a key role protecting against body weight gain and fat deposition (Cipriani et al. 2010), these effects seem to be linked to its action in enteropatic tissues and not a direct effect in adipose tissue.

In summary, our results demonstrate that the natural bile acid CDCA induces a decrease in TG accumulation. This decreased is justified by a conversion of white adipocytes into UCP-1 positive cells. The increase in the mitochondrial protein UCP-1 justifies the subsequent energy dissipation process that is crucial to the reduction of TG accumulation. However, we do not rule out the possibility of CDCA exert a minor role at mitochondria level. CDCA effects seem to be mediated by the Wnt/ β-catenin signaling pathway. This pathway has an important role in the control of TG accumulation in 3T3-L1 adipocytes. The inhibition of the pathway induces a slight decrease in UCP-1 content and, it might play a role in the control of TG accumulation by a PPAR-γ mechanism. Additionally, activation of PPAR-α by CDCA may be the pathway that explains UCP-1 induction. CDCA effects on 3T3-L1 adipocytes are summarized in the Figure 4.1.

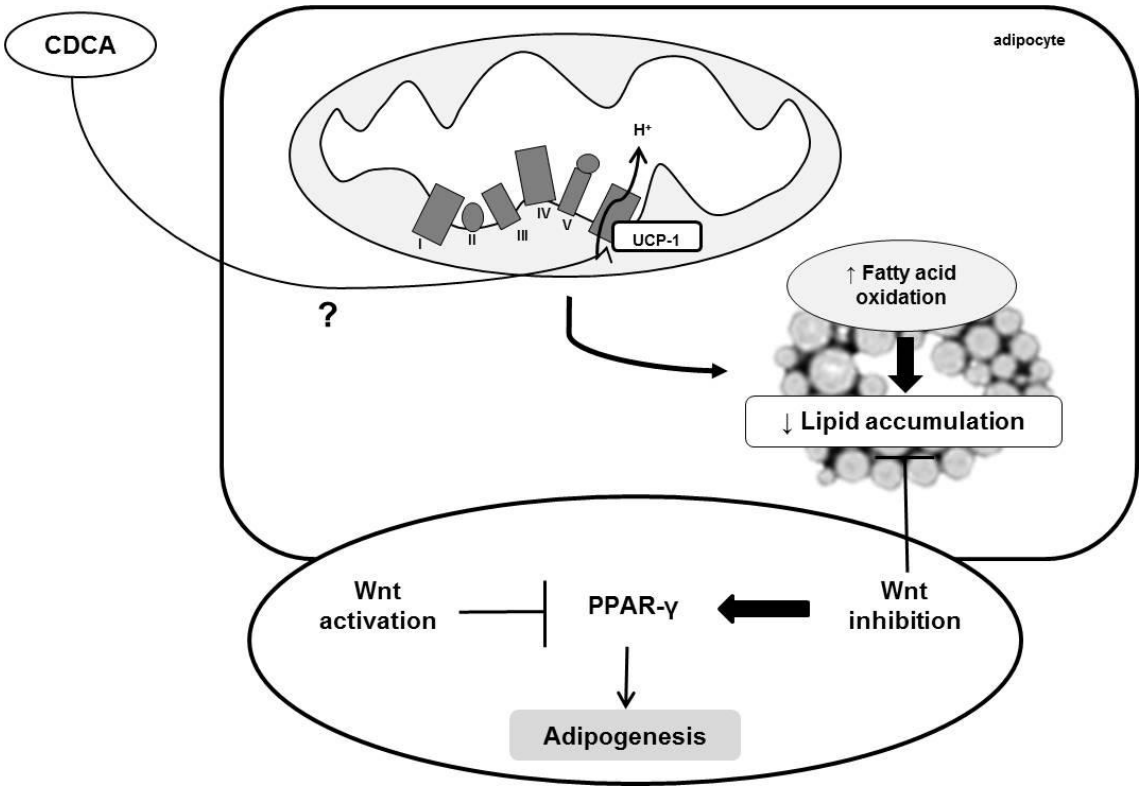

Figure 4.1. Mechanisms of CDCA action in the 3T3-L1 adipocytes. CDCA induces an increase in UCP-1 content in white adipocytes, thereby inducing an increase in mitochondrial uncoupling and an increase in the energy dissipation. This process leads to an increase in fatty acid oxidation, and consequently to a decrease in TG accumulation in 3T3-L1 adipocytes. This effect is mediated by the Wnt/ β-catenin signaling pathway. While the activation of the pathway does not interfere with the decrease in TG accumulation induced by CDCA, an inhibition of the pathway blocks this reduction. The inhibition of the pathway seems to promote the activation of PPAR-γ, the key adipogenic factor in the induction of adipogenesis. Therefore, an increase in lipid accumulation is at least justified by the induction of the adipogenic process.

CHAPTER 5

CONCLUSIONS

In the present study, we evaluated the CDCA effects as well as the role of Wnt/ β-catenin signaling pathway in the modulation of these BA effects in 3T3-L1 adipocytes.

Importantly, using this cell line, we clearly show for the first time that CDCA induced UCP-1 positive cells *in vitro*. Moreover, CDCA induced a decrease in TG accumulation. This effect is maintained when canonical Wnt pathway is activated. However, the reduction in lipid accumulation induced in the presence of CDCA was blocked by the inhibition of the pathway. It was also shown that CDCA effects are partially elicited by an induction of UCP-1 which was reported to be partially reduced by the inhibition of the canonical Wnt pathway. This protein increases energy expenditure and its induction is able to protect adipocyte cells from the deleterious effects of excess of lipid accumulation in fat cells. Although CDCA was found to decrease TG accumulation through an increase in the induction of UCP-1 and fatty acid oxidation, other mechanisms should not be ruled out. Despite no alterations were detected in the mitochondrial functional parameters assessed, CDCA was able to induce an increase in the expression of mitochondrial-encoded protein subunits.

This study demonstrates that CDCA induce changes in adipocytes that fully account to a decrease in lipid accumulation *in vitro*. Moreover, our results demonstrated that the beneficial effects of CDCA treatment were at least dependent of Wnt/ β-catenin signaling pathway. The reduction in lipid accumulation supports the decrease in WAT mass and the improvement of the metabolic profile, promoting weight loss and insulin sensitivity during conditions of high-fat intake *in vivo*. Altogether, this work demonstrates that CDCA induce alterations at the adipose tissue level that can influence the energy balance and prevent dietary-induced obesity.

Thus, CDCA may contribute to the development of an effective strategy in the prevention and in the reversion of the deleterious effects of obesity and associated metabolic disorders. However, further studies are needed to better understand the molecular and cellular mechanisms involved in the actions of CDCA as well as in its modulation by Wnt/ β-catenin signaling pathway.

REFERENCES

Adjeitey, C.N., Mailloux, R.J., Dekemp, R.A., Harper, M., 2013. Mitochondrial uncoupling in skeletal muscle by UCP1 augments energy expenditure and glutathione content while mitigating ROS production. *American Journal of Physiology, Endocrinology and Metabolism*, 305(3), pp.E405-E415.

Anghel, S.I., Bedu, S.I., Vivier, E.D., Descombes, P., 2007. Adipose tissue integrity as a prerequisite for systemic energy balance: a critical role for peroxisome proliferator-activated receptor ϒ. *The Journal of Biological Chemistry*, 282(41), pp.29946–29957.

Arch, J.R.S., 2002. β3-Adrenoceptor agonists: potential, pitfalls and progress. *European Journal of Pharmacology*, 440(2-3), pp.99–107.

Barbatelli, G., Murano, I., Madsen, L., Hao, Q., Jimenez, M., Kristiansen, K., Giacobino, J.P., Matteis, R., Cinti, S., 2010. The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. *Endocrinology and Metabolism: American Journal of Physiology*, 298, pp.1244–1253.

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

Bartelt, A., Bruns, O.T., Reimer, R., Hohenberg, H., Ittrich, H., Peldschus, K., Kaul, M. G., Tromsdorf, U.I., Weller, H., Waurisch, C., Eychmüller, A., Gordts, P.L.S.M., Rinninger, F., Bruegelmann, K., Freund, B., Nielsen, P., Merkel, M., Heeren, J., 2011. Brown adipose tissue activity controls triglyceride clearance. *Nature medicine*, 17(2), pp.200–205.

Bartness, T.J.,Vaughan, C.H. & Song, C.K., 2014. Sympathetic and sensory innervation of brown adipose tissue. *International Journal of Obesity*, 34(0-1), pp.S36-S42.

Baumruk, F., Flachs, P., Horáková, M., Floryk, D., Kopecký, J., 1999. Transgenic UCP1 in white adipocytes modulates mitochondrial membrane potential. *FEBS Letters*, 444, pp.206-210.

Bays, H., Mandarino, L. & DeFronzo, R.A., 2004. Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. *The Journal of Clinical Endocrinology & Metabolism*, 89(2), pp.463-478.

Bennett, C.N., Ross, S.E., Longo, K.A., Bajnok, L., Hemati, N., Johnson, K.W., Harrison, S.D., Macdougald, O.A., 2002. Regulation of Wnt signaling during adipogenesis. *The Journal of Biological Chemistry*, 277(34), pp.30998–31004.

Blanchet, E., Bertrand, C., Annicotte, J.S., Schlernitzauer, A., Pessemesse, L., Levin, J., Fouret, G., Feillet-Coudray, C., Bonafos, B., Fajas, L., Cabello, G., Wrutniak-Cabello, C., Casas, F., 2012. Mitochondrial T3 receptor p43 regulates insulin secretion and glucose homeostasis. *The FASEB Journal*, 26(1), pp.1–11.

Boatright, K.M. & Salvesen, G.S., 2003. Mechanisms of caspase activation. *Current Opinion in Cell Biology*, 15(6), pp.725-731.

Boström, P., Wu, J., Jedrychowski, M.P., Korde, A., Ye, L., Lo, J.C., Rasbach, K.A., Boström, E.A., Choi, J.H., Long, J.Z., Zingaretti, M.C., Vind, B.F., Tu, H., Cinti, S., Gygi, S.P., Spiegelman, B.M., 2012. A PGC1α-dependent myokine that drives browning of white fat and thermogenesis. *Nature*, 481(7382), pp.463–468.

Bouillaud, F., Combes-George, M. & Ricquier, D., 1992. Mitochondria of adult human brown adipose tissue contain a 32 000-Mr uncoupling protein. *Bioscience Reports*, 3(8), pp.775–780.

Brautigan, D.L., Ferguson-Miller, S. & Margoliash, E., 1978. Mitochondrial cytochrome c: preparation and activity of native and chemically modified cytochromes c. *Methods in Enzimology*, 53, pp.128-164.

Cabrero, A., Llaverías, G., Roglans, N., Alegret, M., Sánchez, R., Adzet, T., Laguna, J., Vázquez, M., 1999. Uncoupling protein-3 mRNA levels are increased in white adipose tissue and skeletal muscle of bezafibrate-treated rats. *Biochemical and Biophysical Research Communications*, 260(2), pp. 547-556.

Cai, D., Yuan, M., Frantz, D.F., Melendez, P.A., Hansen, L., Lee, J., Shoelson, S.E., 2006. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nature Medicine*, 11(2), pp.183–190.

Cannon, B. & Nedergaard, J., 2004. Brown adipose tissue: function and physiological significance. *Physiological Reviews*, 84(1), pp.277–359.

Casas, F., Pessemesse, L., Grandemange, S., Seyer, P., Gueguen, N., Baris, O., Lepourry, L., Cabello, G., Wrutniak-Cabello, C., 2008. Overexpression of the mitochondrial T3 receptor p43 induces a shift in skeletal muscle fiber types. *PloS one*, 3(6), p.e2501.

Choi, O.M., Cho, Y-H., Choi, S., Lee, S-H., Seo, S.H., Kim, H-Y., Han, G., Min, D.S., Park, T., Choi, K.Y., 2013. The small molecule indirubin-3'-oxime activates Wnt/β-catenin signaling and inhibits adipocyte differentiation and obesity. *International Journal of Obesity*, pp.1-9.

Choo, H.-J.,Kim, J-H., Kwon, O-B., Lee, C.S., Mun, J.Y., Han, S.S., Yoon, Y-S., Yoon, G., Choi, K-M., Ko, Y-G., 2006. Mitochondria are impaired in the adipocytes of type 2 diabetic mice. *Diabetologia*, 49(4), pp.784–91.

Christodoulides, C., Lagathu, C., Sethi, J.K., Vidal-Puig, A., 2009. Adipogenesis and WNT signalling. *Trends in Endocrinology and Metabolism*, 20(1), pp.16-24.

Chung, S.S., Lee, J.S., Kim, M., Ahn, B.Y., Jung, H.S., Lee, H.M., Kim, J-W., Park, K.S., 2012. Regulation of Wnt/β-catenin signaling by CCAAT/enhancer binding protein β during adipogenesis. *Obesity (Silver Spring, Md.)*, 20(3), pp.482–487.

Church, C.D., Horowitz, M.C. & Rodeheffer, M.S., 2012. WAT is a functional adipocyte?. *Adipocyte*, 1(1), pp.38-45.

Cinti, S., Mitchell, G., Barbatelli, G., Murano, I., Ceresi, E., Faloia, E., Wang, S., Fortier, M., Greenberg, A.S., Obin, M.S. et al., 2005. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *Journal of Lipid Research*, 46(11), pp.2347–2355.

Cinti, S., 2012. The adipose organ at a glance. *Disease Models & Mechanisms*, 5, pp.588–594.

Cipriani, S., Mencarelli, A., Palladino, G., Fiorucci, S., 2010. FXR activation reverses insulin resistance and lipid abnormalities and protects against liver steatosis in Zucker (fa/fa) obese rats. *Journal of Lipid Research*, 51(4), pp.771–784.

Clapham, J.C. & Arch, J.R.S., 2007. Thermogenic and metabolic antiobesity drugs: rationale and opportunities. *Diabetes, obesity and metabolism*, 9(3), pp.259–75.

Claudel, T., Staels, B. & Kuipers, F., 2005. The farnesoid X receptor: a molecular link between bile acid and lipid and glucose metabolism. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 25(10), pp.2020–2030.

Clevers, H., 2006. Wnt/beta-catenin signaling in development and disease. *Cell*, 127(3), pp.469–480.

Cousin, B., Cinti, S., Morroni, M., Raimbault, S., Ricquier, D., Pénicaud, L., Casteilla, L., 1992. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *Journal of Cell Science*, 103 (Pt 4), pp.931–942.

Cristancho, A.G. & Lazar, M.A., 2011. Forming functional fat: a growing understanding of adipocyte differentiation. *Nature Reviews. Molecular Cell Biology*, 12(11), pp.722–734.

Curtis, J.M., Grimsrud, P.A., Wright, W.S., Xu, X., Foncea, R.E., Graham, D.W., Brestoff, J.R., Wiczer, B.M., Ilkayeva, O., Cianflone, K., Muoio, D.E., Arriaga, E.A., Bernlohr, D.A., 2014. Downregulation of adipose glutathione S-transferase A4

leads to increased protein carbonylation, oxidative stress, and mitochondrial dysfunction. *Diabetes*, 59(5), pp.1132–1142.

Cypess, A.M., Chen, Y-C., Sze, C., Wang, K., English, J., Chan, O., Holman, A.R., Tal, I., Palmer, M.R., Kolodny, G.M., Kahn, C.R., 2012. Cold but not sympathomimetics activates human brown adipose tissue in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 109(25), pp.10001–10005.

Cypess, A.M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A.B., Kuo, F.C., Palmer, E.L., Tseng, Y., Doria, A., Kolodny, G.M., Kahn, C.R., 2009. Identification and importance of brown adipose tissue in adult humans. *The New England Journal of Medicine*, 360(15), pp.1509–1517.

Cypess, A.M. & Kahn, C.R., 2010. Brown fat as a therapy for obesity and diabetes. *Current Opinion in Endocrinology, Diabetes and Obesity*, 17(2), pp.1–14.

da-Silva, W.S., Ribich, S., Arrojo e Drigo, R., Castillo, M., Patti, M., Bianco, A.C., 2011. The chemical chaperones tauroursodeoxycholic and 4-phenylbutyric acid accelerate thyroid hormone activation and energy expenditure. *FEBS Letters*, 585(3), pp.539–544.

DeFronzo, R.A. & Tripathy, D., 2009. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes Care*, 32(2), pp.157–63.

Distelmaier, F., Koopman, W.J.H., Testa, E.R., Jong, A.S., Swarts, H.G., Mayatepek, E., Smeitink, J.A.M., Willems, P.H.G.M., 2008. Life cell quantification of mitochondrial membrane potential at the single organelle level. *Journal of the International Society for Analytical Cytology*, 73A, pp.129-138.

Eckel, R.H., Grundy, S.M. & Zimmet, P.Z., 2005. The metabolic syndrome. *Lancet*, 365, pp.1415-1428

Ehrlund, A., Mejhert, N., Lorente-Cebrián, S., Aström, G., Dahlman, I., Laurencikiene, J., Rydén, M., 2013. Characterization of the Wnt inhibitors secreted frizzled-related proteins (SFRPs) in human adipose tissue. *The Journal of Clinical Endocrinology and Metabolism*, 98(3), pp.E503–E508.

Enerbäck, S., 2010. Human brown adipose tissue. *Cell Metabolism*, 11(4), pp.248-252.

Festuccia, W.T., Blanchard, P-G. & Deshaies, Y., 2011. Control of brown adipose tissue glucose and lipid metabolism by PPARγ. *Frontiers in endocrinology*, 2(84), pp.1–6.

Fink, B.D., Reszka, K.J., Herlein, J.A., Mathahs, M.M., Sivitz, W.I., 2005. Respiratory uncoupling by UCP1 and UCP2 and superoxide generation in endothelial cell mitochondria. *American Journal of Physiology - Endocrinology and Metabolism*, 288(1), pp. E71-E79.

Fiorucci, S., Mencarelli, A., Palladino, G., Cipriani, S., 2009. Bile-acid-activated receptors: targeting TGR5 and farnesoid-X-receptor in lipid and glucose disorders. *Trends in Pharmacological Sciences*, 30(11), pp.570–580.

Fischer-Posovszky, P., Wang, Q.A., Asterholm, I.W., Rutkowski, J.M., Scherer, P.E., 2011. Targeted deletion of adipocytes by apoptosis leads to adipose tissue recruitment of alternatively activated M2 macrophages. *Endocrinology*, 152(8), pp.3074–3081.

Frontini, A., Vitali, A., Perugini, J., Murano, I., Romiti, C., Ricquier, D., Guerrieri, M., Cinti, S., 2013. White-to-brown transdifferentiation of omental adipocytes in patients affected by pheochromocytoma. *Biochimica et Biophysica Acta*, 1831(5), pp.950–959.

Fukuchi, K.,Tatsumi, M., Ishida, Y., Oku, N., Hatazawa, J., Wahl, R.L., 2004. Radionuclide imaging metabolic activity of brown adipose tissue in a patient with pheochromocytoma. *Experimental and Clinical Endocrinology & Diabetes*, 112(10), pp.601–603.

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., 2004. Increased oxidative stress in obesity and its impact on metabolic syndrome. *The Journal of Clinical Investigation*, 114(12), pp.1752–1761.

Ghorbani, M., Claus, T.H. & Himms-Hagen, J., 1997. Hypertrophy of brown adipocytes in brown and white adipose tissues and reversal of obesity in rats treated with a β3-adrenoceptor agonist. *Biochemical Pharmacology*, 54(1), pp.121–131.

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

Godpaster, B.H., DeLany, J.P., Otto, A.D., Kuller, L., Vockley, J., South-Paul, J.E., Thomas, S.B., Brown, J., McTigue, K., Hames, K.C., Lang, W., Jakicic, J.M., 2011. Effects of diet and physical activity interventions on weight loss and cardiometabolic risk factors in severely obese adults. *The Journal of American Medical Association*, 304(16), pp.1795–1802.

Goodparter, B., He, J., Watkins, S., Kelley, D.E., 2001. Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *The Journal of Clinical Endocrinology and Metabolism*, 86(12), pp.5755-5761.

Gomes, A., Fernandes, E. & Lima, J.L.F.C., 2005. Fluorescence probes used for detection of reactive oxygen species. *Journal of Biochemical and Biophysical Methods*, 65(2-3), pp.45-80.

Green, H. & Meuth, M., 1974. An established pre-adipose cell line and its differentiation in culture. *Cell*, 3(2), pp. 127-133.

Guerra, C., Koza, R.A., Yamashita, H., Walsh, K., Kozak, L.P., 1998. Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. *The Journal of Clinical Investigation*, 102(2), pp. 412-420.

Guilherme, A., Virbasius, J.V., Puri, V., Czech, M.P., 2008. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nature Reviews Molecular Cell Biology*, 9(5), pp.367–377.

Hall, J.A., Ribich, S., Christoffolete, M.A., Simovic, G., Correa-Medina, M., Patti, M.E., Bianco, A.C., 2010. Absence of thyroid hormone activation during development underlies a permanent defect in adaptive thermogenesis. *Endocrinology*, 151(9), pp.4573–4582.

Hamann, A., Benecke, H., Marchand-brustel, Y.L., Susulic, V.S., Lowell, B.B., Flier, J.S., 1995. Characterization of insulin resistance and NIDDM in transgenic mice with reduced brown fat. *Diabetes*, 44, pp.1266–1273.

Hamann, A., Flier, J.S. & Lowell, B.B., 1996. Decreased brown fat markedly enhances susceptibility to diet-induced obesity, diabetes, and hyperlipidemia. *Endocrinology*, 137(1), pp.21–29.

Henriksen, E.J. & Dokken, B.B., 2006. Role of glycogen synthase kinase-3 in insulin resistance and type 2 diabetes. *Current Drug Targets*, 7(11), pp.1435–1441.

Himms-Hagen, J., Ceresi, E., Barbatelli, G., Cinti, S., Melnyk, A., Zingaretti, M.C., 2000. Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *American Journal of Physiology - Cell Physiology*, 279, pp.C670–C681.

Hondares, E., Rosell, M., Gonzalez, F.J., Giralt, M., Iglesias, R., Villarroya, F., 2011. Hepatic FGF21 expression is induced at birth via PPARα in response to milk intake and contributes to thermogenic activation of neonatal brown fat. *Cell metabolism*, 11(3), pp.206–212.

Hondares, E., Rosell, M., Díaz-Delfín, J., Olmos, Y., Monsalve, M., Iglesias, R., Villarroya, F., Giralt, M., 2011. Peroxisome proliferator-activated receptor alpha (PPARalpha) induces PPARgamma coactivator 1alpha (PGC-1alpha) gene expression and contributes to thermogenic activation of brown fat. *The Journal of Biological Chemistry*, 286(50), pp.43112–43122.

Hotamisligil, G.S. & Spiegelman, B.M., 1994. Tumor necrosis factor alpha: a key component of the obesity-diabetes link. *Diabetes*, 43(11), pp.1271–1278.

Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., Iwahashi, H., Kuriyama, H., Ouchi, N., Maeda, K., Nishida, M., Kihara, S., Sakai, N., Nakajima, T., Hasegawa, K., Muraguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S.

Hanafusa, T., Matsuzawa, Y., 2000. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 20(6), pp.1595–1599.

Huang, S.A., Mishina, Y.M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G.A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S., Hild, M., Shi, X., Wilson, C.J., Mickanin, C., Myer, V., Fazal, A., Tomlinson, R., Serluca, F., Shao, W., Cheng, H., Shultz, M., Rau, C., Schirle, M., Schlegl, J., Ghidelli, S., Fawell, S., Lu, C., Curtis, D., Kirschner, M.W., Lengauer, C., Finan, P.M., Tallarico, J.A., Bouwmeester, T., Porter, J.A., Bauer, A., Cong, F., 2009. Tankyrase inhibition stabilizes axin and antagonizes Wnt signaling. *Nature*, 461(1), pp. 614-620.

Im, E.O., Choi, Y.H., Paik, K.J., Suh, H., Jin, Y., Kim, K.W., Yoo, Y.H., Kim, N.D., 2001. Novel bile acid derivatives induce apoptosis via a p53-independent pathway in human breast carcinoma cells. *Cancer Letters*, 163(1), pp.83-93.

Inokuma, K., Ogura-Okamatsu, Y., Toda, C., Kimura, K., Yamashita, H., Saito, M., 2005. Uncoupling protein 1 is necessary for norepinephrine-induced glucose utilization in brown adipose tissue. *Diabetes*, 54, pp.1385–1391.

James, A.M. & Murphy, M.P., 2002. How mitochondrial damage affects cell function. *Journal of Biomedical Science*, 9(6), pp.475-487.

Jones, L.R., Wilson, C.I. & Wadden, T.A., 2007. Lifestyle modification in the treatment of obesity: an educational challenge and opportunity. *Clinical Pharmacology & Therapeutics*, 81(5), pp. 776-779.

Kahn, B.B. & Flier, J.S., 2000. Obesity and insulin resistance. *The Journal of Clinical Investigation*, 106(4), pp.473-481.

Kajimura, S., Seale, P., Tomaru, T., Erdjument-Bromage, H., Cooper, M.P., Ruas, J.L., Chin, S., Tempst, P., Lazar, M.A., Spiegelman, B.M., 2008. Regulation of the brown and white fat gene programs through a PRDM16/CtBP transcriptional complex. *Genes & Development*, 22(10), pp.1397–1409.

Kang, S., Bajnok, L., Longo, K.A., Petersen, R.K., Hansen, J.B., Kristiansen, K., Macdougald, O.A., 2005. Effects of Wnt signaling on brown adipocyte differentiation and metabolism mediated by PGC-1α. *Molecular and Cellular Biology*, 25(4), pp.1272–1282.

Karamitri, A., Shore, A.M., Docherty, K., Speakman, J.R., Lomax, M.A., 2009. Combinatorial transcription factor regulation of the cyclic AMP-response element on the Pgc-1alpha promoter in white 3T3-L1 and brown HIB-1B preadipocytes. *The Journal of Biological Chemistry*, 284(31), pp.20738–20752.

Kawamata, Y., Fujii, R., Hosoya, M., Harada, M., Yoshida, H., Miwa, M., Fukusumi, S., Habata, Y., Itoh, T., Shintani, Y., Hinuma, S., Fujisawa, Y., Fujino, M., 2003. A G protein-coupled receptor responsive to bile acids. *The Journal of Biological Chemistry*, 278(11), pp.9435–9440.

Kelley, D.E., He, J., Menshikova, E.V., Ritov, V.B., 2002. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes*, 51(10), pp.2944–50.

Kim, J-A., Wei, Y. & Sowers, J.R., 2008. Role of mitochondrial dysfunction in insulin resistance. *Circulation Research*, 102(4), pp.401-414.

Kim, M-B., Song, Y., Kim, C., Hwang, J-K., 2014. Kirenol inhibits adipogenesis through activation of the Wnt/β-catenin signaling pathway in 3T3-L1 adipocytes. *Biochemical and Biophysical Research Communications*, 445(2), pp. 433-448.

Klaus, S., Keipert, S., Rossmeisl, M., Kopecky, J., 2012. Augmenting energy expenditure by mitochondrial uncoupling: a role of AMP-activated protein kinase. *Genes & Nutrition*, 7(3), pp.369-386.

Kopecky, J., Clarke, G., Enerbãck, S., Spiegelman, B., Kozak, L.P., 1995. Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. *The Journal of Clinical Investigation*, 96(6), pp.2914–2923.

Kovacic, P.B., Chowdhury, H.H., Velebit, J., Kreft, M., Jensen, J., Zorec, R., 2011. New insights into cytosolic glucose levels during differentiation of 3T3-L1 fibroblasts into adipocytes. *The Journal of Biological Chemistry*, 286(15), pp.13370-13381.

Kusminski, C.M. & Scherer, P.E., 2012. Mitochondrial dysfunction in white adipose tissue. *Trends in Endocrinology & Metabolism*, 23(9), pp.435-443.

Lancha, A., Frühbeck, G. & Gómez-Ambrosi, J., 2012. Peripheral signaling involved in energy homeostasis control. *Nutrition Research Reviews*, 25(2), pp.223-248.

Langin, D., 2010. Recruitment of brown fat and conversion of white into brown adipocytes: strategies to fight the metabolic complications of obesity?. *Biochimica et Biophysica Acta*, 1801(3), pp.372–376.

Lean, M.E.J., 1989. Brown adipose tissue in humans. *Proceedings of the Nutrition Society*, 48, pp.243–256.

Lee, P., Swarbrick, M.M. & Ho, K.K.Y., 2013. Brown adipose tissue in adult humans: a metabolic renaissance. *Endocrine reviews*, 34(3), pp.413–38.

Lee, Y-K. & Cowan, C.A., 2013. White to brite adipocyte transition and back again. *Nature Cell Biology*, 15(6), pp.568–569.

Lehr, L., Canola, K., Léger, B., Giacobino, J-P. 2009. Differentiation and characterization in primary culture of white adipose tissue brown adipocyte-like cells. *International Journal of Obesity*, 33, pp.680–686.

Li, Y., Park, J-S., Deng, J-H., Bai, Y., 2007. Cytochrome *c* oxidase IV is essential for assembly and respiratory function of the enzyme complex. *Journal of Bioenergetics and Biomembranes,* 38(5-6), pp.283-291.

Liu, J., Wang, H., Zuo, Y., Farmer, S.R., 2006. Functional interaction between peroxisome proliferator-activated receptor gamma and beta-catenin. *Molecular and Cellular Biology*, 26(15), pp.5827–37.

Lo, K.A. & Sun, L., 2013. Turning WAT into BAT: a review on regulators controlling the browning of white adipocytes. *Bioscience Reports*, 33(5), pp.711–719.

Longo, K.A., Wright, W.S., Kang, S., Gerin, I., Chiang, S-H., Lucas, P.C., Opp, M.R., MacDougald, O., 2004. Wnt10b inhibits development of white and brown adipose tissues. *The Journal of Biological Chemistry*, 279(34), pp.35503–35509.

Lowell, B.B. & Flier, J.S., 1997. Brown adipose tissue, β3-adrenergic receptors, and obesity. *Annual Review of Medicine*, 48, pp.307–316.

Lumeng, C.N., Bodzin, J.L. & Saltiel, A.R., 2007. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *The Journal of Clinical Investigation*, 117(1), pp.175–184.

Maassen, J.A., Romijn, J.A. & Heine, R.J., 2007. Fatty acid-induced mitochondrial uncoupling in adipocytes as a key protective factor against insulin resistance and beta cell dysfunction : a new concept in the pathogenesis of obesity-associated type 2 diabetes mellitus. *Diabetologia*, 50, pp.2036–2041.

Mandard, S., Müller, M., Kersten, S., 2004. Peroxisome proliferator-activated receptor α target genes. *Cellular and Molecular Life Sciences*, 61(4), pp. 393-416.

Mano, N., Goto, T., Uchida, M., Nishimura, K., Ando, M., Kobayashi, N., Goto, J., 2004. Bioconversion of 3β-hydroxy-5-cholenoic acid into chenodeoxycholic acid by rat brain enzyme systems. *Journal of Lipid Research*, 45(9), pp.1741–1748.

Martini, C., Gabrielli, M. & Vila, M., 2012. A commercial formulation of glyphosate inhibits proliferation and differentiation to adipocytes and induces apoptosis in 3T3-L1 fibroblasts. *Toxicology in vitro*, 26, pp.1007-1013.

Medina-Gómez, G., 2012. Mitochondria and endocrine function of adipose tissue. *Best Practice & Research Clinical Endocrinology & Metabolism*, 26, pp.791-804.

Meex, R.C.R., Schrauwen-Hinderling, V.B., Moonen-Kornips, E., Schaart, G., Mensink, M., Phielix, E., Weijer, T., Sels, J-P., Schrauwen, P., Hesselink, M.K.C., 2010. Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. *Diabetes*, 59(3), pp.572-579.

Metcalfe, C. & Bienz, M., 2011. Inhibition of GSK3 by Wnt signaling - two contrasting models. *Journal of Cell Science*, 124(Pt 21), pp.3537–3544.

Moran, J.H. & Schnellmann, R.G., 1996. A rapid beta-NADH-linked fluorescence assay for lactate dehydrogenase in cellular death. *Journal of Pharmacological and Toxicological Methods*, 36(1), pp.41-44.

Mori, H., Prestwich, T.C., Reid, M.A., Longo, K.A., Gerin, I., Cawthorn, W.P., Susulic, V.S., Krishnan, V., 2012. Secreted frizzled-related protein 5 suppresses adipocyte mitochondrial metabolism through WNT inhibition. *The Journal of Clinical Investigation*, 122(7), pp.2405-2416.

Murano, I., Barbatelli, G., Parisani, V., Latini, C., Muzzonigro, G., Castellucci, M., Cinti, S., 2008. Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. *Journal of Lipid Research*, 49(7), pp.1562–1568.

Nedergaard, J., Petrovic, N., Lindgren, E.M., Jacobsson, A., Cannon, B., 2005. PPARgamma in the control of brown adipocyte differentiation. *Biochimica et Biophysica Acta*, 1740, pp.293–304.

Neuschwander-Tetri, B.A., 2012. Farnesoid X receptor agonists: what they are and how they might be used in treating liver disease. *Current Gastroenterology Reports*, 14(1), pp.55–62.

Nicholls, D. G., 2013. Bioenergetics. Fourth edition, Academic Press.

Nisoli, E., Clementi, E., Carruba, M.O., Moncada, S., 2007. Defective mitochondrial biogenesis: a hallmark of the high cardiovascular risk in the metabolic syndrome?. *Circulation Research*, 100(6), pp. 795-806.

Nye, C., Kim, J., Kalhan, S.C., Hanson, R.W., 2008. Reassessing triglyceride synthesis in adipose tissue. *Trends in Endocrinology and Metabolism*, 19(10), pp.356–361.

Ogden, C.L., Carroll, M.D., Kit, B.K., Flegal, K.M., 2012. Prevalence of obesity in the United States, 2009-2010. *NCHS data brief*, (82), pp.1–8.

Orava, J., Nuutila, P., Lidell, M.E., Oikonen, V., Noponen, T., Viljanen, T., Scheinin, M., Taittonen, M., Niemi, T., Enerbäck, S., Virtanen, K., 2011. Different metabolic responses of human brown adipose tissue to activation by cold and insulin. *Cell metabolism*, 14(2), pp.272–279.

Palmeira, C.M., Rolo, A.P., Berthiaume, J., Bjork, J.A., Wallace, K.B., 2007. Hyperglycemia decreases mitochondrial function: the regulatory role of mitochondrial biogenesis. *Toxicology and Applied Pharmacology*, 225(2), pp.214-220.

Perez, M.J. & Briz, O., 2009. Bile-acid-induced cell injury and protection. *World Journal of Gastroenterology*, 15(14), pp.1677-1689.

Peschechera, A. & Eckel, J., 2013. "'Browning'" of adipose tissue – regulation and therapeutic perspectives. *Archives of Physiology and Biochemistry*, 119(4), pp.151–160.

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

Pisani, D.F., Djedaini, M., Beranger, G.E., Elabd, C., Scheideler, M., Aihaud, G., Amri, E-Z., 2011. Differentiation of human adipose-derived stem cells into "brite" (brown-in-white) adipocytes. *Frontiers in Endocrinology*, 2(87), pp.1–9.

Porter, A.G. & Jänicke, R.U., 1999. Emerging roles of caspase-3 in apoptosis. *Cell Death and Differentiation*, 6(2), pp.99-104.

Prawitt, J., Abdelkarim, M., Stroeve, J.H.M., Popescu, I., Duez, H., Velagapudi, V.R., Dumont, J., Bouchaert, E., van Dijk, T.H., Lucas, A., Dorchies, E., Daoudi, M., Lestavel, S., Gonzalez, F.J., Oresic, M., Cariou, B., Kuipers, F., Caron, S., Staels, B., 2011. Farnesoid X receptor deficiency improves glucose homeostasis in mouse models of obesity. *Diabetes*, 60(7), pp.1861–1871.

Prestwich, T.C. & MacDougald, O.A., 2007. Wnt/beta-catenin signaling in adipogenesis and metabolism. *Current Opinion in Cell Biology*, 19(6), pp.612–617.

Puigserver, P., Wu, Z., Park, C.W., Graves, R., Wright, M., Spiegelman, B.M., 1998. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell*, 92(6), pp.829–839.

Puigserver, P. & Spiegelman, B.M., 2003. Peroxisome Proliferator-Activated Receptor-gamma Coactivator 1alpha (PGC-1alpha): transcriptional coactivator and metabolic regulator. *Endocrine Reviews*, 24(1), pp.78–90.

Qatani, M. & Lazar, M.A., 2007. Mechanisms of obesity-associated insulin resistance: many choices on the menu. *Genes & Development*, 21(12), pp.1443-1455.

Ramírez-Zacarías, J.L., Castro-Muñozledo, F., Kuri-Harcuch, W., 1992. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with oil red O. *Histochemistry*, 97, pp.493-497.

Ravussin, E. & Galgani, J.E., 2011. The implication of brown adipose tissue for humans. *Annual Review of Nutrition*, 31, pp.33–47.

Ribeiro, M.O., Lebrun, F.L.A.S., Christoffolete, M.A., Branco, M., Crescenzi, A., Carvalho, S.D., Negrão, N., Bianco, A.C., 2000. Evidence of UCP1-independent regulation of norepinephrine-induced thermogenesis in brown fat. *American Journal of Physiology - Endocrinology and Metabolism*, 279, pp.E314–E322.

Richards, W.L., Song, M.K., Krutzsch, H., Evarts, R.P., Marsden, E., Thorgeirsson, S.S., 1985. Measurement of cell proliferation in microculture using Hoechst 33342 for the rapid semiautomated microfluorimetric determination of chromatin DNA. *Experimental Cell Research*, 159(1), pp.235-246.

Rizzo, G., Disante, M., Mencarelli, A., Renga, B., Gioiello, A., Pellicciari, R., Fiorucci, S., 2006. The farnesoid X receptor promotes adipocyte differentiation and regulates adipose cell function in vivo. *Molecular Pharmacology*, 70(4), pp.1164-1173.

Rolo, A.P., Palmeira, C.M. & Wallace, K.B., 2002. Interactions of combined bile acids on hepatocyte viability: cytoprotection or synergism. *Toxicology Letters*, 126, pp.197-203

Rolo, A.P., Palmeira, C.M. & Wallace, K.B., 2003. Mitochondrially mediated synergistic cell killing by bile acids. *Biochimica et Biophysica Acta*, 1637(1), pp.127-132.

Rossmeisl, M., Syrový, I., Baumruk, F., Flachs, P., Janovská, P., Kopecký, J., 2000. Decreased fatty acid synthesis due to mitochondrial uncoupling in adipose tissue. *The FASEB Journal*, 14, pp.1793-1800.

Rousset, S., Alves-Guerra, M-C., Mozo, J., Miroux, B., Cassard-Doulcier, A.M., Bouillaud, F., Ricquier, D., 2004. The biology of mitochondrial uncoupling proteins. *Diabetes*, 53(1), pp.S130-S135

Saito, M., 2013. Brown adipose tissue as a regulator of energy expenditure and body fat in humans. *Journal of Diabetes and Metabolism*, 37(1), pp.22–29.

Saito, M., Okamatsu-ogura, Y., Matsushita, M., Watanabe, K., Yoneshiro, T., Nio-kobayashi, J., Iwanaga, T., Miyagawa, M., Kameya, T., Nakada, K., Kawai, Y., Tsujisaki, M., 2009. High incidence of metabolically active brown adipose tissue in healthy adult humans. *Diabetes*, 58(7), pp.1526–1531.

Sato, H., Macchiarulo, A., Thomas, C., Gioiello, A., Une, M., Hofmann, A.F., Saladin, R., Schoonjans, K., Pellicciari, R., Auwerx, J., 2008. Novel potent and selective

bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. *Journal of Medicinal Chemistry*, 52(6), pp.1831–1841.

Schaap, F.G., Trauner, M. & Jansen, P.L.M., 2014. Bile acid receptors as targets for drug development. *Nature Reviews. Gastroenterology & Hepatology*, 11(1), pp.55-67.

Schölmerich, J., Becher, M.S., Schmidt, K., Schubert, R., Kremer, B., Feldhaus, S., Gerok, W., 1984. Influence of hydroxylation and conjugation of bile salts on their membrane - damaging properties - studies on isolated hepatocytes and lipid membrane vesicles. *Hepatology*, 4(4), pp. 661-666.

Schubring, S.R., Fleischer, W., Lin, J.S:, Haas, H.L., Sergeeva, O.A., 2012. The bile steroid chenodeoxycholate is a potent antagonist at NMDA and GABA receptors. *Neuroscience Letters*, 506(2), pp.322–326.

Seale, P., Kajimura, S. & Spiegelman, B.M., 2009. Transcriptional control of brown adipocyte development and physiological function - of mice and men. *Genes & Development*, 23, pp.788–797.

Senocak, F.S., Si, Y., Moya, C., Russell, W.K., Russell, D.H., Lee, K., Jayaraman, A., 2008. Effect of uncoupling protein-1 expression on 3T3-L1 adipocyte gene expression. *FEBS Letters*, 581(30), pp.5865-5871.

Sharma, R., Long, A. & Gilmer, J.F., 2011. Advances in bile acid medicinal chemistry. *Current Medicinal Chemistry*, 18(26), pp.4029-4052.

Sharp, L.Z., Shinoda, K., Ohno, H., Scheel, D.W., Tomoda, E., Ruiz, L., Hu, H., Wang, L., Pavlova, Z., Gilsanz, V., Kajimura, S., 2012. Human BAT possesses molecular signatures that resemble beige/ brite cells. *PloS one*, 7(11), pp.1–10.

Shen, Y., Song, S.J., Keum, N., Park, T., 2014. Olive leaf extract attenuates obesity in high-fat diet-fed mice by modulating the expression of molecules involved in adipogenesis and thermogenesis. *Evidence-Based Complementary and Alternative Medicine*, 2014, pp.1-12.

Si, Y., Palani, S., Jayaraman, A., Lee, K., 2007. Effects of forced UCP1 expression in 3T3-L1 cells on mitochondrial function and lipid metabolism. *Journal of Lipid Research*, 48(4), pp.826-836.

Si, Y., Shi, H. & Lee, K., 2009. Metabolic flux analysis of mitochondrial uncoupling in 3T3-L1 adipocytes. *PLoS One*, 4(9), pp.1-8.

Silva, J.E., 2006. Thermogenic mechanisms and their hormonal regulation. *Physiological Reviews*, 86(2), pp.435–464.

Sjöström, L., Peltonen, M., Jacobson, P., Sjöström, C.D., Karason, K., Wedel, H., Ahlin, S., Anveden, Å., Bengtsson, C., Bergmark, G., Bouchard, C., Carlsson, B., Dahlgren, S., Karlsson, J., Lindroos, A-K., Lönroth, H., Narbro, K., Näslund, I., Olbers, T., Svensson, P-A., Carlsson, L.M.S., 2012. Bariatric surgery and long-term cardiovascular events. *The Journal of the American Medical Association*, 307(1), pp.56–65.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., Vistica, D., Warren, J.T., Bokesch, H., Boyd, M.R., 1990. New colorimetric cytotoxicity assay for anti-cancer-drug screening. *Journal of the National Cancer Institute*, 82(13), pp.1107-1112.

Smorlesi, A., Frontini, A., Giordano, A., Cinti, S., 2012. The adipose organ: white-brown adipocyte plasticity and metabolic inflammation. *Obesity Reviews*, 13, pp. 83-96.

Stanford, K.I., Middelbeek, R.J.W., Townsend, K.L., An, D., Nygaard, E.B., Hitchcox, K.M., Markan, K.R., Nakano, K., Hirshman, M.F., Tseng, Y-H., Goodyear, L.J., 2013. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. *The Journal of Clinical Investigation*, 123(1), pp.215–223.

Szendroedi, J., Phielix, E. & Roden, M., 2012. The role of mitochondria in insulin resistance and type 2 diabetes mellitus. *Nature Reviews. Endocrinology*, 8(2), pp.92–103.

Tatsugami, M., Ito, M., Tanaka, S., Yoshihara, M., Matsui, H., Haruma, K., Chayama, K., 2012. Bile acid promotes intestinal metaplasia and gastric carcinogenesis. Cancer Epidemiology, *Biomarkers & Prevention*, 21(11), pp.2101-2107.

Tejerina, S., Pauw, A., Vankoningsloo, S., Houbion, A., Renard, P., Longueville, F., Raes, M., Arnould, T., 2009. Mild mitochondrial uncoupling induces 3T3-L1 adipocyte de-differentiation by a PPARγ-independent mechanism, whereas TNFα-induced de-differentiation is PPARγ dependent. *Journal of Cell Science*, 122, pp.145-155.

Teodoro, J.S., Zouhar, P., Flachs, P., Bardova, K., Janovska, P., Gomes, A.P., Duarte, F. V., Varela, A.T., Rolo, A.P., Palmeira, C.M., Kopecký, J., 2014. Enhancement of brown fat thermogenesis using chenodeoxycholic acid in mice. *International Journal of Obesity*, pp.1–8.

Teodoro, J.S., Rolo, A.P. & Palmeira, C.M., 2011. Hepatic FXR: key regulator of whole-body energy metabolism. *Trends in Endocrinology and Metabolism*, 22(11), pp.458–466.

Thomas, C., Pellicciari, R., Pruzanski, M., Auwerx, J., Schoonjans, K., 2008. Targeting bile-acid signaling for metabolic diseases. *Nature Reviews Drug Discovery*, 7(8), pp.678–693.

Timmons, J.A., Wennmalm, K., Larsson, O., Walden, T.B., Lassmann, T., Petrovic, N., Hamilton, D.L., Gimeno, R.E., Wahlestedt, C., Baar, K., Nedergaard, J., Cannon, B., 2007. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proceedings of the National Academy of Sciences of the United States of America*, 104(11), pp.4401-4406.

Tiraby, C., Tavernier, G., Lefort, C., Larrouy, D., Bouillaud, F., Ricquier, D., Langin, D., 2003. Acquirement of brown fat cell features by human white adipocytes. *The Journal of Biological Chemistry*, 278(35), pp.33370–33376.

Tiwari, A. & Maiti, P., 2009. TGR5: an emerging bile acid G-protein-coupled receptor target for the potential treatment of metabolic disorders. *Drug Discovery Today*, 14(9-10), pp.523-530.

Towler, M.C. & Hardie, D.G., 2007. AMP-activated protein kinase in metabolic control and insulin signaling. *Circulation Research*, 100(3), pp.328-341.

Townsend, K.L. & Tseng, Y., 2012. Brown adipose tissue: recent insights into development, metabolic function and therapeutic potential. *Adipocyte*, 1(1), pp.13–24.

Trauner, M., Claudel, T., Fickert, P., Moustafa, T., Wagner, M., 2010. Bile acids as regulators of hepatic lipid and glucose metabolism. *Digestive Diseases*, 28(1), pp.220–224.

Trayhurn, P., 2013. Hypoxia and adipose tissue function and dysfunction in obesity. *Physiological reviews*, 93(1), pp.1–21.

Uldry, M., Yang, W., St-Pierre, J., Lin, J., Seale, P., Spiegelman, B.M., 2006. Complementary action of the PGC-1 coactivators in mitochondrial biogenesis and brown fat differentiation. *Cell metabolism*, 3(5), pp.333–41.

van Marken Lichtenbelt, W.D., Vanhommerig, J.W., Smulders, N.M., Drossaerts, J.M.A.F.L., Kemerink, G.J., Bouvy, N.D., Schrauwen, P., Teule, G.J.J., 2009. Cold-activated brown adipose tissue in healthy men. *The New England Journal of Medicine*, 360(15), pp.1500–1508.

Vernochet, C., Peres, S.B., Davis, K.E., McDonald, M.E., Qiang, L., Wang, H., Scherer, P.E., Farmer, S.R., 2009. C/EBPalpha and the corepressors CtBP1 and CtBP2 regulate repression of select visceral white adipose genes during induction of the brown phenotype in white adipocytes by peroxisome proliferator-activated receptor gamma agonists. *Molecular and Cellular Biology*, 29(17), pp.4714–4728.

Virtanen, K.A., Lidell, M.E., Orava, J., Hegling, M., Westergren, R., Niemi, T., Taittonen, M., Laine, J., Savisto, N., Enerbäck, S., Nuutila, P., 2009. Functional

brown adipose tissue in healthy adults. *The New England Journal of Medicine*, 360, pp.1518–1525.

Vosselman, M.J., Lichtenbelt, W.D.V.M. & Schrauwen, P., 2013. Energy dissipation in brown adipose tissue: from mice to men. *Molecular and Cellular Endocrinology*, 379(1-2), pp.43–50.

Walker, J., 1994. The bicinchoninic acid (BCA) assay for protein quantitation. *Methods in Molecular Biology*, pp.16-19.

Wang, C.,Mao, X., Wang, L., Liu, M., Wetzel, M.D., Guan, K-L., Dong, L.Q., Liu, F., 2007. Adiponectin sensitizes insulin signaling by reducing p70 S6 kinase-mediated serine phosphorylation of IRS-1. *The Journal of Biological Chemistry*, 282(11), pp.7991–6.

Watanabe, M., Houten, S.M., Wang, L., Moschetta, A., Mangelsdorf, D.J., Heyman, R.A., Moore, D.D., Auwerx, J., 2004. Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. *The Journal of Clinical Investigation*, 113(10), pp.1408–1418.

Watanabe, M., Houten, S.M., Mataki, C., Christoffolete, M.A., Kim, B.W., Sato, H., Messaddeq, N., Harney, J.W., Ezaki, O., Kodama, T., Schoonjans, K., Bianco, A.C., Auwerx, J., 2006. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature*, 439, pp.484–9.

Watanabe, M., Horai, Y., Houten, S.M., Morimoto, K., Sugizaki, T., Arita, E., Mataki, C., Sato, H., Tanigawara, Y., Schoonjans, K., Itoh, H., Auwerx, J., 2011. Lowering bile acid pool size with a synthetic farnesoid X receptor (FXR) agonist induces obesity and diabetes through reduced energy expenditure. *The Journal of Biological Chemistry*, 286(30), pp.26913–26920.

Watanabe, M., Morimoto, K., Houten, S.M., Kaneko-Iwasaki, N., Sugizaki, T., Horai, Y., Mataki, C., Sato, H., Murahashi, K., Arita, E., Schoonjans, K., Suzuki, T., Itoh, H., Auwerx, J., 2012. Bile acid binding resin improves metabolic control through the induction of energy expenditure. *PloS one*, 7(8), pp. 1-9.

Whittle, A.J. & Vidal-Puig, A., 2012. NPs - heart hormones that regulate brown fat? *The Journal of Clinical Investigation*, 122(3), pp.1–4.

Willson, T.M., Lambert, M.H. & Kliewer, S.A., 2001. Peroxisome proliferator-activated receptor gamma and metabolic disease. *Annual Review of Biochemistry*, 70, pp.341–367.

Wilson, P.W.F., D'Agostino, R.B.D., Parise, H., Sullivan, L., Meigs, J-B., 2005. Metabolic syndrome as a precursor of cardiovascular disease and type 2 diabetes mellitus. *Circulation*, 112(20), pp.3066–3072.

Wood, I.S., de Heredia, F.P., Wang, B., Trayhurn, P., 2009. Cellular hypoxia and adipose tissue dysfunction in obesity. *Proceedings of the Nutrition Society*, 68(4), pp.370–377.

World Health Organization, Obesity and overweight fact sheet N311 (http://www.who.int/mediacentre/factsheets/fs311/en/).

World Health organization, Global status report on noncommunicable diseases 2010. (http://www.euro.who.int/en/health-topics/noncommunicable-diseases/obesity/data-and-statistics).

Wright, W.S., Longo, K.A., Dolinsky, V.W., Gerin, I., Kang, S., Bennett, C.N., Chiang, S-H., Prestwich, T.C., Gress, C., Burant, C.F., Susulic, V.S., MacDougald, O., 2007. Wnt10b inhibits obesity in ob/ob and agouti mice. *Diabetes*, 56(2), pp.295-303.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R-C., Spiegelman, B.M., 1999. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell*, 98(1), pp.115–124.

Xu, H., Barnes, G.T., Yang, Q., Tan, G., Yang, D., Chou, C.J., Sole, J., Nichols, A., Ross, J.S., Tartaglia, L.A., Chen, H., 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *The Journal of Clinical Investigation*, 112(12), pp.1821–1830.

Yoneshiro, T., Aita, S., Matsushita, M., Okamatsu-Ogura, Y., Kameya, T., Kawai, Y., Miyagawa, M., Tsujisaki, M., Saito, M., 2011. Age-related decrease in cold-activated brown adipose tissue and accumulation of body fat in healthy humans. *Obesity (Silver Spring, Md.)*, 19(9), pp.1755–60.

Zafrir, B., 2013. Brown adipose tissue: research milestones of a potential player in human energy balance and obesity. *Hormone and Metabolic Research*.

Zebisch, K., Voigt, V., Wabitsch, M., Brandsch, M., 2012. Protocol for effective differentiation of 3T3-L1 cells to adipocytes. *Analytical Biochemistry*, 425, pp.88-90.

Zhou, S., Palmeira, C.M. & Wallace, K.B., 2001. Doxorubicin-induced persistent oxidative stress to cardiac myocytes. *Toxicology Letters*, 121(3), pp.151-157.