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Role of neuronal cholesterol in peripheric metabolic tissues

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Role of neuronal cholesterol in peripheric metabolic tissues

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Authorship statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in this text and are listed in the included references.

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Telma Castro
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LIST OF ABREVIATIONS

24OHC – 24-hydroxycholesterol
AD – Alzheimer Disease
AgRP – Agouti-related protein
AHN – Anterior hypothalamic nucleus
AMPK – AMP-activated protein kinase
ARC – Arcuate nucleus
ATP – Adenosine Triphosphate
BA – Bile acids
BAT – Brown adipose tissue
BBB – Blood brain barrier
BP – Blood pressure
CA – Cholic acid
CART - Cocaine and amphetamine regulated transcript
CDCA - Chenodeoxycholic acid
CKD – Chronic kidney disease
CPT1 – Carnitine Palmitoyl-transferase 1
CRH – Corticotropin-releasing hormone
CVD – Cardiovascular disease
DAPK1 – Death-associated protein-kinase 1
DCA – Deoxycholic acid
DM – Diabetes mellitus
DMH – Dorsomedial hypothalamic nucleus
DMH – Dorsomedial nucleus of the hypothalamus
DMV – Dorsal motor nucleus of the vagus nerve
EDHF – Endothelium-derived hyperpolarizing factor
EET – Epoxyeucisatrienoic acids
EFV – Efavirenz
EGF – Epidermal growth factor
F-2,6BP – Frutose-2,6-bi-phosphate
F6P – Frutose-6-phosphate
FAD – Flavin adenine dinucleotide
FBPase 2 – Fructose biphosphatase 2
FFA – Free fatty acids
FMN – Flavin mononucleotide
G6P – Glucose-6-phosphate
$GABA_A$ – gamma-aminobutiric acid
GHSR – Growth Hormone Secretagogue Receptor
GLP-1 – Glucagon-like peptide-1
HD – Huntington Disease
HDL – High-density lipoprotein
HFD – High Fat Diet
HMGR – HMG reductase
HNF4 α- Hepatocyte nuclear factor 4 α
HTT-MUT – Huntinting mutant
LCA – Lithocholic Acid
LDLR – Low-density lipoprotein receptor
LepRb – Leptin receptor type B

LHA – Lateral hypothalamic area
LOX1 – Lipoprotein receptor 1
LXR – Liver X receptors
MC4R – Melanocortin 4 Receptor
ME – Median emince
mnPOA – median Preoptic Area
MS – Metabolic syndrome
NADPH – Nicotinamide adenine dinucleotide phosphate
NPY – Neuropeptide Y
NTS – Nucleus of the solitary tract
OXR – Orexin receptor
PAI-1 – Plasminogen activator inhibitor 1
PBN – Parabrachial nucleus
PCr – Phosphocreatine
PFK-1 – Phosphofructokinase 1
PGE2 – Prostaglandins E2
POA – Preoptic Area
polyQ – Poliglutamine
POMC – Pro-opiomelanocortin
PON – Postoptic nucleus
PPAR – Peroxisome proliferator-activated receptor
PVN – Paraventricular nucleus
RAAS - Renin-angiotensin-aldosterone system
ROS – Reactive oxygen species
rRPA – rostral raphe pallidus
SAT – Subcutaneous adipose tissue
SCA – Spinocerebelar ataxia
SCAP - SREBP cleavage activating protein
SCN – Suprachiasmatic nucleus
sdLDL – small dense low-density lipoprotein
SIM1 – Single-minded homolog 1
SNS – Sympathetic Nervous System
SREBPs - Sterol regulatory element binding proteins
SREBPs – Sterol-Regulatory Binding proteins
TCA – Tricarboxylic acid
TG – Triacylglycerol
TNF-α - Tumor Necrosis Factor α
UCP – Uncoupling protein
VAT – Visceral adipose tissue
VMH – Ventromedial nucleus of the hypothamalus
WAT – White adipose tissue
WHO – World Health Organization
α-MSH - α-melanocyte-stimulating-hormone

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Abstract

The brain possesses 20% of whole-body cholesterol, becoming the richest cholesterol organ in our body. Due to the Blood Brain Barrier, the cholesterol has its own way to synthesize and degrade cholesterol, in order to maintain its homeostasis. Cholesterol is synthesized through *de novo* synthesis in the brain and transported out of the brain in the form of oxysterols, more specifically 24-hydroxycholesterol (24-OHC). This process is acomplished through CYP46A1, a protein of the CYP450 family. Alterations in cholesterol homeostasis can lead to metabolic abnormalities, such as obesity, inflammation and insulin resistance. Previous studies in our laboratory showed that alterations in the expression of *Cyp46A1* gene (more specifically silencing) with different diets, rich and poor in fat, had impacts in whole-body homeostasis. Taking these results into account, the main objective of this study was to evaluate the impact of modulation of *Cyp46A1* gene in the main metabolic organs (liver, pancreas, and adipose tissue), through silencing and overexpressing CYP46A1 in the arcuate nucleus of the hypothalamus. This modulation was made in C57BL/6J mice fed with two distinct diets, one rich in fat (HF diet) and one poor in fat (CHOW diet). The data obtained from microscopy and tissue quantification made it possible to see the physiological changes resultant from the modulation of CYP46A1. It was possible the phenomenon BAT "whithening", an increase in the size of Langerhans islets and lipidic hypertrophy. RT-qPCR was also realized to evaluate the mRNA levels of the different targets in the hypothalamus. The results obtained from RT-qPCR, showed that modulation of Cyp46A1 affected the mRNA levels of the different targets tested (POMC, NPY and TNF-alpha). All this data confirmed our hypothesis, that the modulation of CYP46A1 do alter the whole-body metabolism. Further studies must be conducted to continue this project, in order to investigate if this gene could be a target for genetic therapies.

Keywords: cholesterol metabolism; *Cyp46a1*; hypothalamus; oxysterols; whole-body metabolism.

Resumo

Um dos grandes problemas de saúde atuais da nossa sociedade é a obesidade. Esta condição é um dos principais sintomas da síndrome metabólica sendo consequência, para além da ausência de exercício físico, de uma dieta desequilibrada e rica em gorduras. A obesidade gera várias consequências no corpo, sendo uma delas a inflamação, podendo esta ocorrer no tecido adiposo, fígado e até no cérebro.

O cérebro é o órgão do corpo humano que possui maior teor de colesterol, cerca de 20% do colesterol total corporal. Este é necessário para a formação das bainhas de mielina e membranas celulares. O cérebro possui uma barreira protetora, altamente seletiva que protege o órgão de substâncias tóxicas que possam ser encontradas no sangue, a barreira hematoencefálica. Esta barreira, impede também o colesterol de entrar ou sair do cérebro, pelo que o cérebro possui um metabolismo do colesterol altamente controlado. Este equilíbrio é mantido através da síntese *de novo*, conversão e efluxo. Para que o colesterol possa ser transportado para fora do cérebro, é necessário que seja previamente convertido em oxisterol, um derivado hidrolisado do colesterol, que por sua vez é capaz de atravessar a barreira hematoencefálica. Esta conversão é possível através da enzima Cyp46a1, que converte o colesterol em colesterol-24-hidroxilase, para que possa ser eliminado no fígado.

Diversos estudos mostraram que alterações na homeostasia do metabolismo do colesterol poderiam levar à obesidade e problemas relacionados (resistência à insulina e diabetes tipo 2). Tendo isto em conta, o principal objetivo deste estudo foi investigar os efeitos da modulação do Cyp46A1 no cérebro no metabolismo corporal. Para tal, utilizou-se uma amostragem de 69 ratinhos C57BL/6J, onde 45 ratinhos foram expostos a um silenciamento da expressão do gene *Cyp46A1* e 24 ratinhos a uma sobre expressão deste gene. Para tal, foi realizada uma injeção estereotáxica bilateral no núcleo arqueado do hipotálamo, com os respetivos grupos virais: a sobre expressão, AAV-Cyp46A1 e o silenciamento, AAV-shCyp46A1. Para verificar também a influência da dieta, os ratinhos foram divididos em seis grupos contendo duas dietas diferentes: AAVCyp46A1, AAV-shCyp46A1 e grupo controlo (não injetados) com uma dieta rica contendo 60% de gordura (denominada *High Fat Diet,* HF) e AAVCyp46A1, AAV-shCyp46A1 e grupo controlo com uma dieta que continha 10% de gordura (denominada *Low Fat control diet,* CHOW).

Os resultados obtidos neste estudo, revelaram que a modulação do gene *Cyp46a1* no núcleo arqueado do hipotálamo afetou a morfologia de vários órgãos metabólicos, levando a um aumento no tamanho das ilhotas de Langerhans no pâncreas, a uma acumulação de

gotículas lipídicas no fígado e também a alterações na estrutura do tecido adiposo, nomeadamente hipertrofia. Estes resultados foram também comprovados através da quantificação dos respetivos tecidos.

Foram também realizados vários RT-qPCR, de forma a verificar se a modulação do gene *Cyp46a1* alterou os níveis de mRNA do próprio Cyp46A1 e de outros importantes mediadores metabólicos do núcleo arqueado do hipotálamo, entre eles o POMC e o NPY. Foi possível verificar uma diminuição dos níveis de mRNA do Cyp46A1 no grupo silenciado quando comparado com o grupo controlo, embora esta não seja significativa. Por outro lado, verificou-se que os níveis de NPY, tanto o grupo AAV-shCyp46A1 como o AAV-Cyp46A1 com a dieta HF possuem aumentos significativos nos níveis de mRNA, quando comparados com o grupo de animais não injetados.

Foram estudados também os níveis de mediadores de inflamação, nomeadamente do TNF-alfa. Neste alvo, observou-se claramente um aumento nos níveis de mRNA nos grupos AAV-Cyp46A1 e AAV-shCyp46A1 que possuíam a dieta HF, em relação aos grupos controlo.

Os resultados obtidos sugerem então um papel fundamental do gene *Cyp46a1* no metabolismo corporal, para além do papel desempenhado a nível cerebral. No entanto, será necessário realizar mais estudos de forma a tentar compreender de forma mais completa o papel desta proteína no metabolismo corporal.

Palavras chave: hipotálamo, núcleo arqueado, *Cyp46a1*, oxisteróis, metabolismo, obesidade.

INTRODUCTION

1. CYP family

Every day, the human body is exposed to several number of drugs, food additives, pollution, among other factors, which could compromise its function and homeostasis. For that, several mechanisms are needed to defend the organism against these putative injuries. One of these mechanisms of defence, is the CYP450 proteins family, where *CYP* stands for *cytochrome P450*. These are hemoproteins that are responsible for oxidizing a variable number of molecules in order to become more polar and soluble, and the term "P450" stands for the spectrophotometric peak at the wavelength of maximum absorption, which is 450nm [1]. The evolutionary origin of the P450 superfamily is ancient, way before the accumulation of molecular oxygen in the atmosphere. The number of CYP450 genes vary among species, as for example *Eschericia coli* has no P450 genes, while *Mycobacterium tuberculosis* has 20 genes. In humans exists 55 genes of the P450 family [2]. There are 18 mammalian CYP families. Following recommendations from a nomenclature committee on the basis of amino-acid identity, phylogenetic criteria and gene organization, the word *CYP* is followed by numbers and letters. Through these criteria, it is easier to differentiate the different CYP mutations and separate them accordingly to their function and role on disease (Table 1). In humans, these cytochromes can be found in the inner membrane of mitochondria or in the endoplasmic reticulum and they can be divided into four classes, according on how electrons from NAD(P)H are delivered to the catalytic site. Class I proteins require both an FAD-containing reductase and an iron sulfur redoxin; Class II proteins involve only an FAD/FMN-containing P450 reductase for transfer of electrons; Class III are self-sufficient and don't need transfer of electrons while Class IV receive electrons directly from NAD(P)H [2] [3].

Table 1. CYP genes affected by mutations and their respective function/disease.

Gene	Function	Disease
CYP1B1	Metabolism of eicosanoids	Primary congenital glaucoma
CYP2R1	Vitamin D 25-hydroxylase	Vitamin D 25-hydroxylase deficiency
CYP4A11	Metabolism of eicosanoids, medium- and long-chain fatty acids	Association with hypertension, coronary artery disease
CYP4A22	Unknown Function	Lamellar ichthyosis type 3
CYP4V2	Unknown Function	Bietti crystalline corneoretinal dystrophy
CYP5A1	Eicosanoid metabolism (thromboxane A2 synthase), participates in platelet aggregation	Ghosal haemato-diaphyseal syndrome
CYP7B1	Oxysterol 7α-hydroxylase, neurosteroid 7α-hydroxylase	Neonatal cholestasis; hereditary spastic paraplegia 5A
CYP8A1	Eicosanoid metabolism (prostacyclin (PGI2) synthase), participates in platelet disaggregation	Hypertension
CYP11A1	Cholesterol side-chain cleavage	Congenital adrenal insufficiency with 46, XY sex reversal
CYP11B1	Steroid 11β-hydroxylase	Occasional congenital adrenal hyperplasia (CAH)
CYP11B2	Steroid 11β-hydroxylase and 18-hydroxylase and 18-oxidase	Corticosterone methyl oxidase deficiency type I, type II; increased aldosterone-to-renin ratio
CYP19A1	Androgen aromatase, estrogen synthetase	Aromatase deficiency, aromatase excess syndrome
CYP21A2	Steroid 21-hydroxylase	Causes greater than 95% of all CAH classic and nonclassical types
CYP24A1	Vitamin D 24-hydroxylase	Idiopathic infantile hypercalcemia
CYP26A1	Retinoid acid inactivation (hydroxylase)	Embryo lethal in mouse
CYP26B1	Retinoid acid inactivation (hydroxylase)	Antley-Bixler syndrome
CYP27A1	Bile acid biosynthesis, sterol 27-hydroxylase, vitamin D 25-hydroxylase	Cerebrotendinous xanthomatosis
CYP27B1	25-hydroxy-vitamin D 1-α hydroxylase	Vitamin D-dependent rickets type 1A; infantile hypocalcemia
CYP27C1	Unknown function	Association with avascular necrosis of femoral head
CYP51A1	Lanosterol 14-α demethylase	Embryo lethal GD15 in mouse

Adapted from (Nebert, Wikvall, and Miller 2013).

1.1. Function

CYP enzymes can be found in all tissues, but the highest concentrations are present in the small intestine and liver, probably because they have a fundamental role in bile acid (BA) biosynthesis and abound in the microsomal fraction of the liver [1]. Besides the liver and small intestine, CYP proteins can also be found in the brain but in smaller concentrations (around 0,5%-2% of liver concentrations) [4]. Due to the lower concentrations, they are not able to induce significant influence in the pharmacokinetics of drugs on the body. Instead, CYP proteins have specific functions in the brain, such as maintenance of brain cholesterol homeostasis, regulation of endogenous $GABA_A$ receptor agonists and elimination of retinoids [5]. Additionally, they are also present in the mitochondria inner membranes of steroidogenic tissues, such as testis, ovaries, breast and placenta. They play a major role in vitamin metabolism and cholesterol biosynthesis [1]. As we know, cholesterol is essential for the membrane's synthesis and fluidity, and it can be obtained through diet or synthetized from acetyl CoA *(de novo* synthesis). The cholesterol homeostasis is balanced by its production and its elimination pathways, wherein the principal route is conversion to BA. This could be done in three ways (Figure 1): the classic pathway ("neutral"), the alternative pathway ("acidic") or through oxidation [6]. The classic pathway leads to two primary BA, the cholic acid (CA) and chenodeoxycholic acid (CDCA). The alternative pathway starts with the oxidation of the cholesterol that leads to the final product that is chenodeoxycholic acid (CDCA). The final products of these two pathways (CA and CDCA) are transported to the intestine and then deconjugated and dehydroxylated to the secondary BA deoxycholic acid (DCA) and lithocholic acid (LCA). Additionally, to the classic and alternative pathway, there is the oxidation of cholesterol through CYP46A1. This hydroxylation is mainly found in the brain, where its function is to convert cholesterol into oxysterols, since cholesterol cannot pass the blood-brain barrier. Once they pass this barrier, the product is finally hydroxylated in the liver [7] [8] [9].

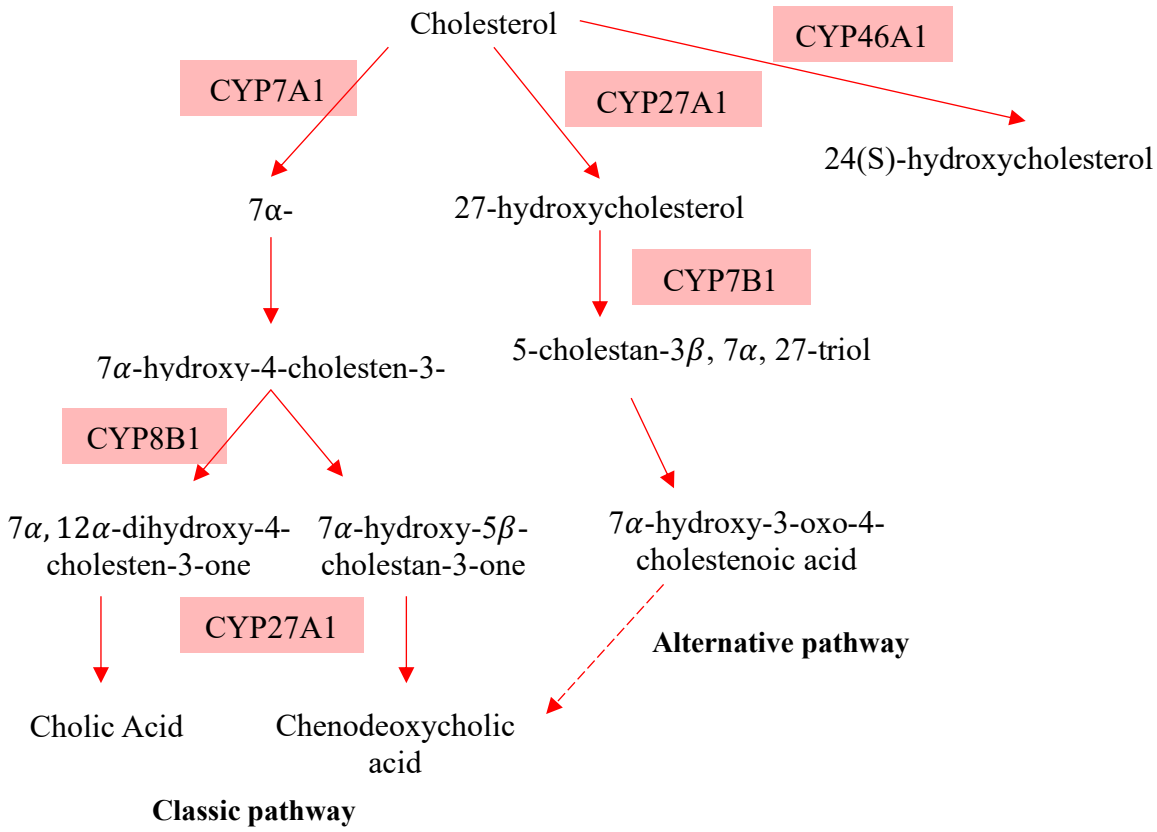

Figure 1. Schematic representation of the classic pathway and alternative pathway.

The main steps of cholesterol and BA biosynthesis pathways. Key regulation enzymes are indicated in the pathways. Cholesterol is converted into two primary BA in liver, CA and CDCA, and then later deconjugated in the intestine. Although the principal route is the classic pathway when disrupted, the alternative pathway becomes a major BA synthesis pathway. Besides the production of BA, it also produces oxysterols, which are strong regulators of lipid homeostasis.

1.2. CYP family and disease

CYP proteins can also be related to numerous diseases, among them diabetes, atherosclerosis, cancer, neurodegenerative diseases, and obesity.

1.2.1. Diabetes *mellitus*

Diabetes mellitus (DM) is a metabolic disease characterized by chronic hyperglycemia that results from defects in insulin secretion, insulin action or both. Insulin is an important anabolic hormone and lack of it could result in metabolic abnormalities in carbohydrates, lipids, and proteins. DM can be divided into type 1, type 2 and gestational diabetes [10]. One of the CYP involved in this disease is CYP2E1, which shows an elevated activity in liver of obese type 2 diabetic patients. This elevated activity of CYP2E1 shows a high ability to produce free radicals that are mostly the cause of liver damage in these patients [11]. DM can also influence these enzymes, and one proof of that is CYP3A4, where DM patients display reduced catalytic activity and protein levels. CYP3A4 is the most expressed CYP in liver and intestine and metabolizes more than 50% of used medication and some endogenous substrates such as cortisol, estradiol, progesterone, and testosterone [12].

1.2.2. Atherosclerosis

Atherosclerosis is a disease characterized by the appearance of fatty deposits, called atheromatous plaques, in the thinner layers of arteries. It begins with the deposition of small cholesterol crystals in the intima and smooth muscle. Then these plaques grow with the proliferation of fibrous tissues and get inside the arteries, which results in a reduction of the blood flow. Ultimately, the uneven surface results in clot formation and thrombosis [13]. CYP enzymes plays a major role in this disease. Cells with CYP epoxygenase activity (more specifically CYP2B, 2C8, 2C9, 2C10, 2J2) can act on arachidonic acid (polyunsaturated fatty acid). This epoxygenase activity consists in the ability to insert oxygen on the arachidonic acid, reducing him to the epoxide form. These forms are called Epoxeicosatrienoic acids (EET) and are synthesized in the endothelium. EET's can activate Ca^{2+}-K^{+} channels, which leads to vasorelaxation, functioning as a endothelium-derived hyperpolarizing factor (EDHF), decreasing the blood pressure [14]. Catalytic reactions of the CYP2C generates reactive oxygen species (ROS) in coronary artery endothelial cells.

On the other hand, CYP7A1 plays a protective role against atherosclerosis, by maintaining the homeostasis of BA synthesis, by improving formation of BA from cholesterol and increases free cholesterol secreted in bile without increasing cholesterol reabsorption by intestinal cells [12].

1.2.3. Cancer

Cancer as we all know, is the disease of the century. The human body has the ability of divide and differentiate cells in order to repopulate organs and tissues. A simple example of that are the cells present in the basal layer of skin, cells composing the epithelial layer of the intestines and cells in bone marrow [15]. Cancer initiates when a cell breaks free from the normal limitations on cell division and starts to proliferate on its own, so cells produced by this "abnormal" cell will also display inappropriate proliferation properties. The originated mass of cells, called tumor, may remain within the tissue of origin or it may begin to invade nearby tissues (invasive cancer). The invasive cancer can be called malignant when cells diffuse into the blood or lymph and are likely to establish new tumors (metastases) [16]. Several CYP enzymes are related with several types of cancer.

A. Renal Cancer: CYP3A was found to be expressed consistently in this type of cancer and CYP3A are suggested to be a cause of multidrug resistance that is observed in this type of cancer [17]. CYP1B1 is also found in this type of cancer (but not detected in normal cells). It has been suggested that CYP1B1 is significantly upregulated in renal cell carcinoma and that promotes cancer progression through down regulation of the death-associated protein-kinase 1 (DAPK1) [18].

B. Breast Cancer: in this type of cancer, CYP2E1 contributes to the generation of ROS. This CYP enzyme can also regulate autophagy, stimulate stress of endoplasmic reticulum and suppress metastatic potential of the breast cancer cells, meaning that overexpression of CYP2E1 could be beneficial for the patients [19].

C. Lung Cancer: in this type of cancer there is a down-regulation in CYP2A13 expression in adenocarcinoma and it is implicated in their development and progression [20]. CYP1B1 is also related to lung cancer, as it is induced by tobacco and implicated in the metabolism of estrogen. Due to the tobacco specific carcinogens induced by CYP1B1, in lung tissues, during the metabolism of estradiol there is formation of adducts and ROS, which may cause DNA damage and consequently, be mutagenic [12].

D. Prostate Cancer: it has been suggested that CYP27B1 controls the growth of prostate cancer cells through its role in vitamin D metabolism. CYP27B1 is regulated by the Epidermal Growth Factor (EGF), and this CYP enzyme has anti-tumor activity and once deregulated by the EGF, the prostate cancer develops [21]. Another CYP isoform of interest is CYP17A1, that is involved in androgen biosynthesis and has been implicated in prostate cancer proliferation. A recent strategy for treatment of castration-resistant prostate cancer is the use of CYP17A1 inhibitors [22].

E. Brain Cancer: glioblastoma cells have an excessive cholesterol accumulation caused by CYP46A1 down-regulation, which leads to 24-hydroxycholesterol (24OHC) production that in turn cause a suppression of Liver X Receptors (LXR) signaling and an induction of Sterol-Regulatory Binding Proteins (SREBPs) targets. A recent study found out that Efavirenz (EFV), an anti-HIV medication known to activate CYP46A1 activity through binding to the enzyme's allosteric site, inhibits glioblastoma growth [23].

1.2.4. Obesity

Apart from cancer risk and diabetes, the western diet is also associated with an increased prevalence in obesity. This metabolic syndrome is now a recognized pathological entity that includes insulin resistance, type II diabetes and cardiovascular disease [24]. There is a number of studies that have assessed the impact on diets, that are rich in saturated fats, on hepatic CYP expression. CYP2E1 is an ethanol-inducible enzyme present in the liver responsible for deactivating most of the drugs. Induction of CYP2E1 protein and activity was reported after ingestion of 70% lard for 3 days and energy-dense diets containing 60% shortening over a 12-month period, which are experimental models of obesity [24]. Corn oils also increased hepatic CYP2E1 activity greater than lard or olive oil, containing saturated and monounsaturated fatty acids, respectively. This suggests that the nature and quantity of dietary lipid could influence the extent to which CYP2E1 is upregulated by these fats [25]. Also, in the field of drug-metabolism, CYP2D6 is responsible for metabolizing around 25% of marketed drugs. Obesity leads to modified expression/activity of different transcriptional regulators of CYP2D6. In particular, high-fat diet (HFD) feeding in mice showed a decrease in hepatocyte nuclear factor 4 α (HNF4α) expression. HNF4α transactivates CYP2D6 promoter by binding to a proximal promoter region [26].

1.2.5. Neurodegenerative diseases

A cholesterol supply is important for synapses, dendrites formation and for axonal guidance. With that said, cholesterol depletion leads to synaptic and dendritic spine degeneration and failing neurotransmission, which is observed at an early stage in several neurogenerative disorders, including Huntington Disease (HD) [27]. HD is a neurodegenerative disorder caused by a dominantly inherited CAG trinucleotide repeat expansion in the huntingtin gene on chromosome 4. Mutant huntingtin results in neuronal dysfunction and death, through disruption of the proteostasis, transcription and mitochondrial function. HD is characterized by cognitive, motor and psychiatric disturbance [28]. Previous studies have shown that CYP46A1 expression reduces the number and size of intranuclear aggregates within the striatum of HD mouse models and improved motor impairment, showing the importance of neuroprotective effect conferred by CYP46A1 in HD mice [29]. More recently, a study by Nóbrega *et al*, showed that CYP46A1 is capable to decrease the number and size of huntingtin mutant (HTT-MUT) aggregates within a neuroblastoma cellular model of HD. Also, autophagy in HD is impaired and activating autophagy though expression of CYP46A1, could in part explain the reduction of inclusions and protein levels [30]. Dysregulation of cholesterol metabolism is also associated with Spinocerebellar Ataxias (SCAs). They are progressive disorders in which the cerebellum slowly degenerates, normally accompanied by degenerative changes in the brainstem and other parts of the central nervous system [31]. SCAs can fit into three major genetic categories: expanded CAG/polyQ ataxias, non-protein coding repeat expansion ataxias, and ataxias caused by conventional mutations (such as missense, deletion, insertion and duplication). Although they are different in origin and clinical manifestations, there is one feature that unifies all SCAs that is the pattern of neurodegeneration [31]. SCA3, also known as Machado-Joseph Disease, is the most common dominantly inherited ataxia in the world. SCA3 is caused by a CAG-repeat expansion in the coding region of the *ATXN3/MJD1* gene, that results in an expanded polyglutamine (polyQ) tract in the coding region of the ataxin-3 protein [32]. CYP46A1 levels are reduced in cerebellar samples from SCA3 patients and SCA3 mouse models, which leads to impaired brain cholesterol metabolism [33]. The demonstration that CYP46A1 is decreased, in patients and mouse models of Alzheimer's Disease (AD) and HD, and in polyQ-SCAs, together with impaired brain cholesterol metabolism, strongly suggests a central role for CYP46A1 and the cholesterol pathway in the physiopathology in these diseases. It was proposed that CYP46A1 deficits could

represent a direct cause in the neurodegenerative process, meaning that cholesterol metabolism could be a critical path to neurodegeneration, despite of the initiation mechanism [33]. AD is also another neurodegenerative disease that could be related to cholesterol metabolism. AD is a neurodegenerative disorder featuring gradually progressive cognitive and functional deficits as well as behavior changes. It is correlated with accumulation of amyloid and tau deposits in the brain. AD cognitive symptoms include deficits in short-term memory, executive and visuospatial dysfunction and praxis [34]. The data collected showed the similar results as for the above-mentioned neurodegenerative disorders. Accumulation of cholesterol in the brain may trigger endoplasmic reticulum stress, which may in turn contribute to neurodegeneration or aggravate the neuronal death induced by neurodegenerative processes [35].

2. Hypothalamus

The hypothalamus is located ventrally to the thalamus and dorsally to the anterior pituitary, surrounding the third ventricle and it is composed by several nuclei that work in a coordinated way to regulate physiological functions [36]. It can be divided into three sections (Figure 2), the periventricular zone that is formed by the preoptic area (POA), suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN), arcuate nucleus (ARC) and the posterior nucleus. The medial zone formed by the medial postoptic nucleus (PON), anterior hypothalamic nucleus (AHN), ventromedial nucleus. At last, the lateral hypothalamic area (LHA) formed by the lateral preoptic nucleus, lateral hypothalamic nucleus, tuberomammillary nucleus and supraoptic nucleus. In the middle-basal hypothalamus and in the third ventricle walls it is located the median eminence (ME), which contacts the cerebrospinal fluid [37].

2.1. Preoptic Area

The POA is the main brain nucleus sensible to temperature, so the main function of this area is to control physiological adaptations to temperature changes, which also includes variation in energy expenditure and food intake [38]. Although the POA is not directly related to body weight control, thermogenesis involves adipose tissue, such as Brown Adipose Tissue (BAT) and White Adipose Tissue (WAT), which are important metabolic organs involved in obesity and metabolic diseases. The adipose tissue is a metabolic dynamic

organ that is the primary site of storage for excess energy. This tissue can also serve as an endocrine organ that is able to synthesize a number of compounds that regulates metabolic homeostasis, classified into two types of fat, BAT and WAT [38]. Thermoregulation begins with detection of cold signals from the skin and thermosensitive neurons by the median preoptic area (mnPOA), which makes the POA to connect directly with the ventromedial nucleus of the hypothalamus in response to cold, meaning activation of BAT thermogenesis. POA can also control febrile response, since it contains prostaglandins E2 (PGE2) and prostaglandins receptors subtype EP3 that are connected to the dorsomedial nucleus of the hypothalamus (DMH) along with rostral raphe pallidus (rRPA), controlling thermogenesis in BAT to induce fever through a mechanism that involves cAMP [39]. The POA is also important in reproduction, since the LepRb (the receptor for the adipose tissue derived hormone leptin) is highly expressed here. The hormone communicates with the brain as a signal for acceptable levels of energy for reproduction, meaning that low levels of leptin prevent reproduction [40].

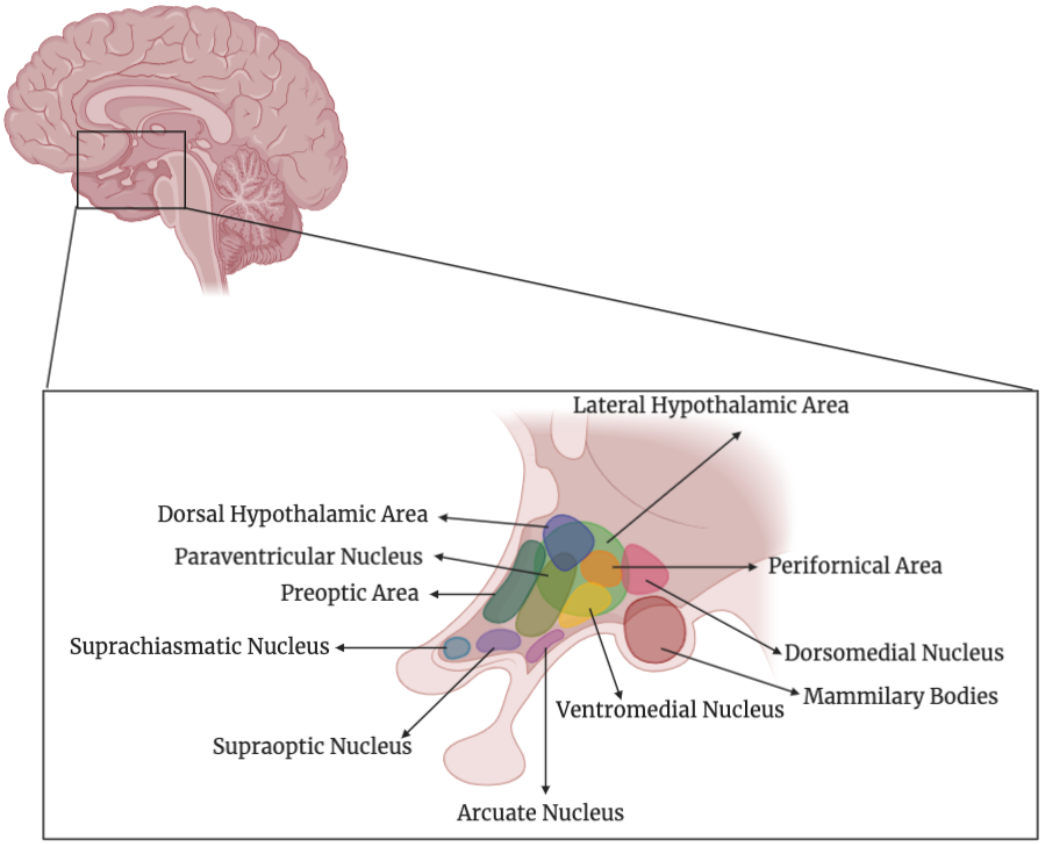

Figure 2. Schematic representation of the hypothalamus highlighting its main regions..

Anatomical localization of the hypothalamus and hypothalamic nuclei. In this figure it is represented the three sections of the hypothalamus. The nuclei act in tune in order to regulate the energy balance, thermogenesis and feeding behaviour, to maintain the homeostasis.

2.2. Arcuate Nucleus

The ARC is located above the median eminence where the Blood Brain Barrier (BBB) is permeable, which gives access of peripheral signals to the brain, making this region one of the gates to the brain [39]. The ARC contains neurons that produce different neuropeptides in order to respond to different signals; some of them are orexigenic, like the agouti related peptide (AgRP) and neuropeptide Y (NPY), that promote the feeding behaviour. On the other hand, the anorexigenic neuropeptides, that inhibit the feeding behaviour, such as proopiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) [41]. To promote or inhibit the feeding behaviour, there must be a flow of signals, which is accomplished by the leptin and ghrelin signalling. The Growth Hormone Secretagogue

Receptor (GHSR, ghrelin receptor) is expressed mostly on NPY/AgRP neurons and in less extent in POMC/CART neurons. These populations of neurons interact among them in order to maintain the balance to regulate body weight. Ghrelin binds directly to the receptors of NPY/AgRP neurons to activate them, and the activation of these neurons have three major effects: 1) the NPY is secreted, which results in increased food intake and decreased energy expenditure; 2) consequently AgRP is also released, which functions both as a competitive antagonist and an inverse agonist of the melanocortin 4 receptor (MC4R), inhibiting downstream melanocortin pathways (that otherwise would decrease food intake and increase energy expenditure); and 3) at last, it is released an inhibitory neurotransmitter of GABA which causes a decreased release of α-melanocyte-stimulating-hormone (α-MSH, endogenous ligand of MC4R) [42]. On the other hand, the main targets of leptin are the POMC (including POMC and CART) and AgRP neurons. Leptin acts via LEPRb (leptin receptor) in order to stimulate the synthesis of POMC that in turn generates α-MSH (Figure 3). This hormone can reduce body weight by binding and activating MC3R and MC4R [43]. Besides the control on feeding behaviour, the ARC also plays an important role in energy expenditure, as a mediator in leptin signalling. Activation of the orexigenic neurons, more particularly NPY, decrease BAT thermogenesis, while on the other hand, anorexigenic neurons (POMC) are related to an increase in thermogenesis [39].

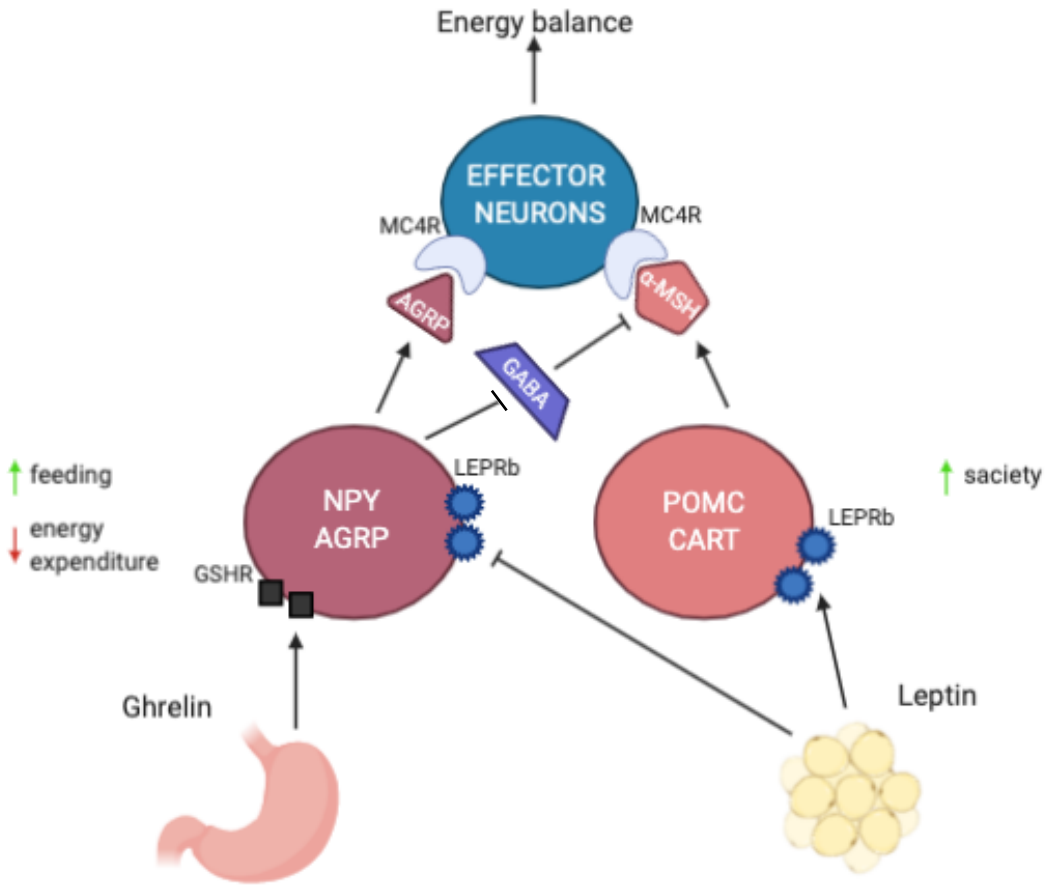

Figure 3. Hunger/Satiety circuit and energy balance.

Ghrelin binds to GSHR in orexigenic receptors in order to activate them. Consequently, AGRP is released, acting as an inverse agonist of MC4R, which then inhibits downstream melanocortin pathways. NPY is also released to increase food intake and lower the energy expenditure. It is also released an inhibitory neurotransmitter of GABA leading to a decrease in α-MSH. On the other hand, leptin binds to POMC/CART neurons through LEPRb to generate α-MSH, through POMC, leading to satiety. Leptin can also bind to LEPRb in NPY/AGRP neurons in order to inhibit them, when there is no need to increase food intake.

LEPRb- Leptin Receptor, GHSR- Growth Hormone Secretagogue Receptor, MC4R- Melanocortin 4 Receptor, GABA- Gamma Aminobutyric Acid.

2.3. Dorsomedial nucleus of the hypothalamus

The main function of DMH is to control thermogenesis in brown and white adipose tissue over sympathetic transmission. Is also involved in febrile response. DMH also has NPY-expressing neurons, capable of regulating thermogenesis. The disruption of NPY in DMH causes an increase in BAT thermogenesis and "browning" of WAT [39]. Under some circumstances, such as exercise, diet-induced obesity or under cold exposure, NPY in the DMH increases the thermogenic process. This happens in a different way from what happens in ARC, where NPY expression is reduced after cold exposure and NPY reduces BAT activity [39]. Neurons in DMH receive GABAergic inputs from warm-sensitive neurons in the preoptic area, so disinhibition of DMH neurons by infusing an antagonist to the $GABA_A$ receptor increases the BAT activity and core body temperature, which is dependent of the activation of downstream rRPA neurons [44].

2.4. Ventromedial nucleus of the hypothalamus

The ventromedial nucleus of the hypothalamus (VMH) has an important role in feeding, energy balance, cardiovascular function and regulating female sexual behaviour. It plays an essential role in the regulation of thermogenesis as well, through the integration of a variety of peripheral signals to coordinate the thermogenic response, particularly BAT and WAT. Thermogenesis can be modulated through AMP-activated protein kinase (AMPK), an energy sensor that in fasting or energy deficiency, inhibits anabolic pathways and promotes catabolic routes, with the point of re-establish the cell energy status [45]. A variety of hormones, like leptin and glucagon-like peptide-1 (GLP-1) analog can control BAT thermogenesis by acting on this axis (AMPK-SNS-BAT) [46]. One of the most important signalling pathways downstream AMPK is the acetyl-CoA/malonyl-CoA pathway. In the hypothalamus, malonyl-CoA levels oscillate in response to the nutritional status, acting as signal of energy excess. Malonyl-CoA is the inhibitor of carnitine palmitoyl-transferase 1 (CPT1) enzymes, which its main function is to catalyse the transport of long chain fatty acids into the mitochondria. One of the isoforms, CPT1C, is located in the endoplasmic reticulum of neurons and their expression is high in neurons of hypothalamic areas where the regulation of feeding is involved, such as the ARC, PVH and VMH. A recent study demonstrates that CPT1C plays an essential role in hypothalamic regulation of BAT thermogenesis, more specifically in response to metabolic challenges activating BAT, such as leptin and short-term diet [47]. Although VMH plays a key role regulating BAT, the

involvement of the VMH in WAT browning has not been clearly explored. A study showed that the administration of liraglutide (a GLP-1 analog) within the VMH not only induces BAT thermogenesis, but also activates WAT through hypothalamic AMPK inhibition [48].

2.5. Lateral hypothalamic area

In this area, a specific type of neurons is expressed, the orexins, also orexigenic neuropeptides. Orexin A and B binds to two receptors, orexin-receptors 1 and 2 (OXR1 and OXR2) in order to regulate sleep-wake cycles, physical activity and appetite. These neurons are, curiously, essential to the correct development, differentiation and function of brown adipocytes (Figure 4) [49] [50]. Lesions in the LHA produce intense anorexia, loss of body weight and increase in metabolism. Some investigations on the role of LHA neurons in energy homeostasis suggested that they function to promote energy storage by increasing the drive for food consumption and by inhibiting energy expenditure. The loss in body weigh due to lesions in LHA is accompanied by hyperthermia and increased energy expenditure, in which an enhanced metabolism in BAT was seen as one key factor, theoretically contributing to a hypermetabolic state that would sustain a lower body weight [51].

2.6. Paraventricular nucleus of the hypothalamus

The paraventricular nucleus (PVH) has also an important role in the regulation of feeding behaviour and energy homeostasis. From all the nucleus of the hypothalamus, the PVH seems to be the centre of the melanocortin system. Here, MC4R is expressed and projected to the brainstem, more specifically, the parabrachial nucleus (PBN) and dorsal motor nucleus of the vagus nerve (DMV). Activated MC4R neurons transport feeding-inhibiting signals, while inhibition of the MC4R neurons results in feeding-promoting signals. For that, MC4R neurons express NPY receptors in order to transfer the feeding-promoting signal. In addition, oxytocin neurons in the PVH also sense leptin signalling and project to the nucleus of the solitary tract (NTS), elevating the response to satiety signalling [52]. The association between PVH and BAT is supported by the fact that the administration of some molecules in the PVH (such as corticotropin-releasing hormone (CRH), glutamate, CART, PGE2, leptin, among others) leads to the activation of BAT thermogenesis (Figure 4) [39]. Another evidence that suggests that PVH induces BAT thermogenesis, is that genetic ablation of single-minded homolog 1 (SIM1), necessary for the correct development of this nucleus, decreases thermogenesis in BAT, which leads to obesity [53]. PVH can also induce

browning of the WAT. CART administration into this nucleus, induces UCP1, UCP2 and UCP3 expression in brown and beige adipocytes [54]. On the other hand, a study reported a negative link between oxidative stress in the PVH and thermogenesis, being that inhibition of NADPH-oxidase, a ROS inducer, only in the PVH of obese mice, boosted BAT thermogenesis and browning of WAT through sympathetic activation protecting against diet-induced obesity [55]. Still, the exact role in the control of thermogenesis is unclear.

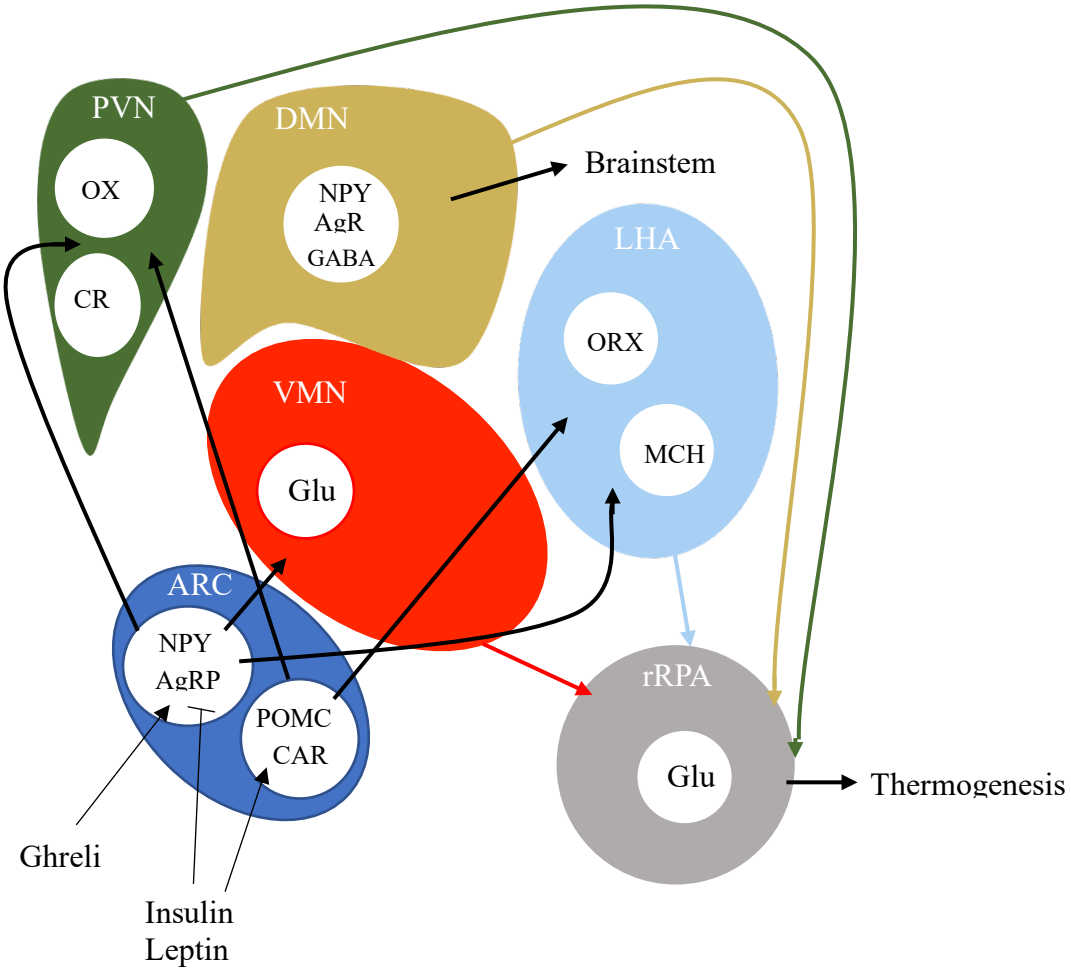

Figure 4. Hormonal signals such as leptin, ghrelin and insulin from metabolic organs lead to the activation of BAT thermogenesis.

These hormones modify the activity of two divergent neuron populations: Agouti-related peptide (AgRP)/NPY-expressing neurons and on the other hand the proopiomelanocortin (POMC) and cocaine-amphetamine-regulated transcript (CART). Subsequently, neuronal projections from the NPY/AgRP and POMC/CART neurons gather to the hypothalamic nuclei, including the PVH, LHA and VMH. The activation of BAT thermogenesis and browning of the WAT comes through projections from the nuclei to rostral raphe nucleus

(rRPA). OXY: oxytocin; CRH: corticotrophin-releasing hormone; Glu: Glutamate; ORX: orexin; MCH: melanin-concentrating hormone. Adapted from [39].

3. Metabolism

Metabolism can be defined as the sum of biological processes in living organisms that either produce or consume energy [56]. During the evolution, humans and animals have created mechanisms that promotes the accumulation of fat during periods of feast in order to survive. However, what was a need become an exaggeration leading to one of the biggest problems that we face nowadays, obesity [57]. Obesity results from an imbalance between energy intake and energy expenditure and in order to maintain the balance, the metabolic mediators must be in line (Table 2).

3.1. Adipose Tissue

The BAT is composed by multi-locular lipid vacuoles with abundance of mitochondria, while the WAT is characterized by a single large lipid vacuole and few mitochondria. Lately, it has been described a new type of fat, called “beige” or “brite” (brown + white), as these cells demonstrate an intermediate phenotype between BAT and WAT (Figure 5) [58]. BAT is the only tissue capable of burning fat due to high concentrations of UCP1 (uncoupled protein 1 or thermogenin). This protein is found in the mitochondria and it generates heat by non-shivering thermogenesis [38]. This type of thermogenesis is accomplished with help from UCP1, which induces a flux of protons across the inner membrane of mitochondria dissipating the excess of energy as heat and avoiding ATP synthesis [46]. On the other hand, the main function of WAT is energy storage, serving as a mechanical buffer and heat insulator in the skin. This tissue can undergo a phenomenon called white fat “browning”, where certain white adipose tissue deposits significantly increase gene expression for the UCP1, and consequently, acquire thermogenic fat-burning properties [59] [60].

Table 2. Main Features of Metabolic Mediators.

Metabolic Mediator	Site of Production	Final destination	Function
Neuropeptide Y (NPY) [61]	Arcuate Nucleus (NPY/AgRp neurons)	PVN, DMN, VMN and median preoptic area	- Stress - Modulation of neuroendocrine systems - Appetite regulation
Agouti-Related Protein (AgRP) [62] [63]	Arcuate Nucleus (NPY/AgRP neurons)	Melanocortin receptors 3 and 4 (MCR3 & MCR4)	- Circadian rhythm - Food intake
Cocaine-Amphetamine-regulated Transcript (CART) [64]	Arcuate Nucleus (CART/POMC neurons)	LHA, PVN, nucleus accumbens (Acb)	- Inhibitor of appetite and food intake - Modulation of energy expenditure
Proopiomelanocortin (POMC) [65]	Arcuate Nucleus (ARC – CART/POMC neurons) and nucleus tractus solitarius (brainstem)	PVN, LHA, DMH, Supraoptic nucleus, VMH, Periventricular nucleus, nucleus accumbens and amygdala	- Appetite regulation - Skin - Stress response - Immune system - Sexual function
Leptin [66]	White Adipose Tissue	Preoptic Area, PVH, ARC, VMH, LHA	- Regulation of energy homeostasis - Neuroendocrine function - Metabolism
Ghrelin [67]	Stomach	Growth Hormone Receptor in the pituitary gland and ARC (POMC/CART neurons)	-Regulation of food intake
Insulin [68] [69]	β-cells of the pancreatic islets of Langerhans	Insulin receptors in liver, muscle, and WAT	- Suppression of the appetite - Promote cellular glucose uptake
Glucagon [70]	α-cells of the pancreatic islets of Langerhans	Glucagon receptors (G protein-coupled receptors)	-Maintains the blood glucose levels (during fasting)

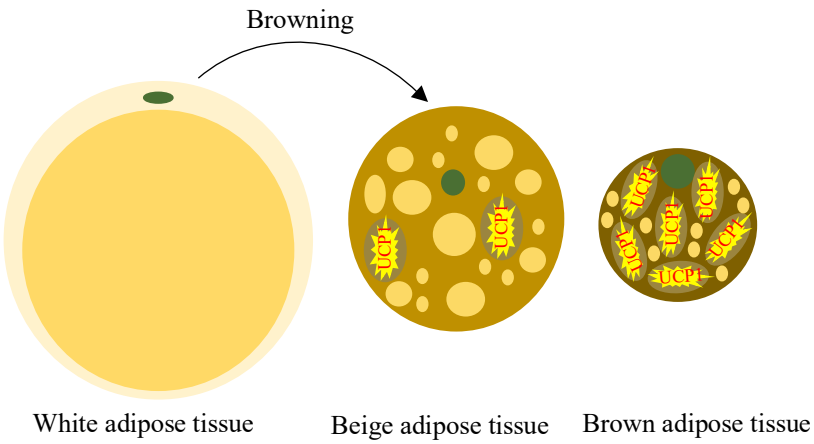

Figure 5. Different types of adipose tissue.

WAT contains only one lipid droplet that fills almost all the cytoplasm, acting as an energy store. Beige adipose tissue emerges in located depots of WAT and expresses some thermogenic markers. BAT is characterized by the presence of small lipid droplets and many mitochondria expressing UCP1.

The adipose tissue plays a major role in metabolism by acting as a caloric reservoir. It is a dynamic organ in response to energy status, through changes in the number and/or size of adipocytes. Energy storage can vary among species, but in humans it is divided into two major types of adipose tissues, BAT and WAT. Anatomically, WAT is composed by two major depots, subcutaneous WAT (SAT) and visceral WAT (VAT) around internal organs [71]. VAT can be divided into mesenteric, omental, perirenal and peritoneal depots (Figure 6). Although the key physiological function of WAT is energy storage; in obesity, excess of VAT is linked to metabolic complications such as, insulin resistance and type 2 diabetes [72]. Mesenteric and omental adipose tissues are important for hepatic insulin resistance and steatosis, since liver is directly exposed to releasing factors directly from these adipose tissues via the portal vein [72].

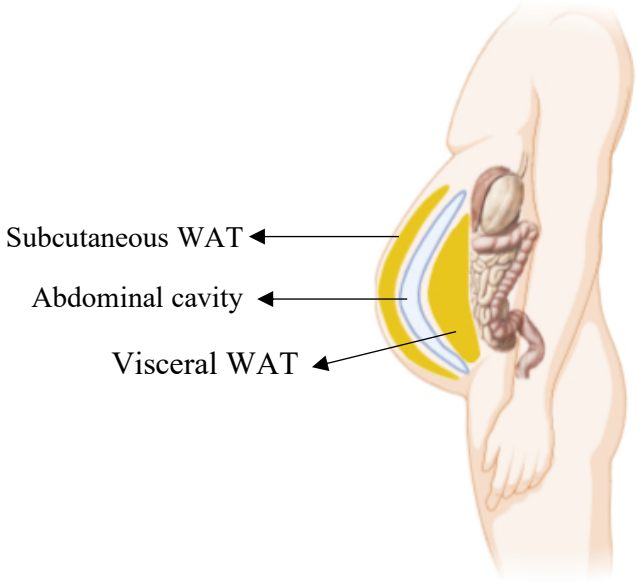

Figure 6. VAT and SAT location in human body.

VAT surrounds intra-abdominal organs, whereas SAT spreads through the body beneath the skin. These fat depots secrete adipokines that regulate the energy homeostasis. VAT is strongly associated with obesity induced metabolic disorders, since is closer to the organs, than SAT.

3.1.1. Leptin, Ghrelin, and Insulin

Leptin is produced and secreted mainly from adipose tissue into circulation. The expression and circulating levels of leptin, exhibit circadian fluctuations and changes with nutritional state. Fasting, decreases circulating leptin levels, while feeding or obesity increases leptin levels [73]. The expression and secretion of leptin are controlled by many factors including inflammatory cytokines, glucocorticoids and insulin. Leptin is too large to cross the BBB, so is instead transported across the barrier by a saturable transport system, which includes several steps. First, fasting can cause the fenestrated capillaries to extend from the ME to proximal parts of the ARC, making possible the diffusion of leptin to neurons at the ARC-ME border. Next, tanycytes (highly specialized glial cells that shield the ARC from the circulation and the adjacent median eminence) transport leptin into the cerebrospinal fluid from where leptin reaches LepRb target cells. Finally, several ARC LepRb neurons send projections across the tanycytes barrier and into the ME, thus gaining access to circulating leptin levels, that together with insulin control the energy balance in a negative way [73]. Therefore, leptin is able to induce POMC/CART neurons and inhibit NPY/AgRP neurons, and consequently, the same inhibitory effect can be seen with insulin

[74]. On the other hand, stomach hormone ghrelin, also called “the hunger hormone”, is an endogenous agonist at the GHSR. Besides stimulating Growth Hormone secretion and gastric motility, it also stimulates appetite and induces a positive energy balance leading to body weight gain. Accordingly, ghrelin is able to induce NPY/AgRP neurons in order to induce food intake, decreasing energy expenditure and also exerting effects in peripheral tissues, including stimulation of glucocorticoid and insulin secretion. This favour further deposition of triglycerides in white adipose tissue. For that, leptin and ghrelin are complementary, yet antagonistic [75].

3.2. Liver

The liver is an important metabolic organ that acts as a metabolic connection between skeletal muscle and adipose tissue. After a meal, the components essential to our survival, such as glucose, fatty acids and amino acids, are absorbed and transported to the liver [76]. This organ is responsible to maintain the glucose homeostasis during the *starved-fed* cycle. Blood glucose penetrates in hepatocytes through a glucose transporter, GLUT2. There, is phosphorylated by the enzyme glucokinase forming glucose-6-phosphate (G6P). This will result in a reduction in glucose concentration and consequently in an increased glucose uptake. When glucose is in its phosphorylated form, it can’t be transported, so it stays retained inside the hepatocytes. To be transported, glucose has three alternatives: isomerization into glucose-1-phosphate, isomerization into fructose-6-phosphate and the pentose phosphate pathway (Figure 7) [77]. In the fed state, G6P acts as precursor for glycogen synthesis, following the route into isomerization into glucose-1-phosphate. This state provokes insulin release, which stimulates the storage of fuels and synthesis of proteins [78]. When short fasting periods occurs the main source of glucose, produced by the liver, will be glycogenolysis, which consists in the disintegration of glycogen to glucose. When glycogen runs out, in order to maintain the blood glucose levels, gluconeogenesis is activated [79].

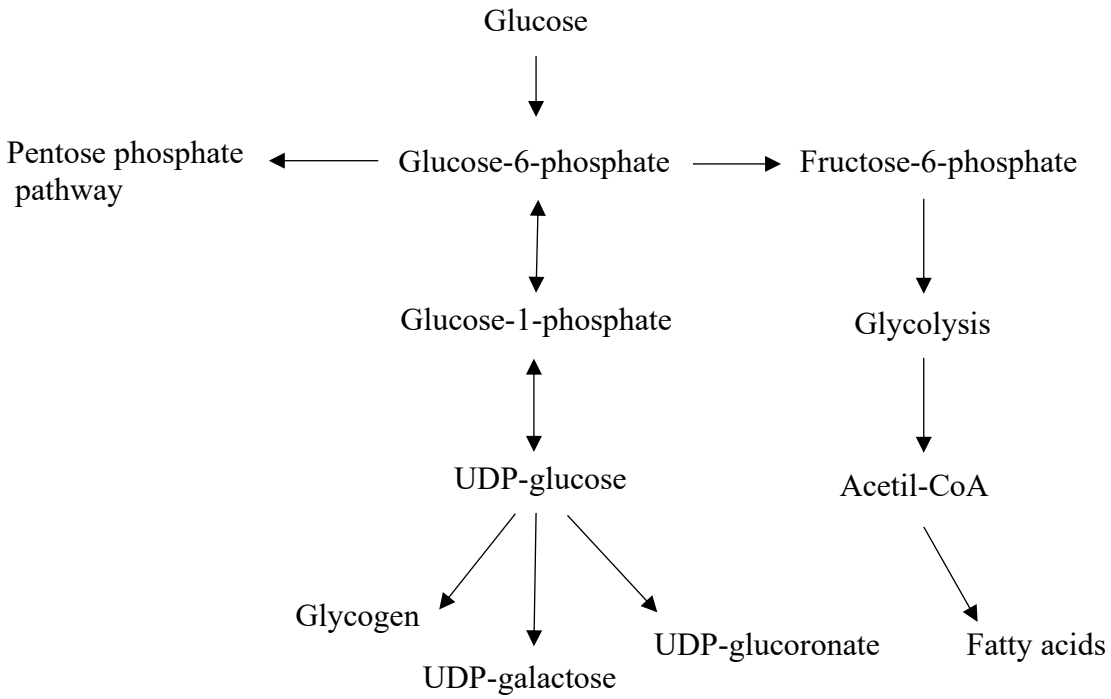

Figure 7. Glucose metabolism in the liver.

Glucose in the liver is converted to G6P, and can follow three routes: Pentose phosphate pathway, Glucose-1-phospate and Fructose-6-phosphate. Is metabolized via the pentose phosphate pathway in order to generate NADPH, which is required for lipogenesis and biosynthesis of bioactive molecules. G6P can act as a precursor of Glycogen synthesis. G6P it is also metabolized into F6P to generate pyruvate through glycolysis. This pyruvate is channelled to the mitochondria and completely oxidized to produce ATP. Alternatively, pyruvate can be used to synthetized fatty acids through lipogenesis.

3.2.1. Glycogen Metabolism

Although most tissues have some glycogen, the two major sites of glycogen storage are the liver and skeletal muscle [80]. The principal organ for glucose storage, in the form of glycogen, is the liver [79]. In the cell, when it is required great amounts of ATP, there will be oxidation as complete as possible of G6P. This means that, in the fed state, glucose will enter hepatocytes, through GLUT2, to be phosphorylated and produce glycogen. On the other hand, when glucose levels are low, glycogen is hydrolysed by glycogen phosphorylase to produce glucose [76]. Phosphorylated glucose, as a product of glycogen breakdown, is unable to be transported out of the cells. So, the liver contains an enzyme, glucose-6-

phosphatase, that cleaves the phosphoryl group to create free glucose. This will make it possible for glucose to leave the cells [80]. As any other mechanism, glycogen metabolism needs to be controlled and the focus of this control is relied on the enzyme glycogen phosphorylase. This enzyme is regulated by allosteric factors, which senses the energy state of the cell, being responsive to hormones, such as insulin, epinephrine and glucagon. The hormones responsible for the breakdown of glucose are glucagon and epinephrine (Figure 8). However, the liver is more sensitive to glucagon, a hormone secreted by the α cells of the pancreas. This hormone is secreted when glucose levels are low, which means that glucagon represent starvation. Epinephrine (more known as adrenaline) is released when there is muscle activity. This hormone acts mainly in the muscle, to stimulate glycogen breakdown. When blood-glucose levels are high, insulin triggers the synthesis of glycogen. This process is achieved by inactivation of glycogen synthase kinase, the enzyme that maintains glycogen synthase in its phosphorylated form, which means inactive. To prevent wasteful depletion of glycogen, when glucose levels are satisfied, the signal-transduction pathway is shut down when the initiating hormone is no longer present [80, 81].

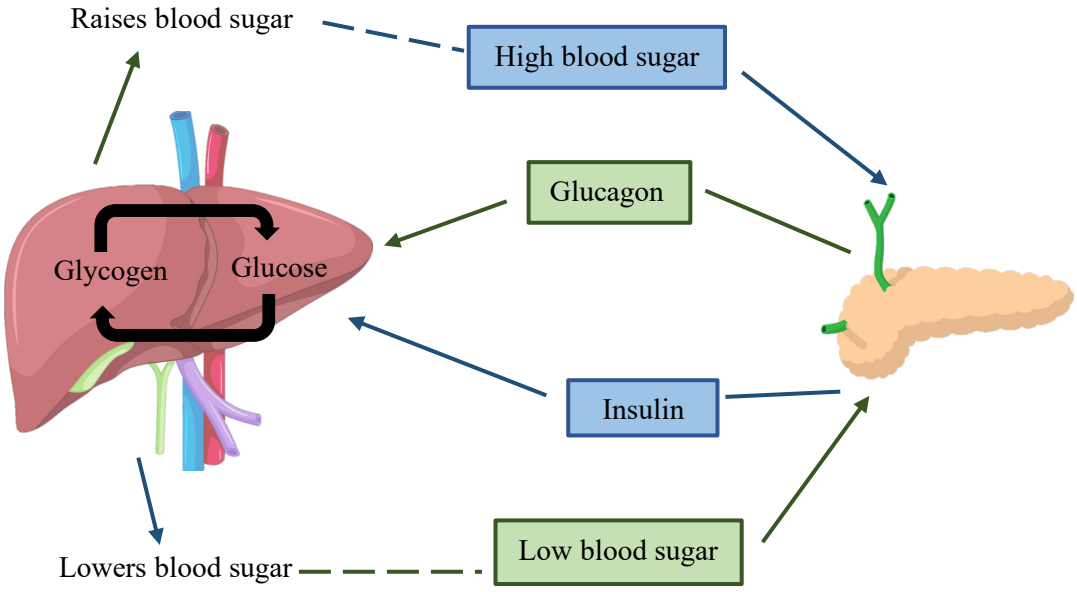

Figure 8. Regulation of blood-glucose levels.
High blood sugar promotes insulin release from pancreas, that stimulates glycogen formation and glucose uptake from food which lowers the blood sugar. On the other hand, low blood sugar stimulates glucagon release that stimulates glycogen breakdown in order to produce glucose to increase blood sugar.

3.2.2. Gluconeogenesis

It is extremely important to maintain the levels of glucose, as the brain for example is highly dependent on these levels. The direct reserves of glucose are sufficient for a day, however during starvation, gluconeogenesis is extremely important. This process is modulated by several external factors, including nutrient and energy conditions, exercise and stress reaction. Glucose is synthesized through gluconeogenesis, during long periods of fasting, using lactate, pyruvate, glycerol, and amino acids [76]. These two processes, gluconeogenesis, and glycolysis must be coordinated, so that when a pathway is active the other must remain inactive. So, when energy is required, glycolysis will prevail, and when there is an excess of energy, gluconeogenesis will take over (Figure 9) [81].

3.3. Muscle

Muscle contraction and exercise in general are highly dependent of Adenosine TriPhosphate (ATP). It is easy to think that muscle, like all cells, would benefit of a storage of ATP, in order to maintain the muscle contraction. However, it is not how it works [82]. The human body can store approximately 20-25 mmol/kg of ATP in the muscle. However, in activity, with peaks of around 15 mmol/kg per second, this storage is enough to fuel only 1-2 seconds of effort [83]. As an alternative, the muscle possesses a sensitive system, that has the ability to increase metabolism when energy is needed [82]. This means that the primary control of muscle glycolysis is the energy charge of the cell, the ratio AMP/ATP. There are three major systems that are responsible for the production of ATP: the phosphagen system, the glycolytic system and mitochondrial respiration [82].

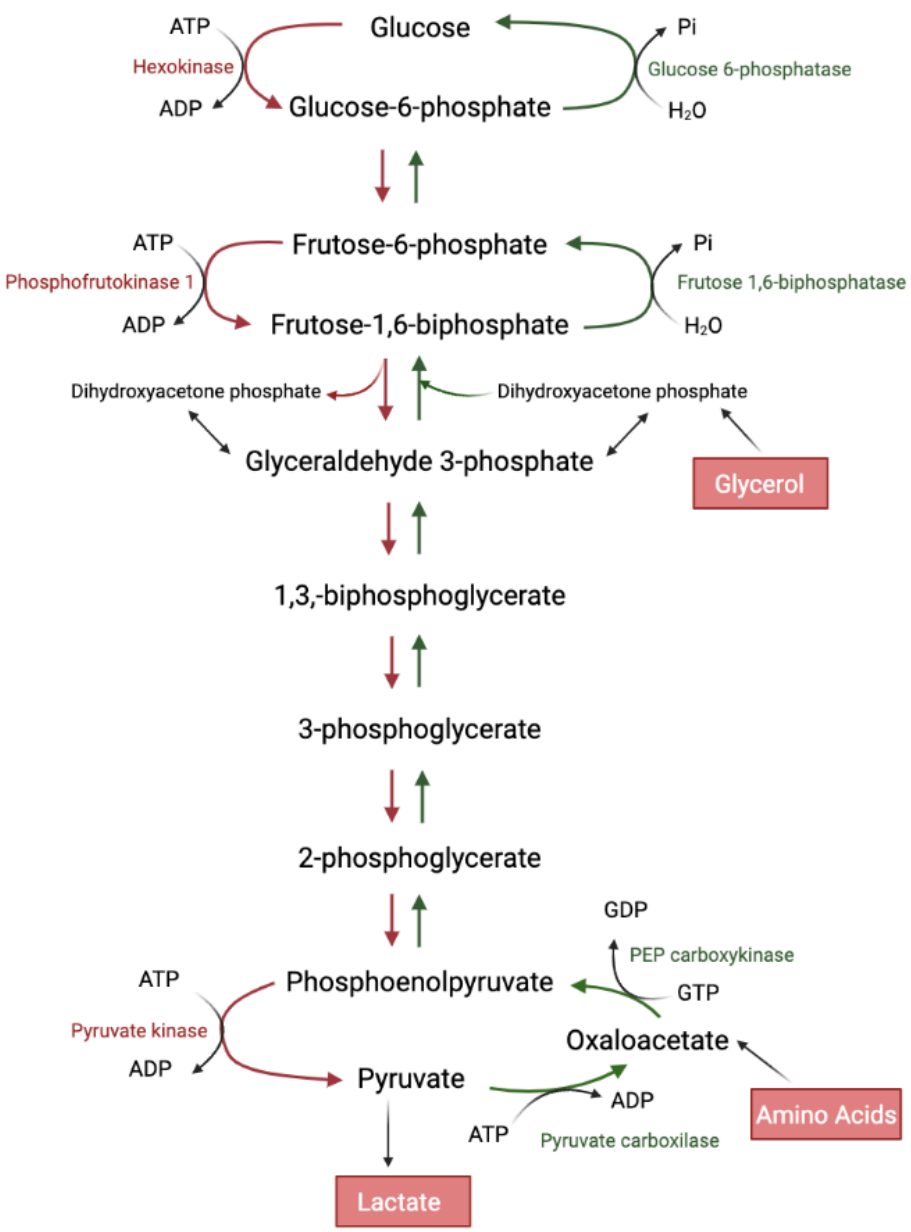

Figure 9. Schematic representation of Glycolysis and Gluconeogenesis.

Gluconeogenesis is not a reversal of glycolysis. Some reactions must differ because of their equilibrium. In ten reactions of gluconeogenesis, three are deviated by enzymes that catalyse irreversible steps, in direction of glucose production. This process assures that the metabolic pathway stays irreversible.

3.3.1. Phosphagen system

Intense muscular activity leads to exhaustion approximately in three minutes, which increases the activity of the enzymes that hydrolyze ATP to extreme levels. It occurs when oxygen is in short quantity, as cardiovascular system tries to deliver oxygen to the muscles, trying to match the metabolic need. Some measurements proved that great amounts of anaerobically derived ATP can be produced from phosphocreatine (PCr) degradation [84]. Creatine is an amine that can be obtained through diet or synthesized by liver, kidneys and pancreas. About 95% is stored in skeletal muscle on both forms (free and phosphorylated). The principal role of creatine is to rapidly convert energy, in a reaction catalyzed by creatine kinase (Figure 10). Supplementation with this nitrogen amine rises phosphocreatine stores and improves the energy supply from the phosphagen systems. Consequently, it increases the maximum capacity to resynthesize ATP by anaerobic pathways during intense muscular activity [85].

$$\text{CrP} + \text{ADP} + \text{H}^+ \longrightarrow \text{creatine kinase} \longrightarrow \text{ATP} + \text{Cr}$$
$$\text{ADP} + \text{ADP} \longrightarrow \text{adenylate kinase} \longrightarrow \text{ATP} + \text{AMP}$$
$$\text{AMP} + \text{H}^+ \longrightarrow \text{AMP deaminase} \longrightarrow \text{IMP} + \text{NH}_4^+$$

The three reactions that comprise the phosphagen system. The creatine and adenylate kinase reactions both produce ATP, but the creatine kinase reaction has by far the greater capacity for ATP regeneration. The proton (H^+) consumption during the creatine kinase reaction accounts for the alkalinization of muscle. As the acid production increases, it activates AMP deaminase leading to the production of ammonia (NH_4^+). CrP – Phosphocreatine, ADP – Adenosine Diphosphate, ATP- Adenosine Triphosphate, Cr – Creatine, IMP – Inosine Monophosphate.

One spotlight of the phosphagen system is the synthesis of AMP. AMP is an activator of two enzymes that influence glycolysis. It activates phosphorylase, leading to an increase in glycogenolysis and consequently G6P, leading to glycolysis. AMP also activates phosphofructokinase (PFK) in glycolysis, which will increase the rate of ATP, thanks to the increasing flux of G6P. At last, the production of ammonia, which is a toxic component and

so it is important to remove it to the liver, in order to be converted to urea, in a process known as urea cycle [82].

3.3.2. Glycolytic System

As seen in Figure 9, the glycolytic pathway starts with glucose and ends with two molecules of pyruvate. When glucose is catabolized during intense muscular activity, only limited oxidation occurs. This happens as pyruvate production develops at rates that overcome the capacity of the mitochondria to pick up pyruvate. In order to prevent the pyruvate production and consequently the decline in the rate of ATP regeneration, this product must be removed from the cytosol, as much quantity as possible. The majority of it is converted to lactate through lactate dehydrogenase, while the rest is transported out of contracting muscle [86].

$$\text{Pyruvate} + \text{NADH} + H^{+} \longrightarrow \text{lactate dehydrogenase} \longrightarrow \text{Lactate} + NAD^{+}$$

The production of lactate during intense muscular activity is profitable to remove pyruvate, keep glycolysis working at full speed and reestablish NAD+, which will be necessary in the glyceraldehyde-3-phophate reaction in glycolysis [82].

3.3.3. Mitochondrial respiration

As said before, glucose is transformed into pyruvate through glycolysis. When oxygen supply is insufficient or null, the pyruvate is converted into lactate or ethanol. In aerobic circumstances, the pyruvate must be transported into the mitochondria. In the mitochondrial matrix, pyruvate is decarboxylated to form Acetyl CoA [87]. This product will be used in the Citric Acid Cycle (or Krebs cycle) to form citrate. To maintain the cycle working, the citrate is oxidized back to oxaloacetate, as the excess carbon is released as carbon dioxide and the electrons passed to cofactors NADH and $FADH_2$ to participate in the mitochondrial electron transport chain (Figure 11) [88]. This chain, also known as respiratory chain, occurs at the inner mitochondrial membrane, where electrons resulting from the citric acid cycle are used to give energy to pump protons from the matrix to the intermembrane space. This process will produce a potential difference across the membrane, which will be used to power the synthesis of ATP in oxidative phosphorylation [88].

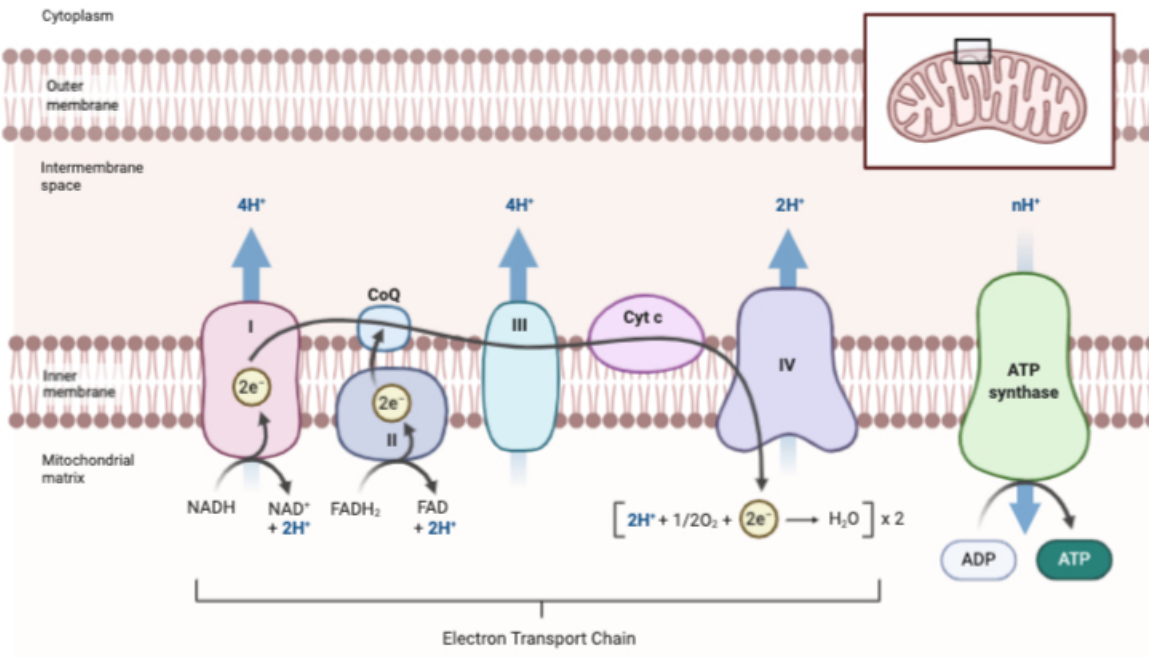

Figure 10. Mitochondrial Electron Transport Chain.

NADH brings free energy to the electron transport chain by binding to the largest of the respiratory complexes, the NADH dehydrogenase, or complex I. NADH donates two electrons, carried away from the citric acid cycle. While NADH must diffuse to complex I in order to feed the electrons into the electron transport chain, the succinate dehydrogenase, also knowns as complex II, catalyzes the reduction of FAD to $FADH_2$. Coenzyme Q, reduced by either complex I or complex II, is free to diffuse through the inner mitochondrial membrane to donate its electrons to the complex III of the electron transport chain, cytochrome c reductase. The last fate of the electrons passed along this chain, is in the conversion of oxygen to water, that occurs at complex IV, cytochrome c oxidase. Alongside the reaction, protons are pumped from the mitochondrial matrix into the intermembrane space. (Source: Biorender)

3.3.4. Cori Cycle

When the muscle is in effort, the ratio by which glycolysis produce pyruvate is greater than the ratio that citric acid cycle oxidizes it. The conversion of pyruvate to lactate in muscle and their release, makes possible for the muscle to generate ATP in anaerobic conditions and also deviates metabolizing lactate from muscle to other organs, for example the liver [81]. In the liver, the lactate is converted to glucose by the gluconeogenic pathway (Figure 13). Consequently, the liver makes it possible to restore the level of glucose that is necessary for the muscle in activity, releasing ATP from conversion of glucose into lactate [81].

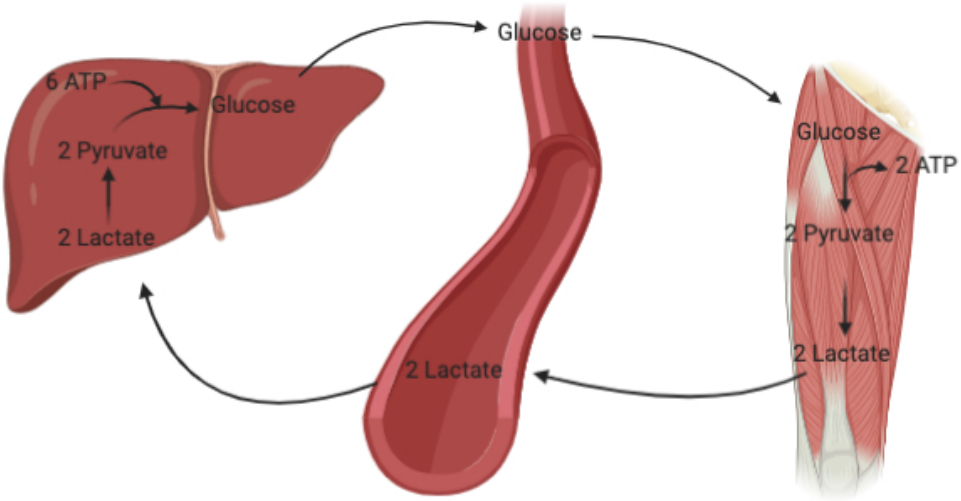

Figure 11. Cori cycle.

Connection between glycolysis and gluconeogenesis. This cycle is mainly active in short vigorous exercise situations when oxygen supply is deficient. In anaerobic conditions, the pyruvate follows the fermentation pathway to produce lactate. However, this product cannot be used by muscle cells, so it has to be transported to liver, where it can undergo gluconeogenesis, to synthesize glucose, so it can be used as an energy by muscle.

3.4. Pancreas

The pancreas is fundamental to regulate metabolism through the release of digestive enzymes and hormones. The most part of this organ consists in exocrine cells, which secrete the pancreatic liquid, which contains important digestive enzymes and is released into the ducts. On the other hand, the endocrine cells secrete pancreatic hormones directly into the blood stream. These cells are aggregated to form the islets of Langerhans [89]. These islets are composed by α-cells that produce glucagon, β-cells that secrete insulin, delta cells that produce somatostatin and at last PP cells that produce the pancreatic polypeptide [90]. Every hormone has a different function and some of them are antagonist, such as insulin and glucagon. Somatostatin has the ability to inhibit both insulin and glucagon and PP cells regulate the secretion activity of the pancreas, both exocrine and endocrine. To maintain the homeostasis, glucagon and insulin release must be in balance. When the blood glucose levels get low, like during sleep or between meals, glucagon is secreted to promote gluconeogenesis in the liver, so the levels of glucose get restored (Figure 13). On the other hand, insulin is stimulated when blood glucose levels are elevated, such as after a meal. After insulin reaches their target (muscle and adipose tissue), it will set up the uptake of glucose into these tissues, which in turn, will lower the glucose levels [89].

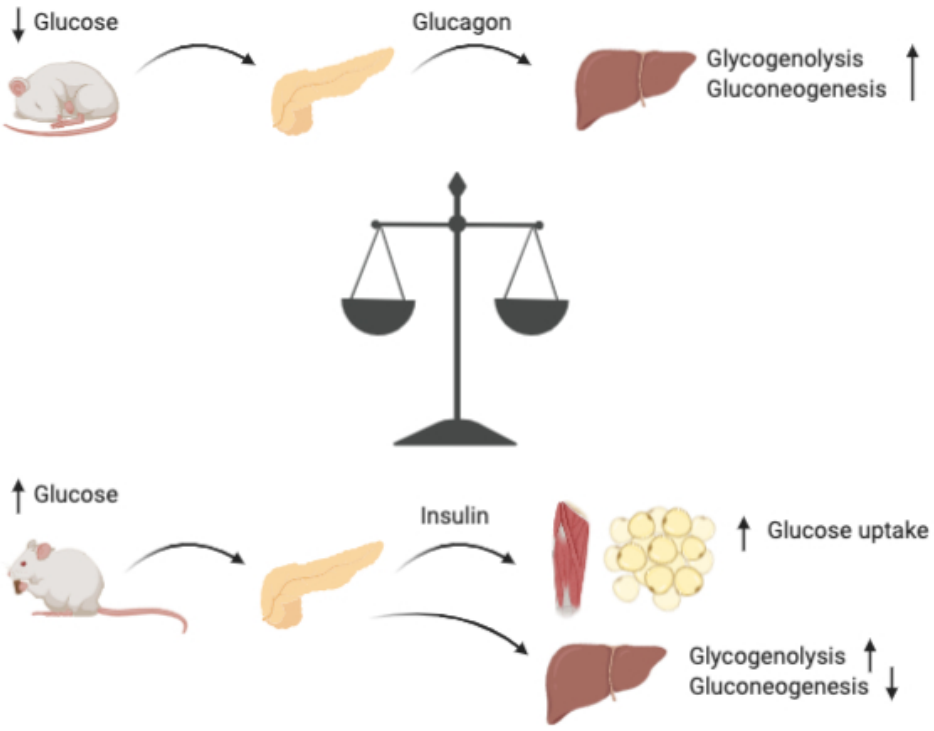

Figure 12. Balanced actions of glucagon and insulin.

During long periods of fasting (sleep), blood glucose levels lower so the pancreas secretes glucagon. This hormone will activate gluconeogenesis in order to produce glucose to rise the levels of blood glucose and the balance is accomplished. When glucose levels are high (after a meal), pancreas secretes insulin, that will dock in his targets (muscle and adipose tissue) to promote glycogenesis and inhibit gluconeogenesis. The blood sugar levels get lower, and the balance is restored.

4. Metabolic Syndrome

Metabolic Syndrome (MS), also called "the deadly quartet", is an aggregate of metabolic abnormalities that covers hypertension, central obesity, insulin resistance and atherogenic dyslipidemia (Table 3). This syndrome is associated with an increased risk of establishing cardiovascular disease (CVD) [91]. The first internationally recognized definition of MS was created by the World Health Organization (WHO) in 1998 but has suffered some alterations through the years. The WHO defined MS as the presence of insulin resistance (type II diabetes *mellitus*) in addition to two of the risk factors: obesity, hyperlipidemia, hypertension or microalbuminuria [91].

Table 3. Definitions of Metabolic Syndrome in 1998 vs 2005.

Clinical measure	World Health Organization 1998	American Heart Association/ National Heart, Lung and Blood Institute 2005
Criteria	IR + any other 2	Any 3 of 5
Insulin resistance	IGT/IFG IR	-
Blood glucose	IFG/IGT/T2DM	≥100 mg/dL
Dyslipidemia	TG≥1,69 mg/dL and HDL-C Men<0,90 mg/dL Women<1,01 mg/dL	TG≥1,69 mg/dL or on TG treatment HDL-C Men<1,03 mg/dL Women <1,29 mg/dL Or HDL treatment
Blood pressure	≥140/90 mmHg	≥130/85 mmHg or on antihypertensive medication
Obesity	Waist: hip ratio Men>0,9 and women>0,85 And/or BMI>30kg/m^2	WC men≥102 cm women≥88 cm
Other	Microalbuminuria	-

IR- Insulin Resistance; IGT- Impaired Glucose Tolerance; IFG- Impaired Fasting Tolerance; T2DM- Type 2 diabetes mellitus; TG- Triglycerides; HDL-C- High density lipoprotein cholesterol; WC- waist circumference; BMI- Body mass index.

4.1. Hypertension

Hypertension is described as continual high blood pressure (BP) in the systemic arteries. Hypertension can be divided into primary and secondary. The great majority of individuals (90-95%) suffers from primary hypertension (or essential hypertension). It results as a combination of genetic, and environmental factors, so its etiology cannot be completely determined [92]. Our body has mechanisms that are able to compensate any changes in blood pressure, in order to maintain the homeostasis. Hypertension results when the balance between factors that tend to increase blood pressure (mutations for example) and the ones that try to normalize, is disturbed. Besides genetic factors, diet and lifestyle also increase the risk of hypertension [93]. Secondary hypertension results of another conditions, such as neuroendocrine tumors on the adrenal glands or primary aldosteronism. In cardiovascular disease (CVD), hypertension is the most preventable risk. Prevention and treatment are fundamental to reduce disease burden. So, in order treat hypertension successfully, it is important to consider the person predicted atherosclerotic CVD risk more than the BP alone, as people with high atherosclerotic risk profit from BP-lowering treatment. Globally, 3.5 billion adults have non-optimal systolic BP (>110-115 mmHg), which means that one in four adults has hypertension [94].

4.1.1. Blood pressure regulation

BP is associated to cardiac output, which means heart rate per stroke volume, and total peripheral vascular resistance [95]. The preservation of BP levels needs reciprocity of several elements of neurohormonal system. That system includes renin-angiotensin-aldosterone system (RAAS), the sympathetic nervous system (SNS) and immune system. Any disruption of a component in this system, will lead, directly or indirectly, to increases in mean BP, its variability, or both. With time that can result in target-organ damage, such as left ventricular hypertrophy for example [94].

4.1.1.1. Renin-Angiotensin-Aldosterone System

This system is present in many organs, at cellular level. However, is essential to regulate pressure-volume homeostasis in the kidney [94]. Angiotensin II is formed through an enzyme activation that leads to generation of reactive oxygen species (ROS). ROS is prejudicial, producing effects such as endothelial injury, oxidation of LDL and expression

of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1). RAAS, ROS and LOX-1 possess a positive feedback loop, that leads to inflammation, endothelial damage and fibroblast proliferation. These consequences contribute to the development of hypertension, diabetes, CVD, and cardiac hypertrophy [91].

4.1.1.2. Sympathetic Nervous System

The body possess mechanoreceptors that sense variations in the pressure of circulatory systems (also known as baroreceptors), located in diverse positions along the arterial tree, near the carotid artery. When the blood pressure is elevated, the carotid artery stretches, and the nerve clusters projected it to the brain. The brain receives these messages to reduce the sympathetic outflow of nerve impulses, and consequently, blood pressure reduces [94]. Although the development of sophisticate techniques for the direct assessment of adrenergic activity has changed our conception about the role of SNS in the regulation of BP, nowadays there is only one theory. The established assumption is that SNS hyperactivity contributes to initiation, maintenance, and progression of hypertension [96].

4.1.1.3. Immune System

The immune system plays a significant role by inducing inflammatory processes in the central nervous, the cardiovascular and renal systems. The magnitude and extent of these responses could vary in different models of hypertension [97]. Innate immune response, specially mediated by macrophages, have been associated to hypertension, induced by the RAAS system. Hypertension is also linked to adaptative immune response, via T-cells. Anomalies in T-cells, both pro-inflammatory and anti-inflammatory, are connected to hypertension-induced target-organ damage, because these cells regulate the inflammatory processes in the kidney and vasculature that control hypertension-induced kidney disease [94].

4.2. Central Obesity

Obesity can be simply defined as excess of body weight for height. Although underlies on complex phenotypes, such as excess adiposity, it can be manifested metabolically and not only in terms of body size. Obesity increases the risk of chronic disease morbidity, like

type 2 diabetes, CVD, and mortality [98]. Obesity pathogenesis is related to many factors, including genetics, epigenetics, and preventable ones (lifestyle factors) [99].

As known from previous sections, WAT is a complex organ and plays a major role in energy homeostasis control. Besides adipocytes acting as a storage for energy, they also sense the energy demands and secrete paracrine factors. In obesity, WAT may not expand properly to store the energy excess, becoming severely dysfunctional [99].

In a state of obesity, WAT expansion is accomplished by recruiting and differentiating adipose precursor cells instead of infiltrating fat into adipocytes. When the capacity of SAT to storage fat is surpassed, the excess fat is concentrated in the liver, skeletal muscle and heart. This leads to numerous complications, like local inflammation and insulin resistance [99]. Since insulin resistance is associated with obesity, it is probable that adipose tissue dysfunction turns out to be an essential contributor to associated complications in obese patients. Consequently, the image of metabolic syndrome phenotype is weight gain [100].

4.3. Insulin Resistance

As obesity and diabetes increase to pandemic proportions, the role of insulin resistance in both conditions gain importance. Insulin resistance can be defined as when your cells in muscle, fat and liver do not respond to insulin action. Consequently, the pancreas produces more insulin to get glucose into the cells. The inability of insulin action, leading to hyperinsulinemia, promote the dysfunction of pancreatic β-cells and irregular glucose intake. In the context of obesity, as the food consumption rises, the pancreatic β-cell increases the insulin secretion to maintain the normal glycemia [68]. However, if the food consumption continues to increase, the β-cell become impaired and the secreted insulin becomes insufficient for response to glucose stimuli, leading to type 2 diabetes and hyperglycemia [101]. Some possible mechanisms of insulin resistance include down-regulation (or genetic polymorphisms) of tyrosine phosphorylation of the insulin receptor or may involve abnormalities of GLUT4 function [102].

The effects of insulin resistance can vary accordingly to the physiological function of the tissue and their dependence on insulin. Although insulin actions are widespread, the mainly affected tissues are muscle and adipose tissue since they are defined as insulin dependent [68].

4.3.1. Muscle

Glucose uptake into muscle is mainly insulin dependent via GLUT4 and muscle itself accounts for about 60-70% of whole-body insulin mediated uptake [103]. In normal skeletal muscle metabolism, muscle uses both glucose and fatty acids (FA) as sources for energy production. As said before, insulin resistance in skeletal muscle is linked to lipid accumulation. That results in defects of FA metabolism, which include alterations in muscle FA uptake, FA oxidation, triacylglycerol (TG) synthesis/breakdown, and even a combination of them [104]. Once the lipid metabolism is altered, it leads to an increased level of circulating FA and TG, and consequently to an accumulation of lipid intermediates that will make defects in the insulin signaling cascade. This accumulation will lead to a reduction in glucose uptake by muscle cells (besides fat and liver) and insulin resistance is developed [104].

4.3.2. Adipose Tissue

Despite skeletal muscle accounts for most of the whole-body insulin, it is estimated that adipose tissue uses at least 10% of insulin stimulated whole-body glucose uptake. Insulin, besides stimulating glucose uptake, also promotes lipogenesis while suppressing lipolysis and consequently free fatty acid flux into the blood flow [68]. Adipose tissue can also secrete cytokines that have effects on insulin resistance, namely by increasing it. These are TNF-α (Tumor Necrosis Factor α), IL-6, plasminogen activator inhibitor 1 (PAI-1), angiotensin and leptin [68]. In a state of obesity, adipose tissue gets overburden, and their buffering capacity gets exceeded, which makes it uncapable to protect other tissues from an exorbitant flux of fatty acids. The accumulation of TG in insulin dependent tissues, such as skeletal muscle, liver and pancreatic β-cells, alters the normal sensitivity of glucose metabolism to insulin. When the buffering capacity is exceeded, through consistent intake of fat above the oxidation rate, the dietary fat needs to go somewhere, and its deposition in other tissues leads to insulin resistance [105].

4.4. Atherogenic dyslipidemia

Atherogenic dyslipidemia consists of elevated blood concentrations of small dense low-density lipoprotein (sdLDL), low high-density lipoprotein (HDL) and high triglycerides.

Atherogenic dyslipidemia embrace a triad of increased blood concentrations of small dense low-density lipoprotein (sdLDL) particles, decreased high-density lipoprotein (HDL) particles and increased triglycerides. This metabolic abnormality is a typical feature of obesity, insulin resistance and type 2 diabetes mellitus [106]. This process begins at childhood, as obese children and adolescents present an adverse lipid profile versus normal body weight [107]. Although atherosclerotic cardiovascular disease is rare in children, the atherosclerotic process begins at a young age, and so the risk factors associated with its development [108]. Among all lipid particles, sdLDL may have a predominant role in atherosclerotic disease [109]. sdLDL have more susceptibility to oxidation and consequently are more prone to initiate the inflammatory process in the vascular endothelium. sdLDL also has the ability to penetrate arterial wall easily. They also have lower affinity for the LDL receptor, leading to a reduced cellular uptake. This leads to more time in the bloodstream resulting in a major influence on the atherosclerotic process [106].

4.5. Cholesterol metabolism and implications on body homeostasis

Cholesterol is a lipophilic molecule that is essential for life. It is important for neuronal physiology, both during development and in adult life, as a key component of cell membranes and precursor of steroid hormones. Cellular cholesterol synthesis is a complex process, which starts with the conversion of Acetyl-CoA and many enzymatic reactions later to cholesterol (Figure 14) [110]. Cholesterol homeostasis is accomplished through a feedback regulatory system that senses the levels of cholesterol, to normalize it. That is achieved by membrane-bound transcription factors known as sterol regulatory element-binding proteins (SREBPs), which regulate the transcription of genes encoding enzymes involved in cholesterol and fatty acid biosynthesis (Figure 14) [111]. Cholesterol is present in brain, where a large fraction is in the myelin sheath to insulate axons. Due to the blood-brain barrier, all cells in the brain are cut off from cholesterol prevenient from blood supply, so they must produce their own. Consequently, defects on cholesterol metabolism and transport, can lead to or be part of brain disfunction. Alterations in brain cholesterol can lead to several neurodegenerative disorders such as Alzheimer's disease, Huntington's disease, Parkinson's disease, and Spinocerebellar Ataxia type 3/Machado-Joseph disease

(SCA3/MJD) [112] [33]. To avoid an accumulation of excess cholesterol, the brain requires specific mechanisms to prevent it.

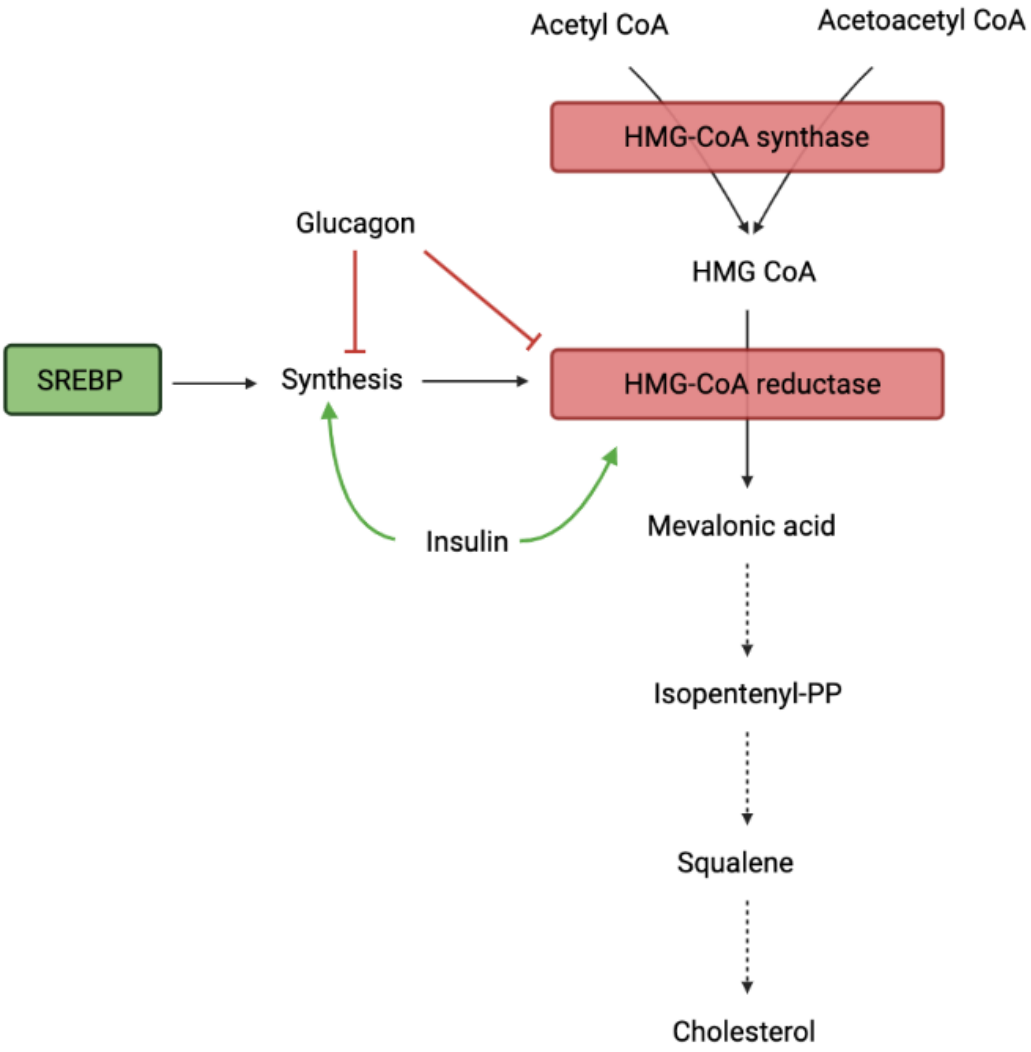

Figure 13. Schematic cholesterol synthesis.

The two beginning precursors of cholesterol synthesis are Acetyl CoA and Acetoacetyl CoA. They come together with help from enzyme HMG-CoA synthase to produce HMG CoA. This product can be used to produce not also cholesterol, but ketone bodies, depending on if it is in the mitochondria or in the cytosol. When located in the cytosol, it undergoes through the enzyme HMG-CoA reductase to produce Mevalonic Acid and following the mevalonate pathway will lead to our final product, cholesterol. This enzyme is especially important in cholesterol synthesis once it is heavily regulated by Glucagon and Insulin. Glucagon can inhibit the enzyme itself or its synthesis, while insulin has a positive influence, by inducing it. SREBPs also play an important role in cholesterol synthesis, since this transcription

factors induce the synthesis of HMG-CoA reductase, essential for cholesterol synthesis. Adapted from [113].

4.6. Cholesterol in Muscle

Skeletal muscle reports around 40% of adult total body weight and is a site of glucose and fatty acid oxidation [114]. Insulin plays an important role in this tissue once it regulates the balance between glucose and fatty acids. Members of the superfamily of ligand-dependent transcription factors were identified as fundamental regulators of the genes involved in cholesterol and lipid metabolism, the liver X receptor (LXR) and peroxisome proliferator-activated receptor (PPAR). They are transcription factors whose activity can be controlled by the direct binding of fatty acids (PPARs and LXRs) and cholesterol derivates (LXRs). Oxysterols, including CYP46A1, are activators of LXR and increase transcription of genes involved in sterol [115]. LXR under circumstances of elevated cholesterol, promote the transfer of cholesterol from the periphery to the liver for catabolism and excretion [116].

4.7. Cholesterol in Liver

The liver is the principal location for cholesterol biosynthesis, through HMG reductase (HMGR) activity. LDLR plays an important role in cholesterol homeostasis since it binds plasma LDL particles, and with that reduces plasma cholesterol levels [117]. When cells are sterol deprived, genes essential for synthesis of cholesterol are upregulated thought the action of SRE (Sterol Regulatory Elements). SREBPs recognize these sequences and work as regulators of lipid homeostasis. On the other hand, when cells have high levels of sterol, SREBPs are inactive, which means that no transcription of the genes that synthesize cholesterol occurs, and therefore sterol levels return to normality [118].

4.8. Cholesterol in Pancreas

Accumulation of cholesterol in β-cells is associated with an increase of cardiovascular risk. Pancreatic β-cells can synthesize cholesterol, using the mevalonate pathway, which controls the physical properties of the membranes, as fluidity and curvature. Cellular cholesterol accumulation in pancreas can lead to pancreatic β-cell dysfunction. A group of lipid abnormalities linked to cholesterol and fatty acid accumulation has detected in individuals with type 2 diabetes, contributing to degeneration of pancreatic islets [119].

Taking this in consideration, islet cholesterol levels affect glucose stimulated insulin secretion, independently of fatty acid levels. This implies the existence of a mechanism that links hyperglycemia and type 2 diabetes, independent of FA. It also proposes a potential target for therapeutic intervention, the regulation of cholesterol directed to preserve glucose stimulated insulin secretion in pancreatic cells. High β-cells with high cholesterol levels can also affect glucose metabolism by inhibiting glucokinase activity and reducing the efficacy of the insulin secretory apparatus [120].

Previous work

Some of the work for the silencing or overexpression of Cyp46A1 was previously performed. The animals were fed with the respective diets and weighted twice a week, during the study. The stereotaxic surgery was performed in the 4th week of study, with the purpose to deliver the vectors (AAVCyp461 and AAV-shCyp46A1) into the hypothalamus of the mice. All mice were subjected to a GTT and ITT test at the 4th and 12th week of study. The animals were sacrificed at 12 weeks. It was also performed some animal behavioral tests to assess the locomotor and anxiety-like behavior. The liver and BAT were quantified; the liver was weighted and saw the hepatic steatosis score and for BAT was measured the adipocyte area.

OBJECTIVES

Obesity is one of the most alarming health issues of this century. Diet and cholesterol metabolism, and consequently oxysterols, are at the bottom of this concern, since low HDL cholesterol levels are one of the criteria to detect the presence of metabolic syndrome. Several organs are affected by the disruption of this homeostasis, for example the liver, which is the organ responsible for the cholesterol metabolism [121]. Previous work in our laboratory investigated the impact of *in vivo Cyp46a1* gene silencing in the hypothalamus, which is important in neuronal cholesterol homeostasis (Arrulo, 2019). In that work, it was found that the reduction of Cyp46a1 levels in the hypothalamus: i) increased body weight, ii) altered food and water intake, iii) reduced glucose tolerance and insulin sensitivity, and iv) led to modifications in several metabolic organs compared to control animals.

Based on those results, the overall goal of this project was to further investigate the impact of Cyp46a1 modulation in hypothalamus in whole-body metabolism. Specifically, samples from different experimental groups (CHOW diet (Non-injected, AAVCyp46A1, AAV-shCyp46A1) and HF diet (Non-injected, AAVCyp46A1, AAV-shCyp46A1)) were analyzed in order to understand the relation between neuronal cholesterol in the hypothalamus and whole-body metabolism. From this main goal, specific aims were outlined:

1) To investigate the impact of hypothalamic Cyp46A1 modulation in the integrity of BAT.
2) To study the impact of modulation of Cyp46A1 in the hypothalamus in the integrity of the liver.
3) To investigate how the impact of Cyp46A1 modulation affects pancreas.
4) To analyze if the modulation of Cyp46A1 in the hypothalamus alters the expression levels of molecular mediators related to metabolism and inflammation.

MATERIAL AND METHODS

1. Animals, diets, and tissue collection

Both sections 1 and 2 were made by Adriana Arrulo in *Centro de Neurociências e Biologia Celular* in Coimbra. From previous work (Arrulo, 2019), C57BL/6J wild-type mice were acquired from in-house breeding and kept in the animal pathogen-free facility of the University of Algarve. In this experiment were used both male and female mice. During this time, twelve weeks old mice, with average body 20-25g, were kept about two-three animals per cage, in a controlled environment in a cycle of 12 hours light – 12 hours dark (with temperature and humidity under control, 22±2ºC).

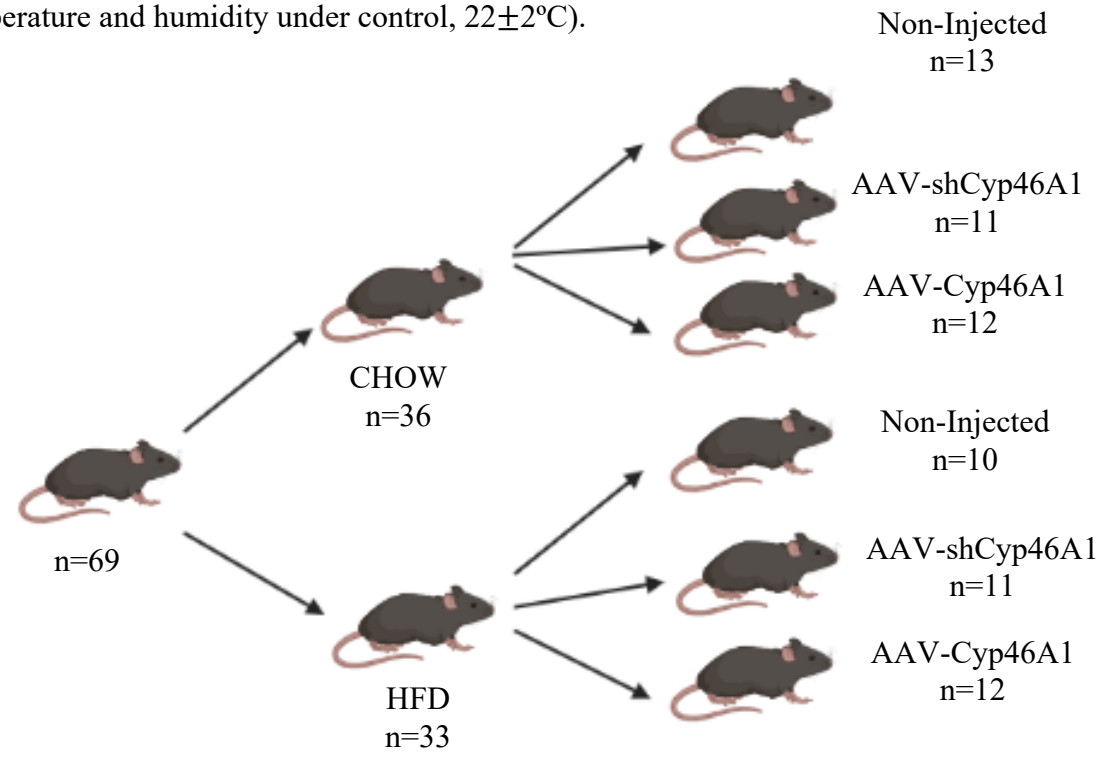

Figure 14. Schematic representation of the total cohort of animals.

The total cohort of animals (n=69) was randomly divided into two diets. One group, (n=36) was given a low-fat control diet (CHOW, D12450J, 10% fat, Research Diets, USA) and the other group (n=33) had access to a high-fat diet (HFD, D12492, 60% fat, Research Diets, USA). The mice followed the respective diets for four weeks, until the stereotaxic injection, in which were injected: AAV-shCyp46A1, to silence the expression of mouse endogenous Cyp46a1, and AAV-CYP46A1, to increase the levels of this protein in the hypothalamus. Additionally, two groups of non-injected mice were also used as control. In the total, 6 experimental groups were constituted: CHOW diet (Non-injected, AAVCyp46A1, AAV-

shCyp46A1) and HFD diet (Non-injected, AAVCyp46A1, AAV-shCyp46A1). After the injection the animals were maintained for 8 weeks before sacrifice.

The AAV vectors were produced and purified by Atlantic Gene Therapies. The AAV-shCyp46A1-GFP contained the expression cassette of a short hairpin RNA (shRNA) targeting the mouse Cyp46A1 gene, driven by a human U6 promoter and a GFP reporter gene driven by the phosphoglycerate kinase 1 (PGK1) promoter in the adeno-associated viral vector serotype 5 (AAV5) [122].

As for AAV-CYP46A1 vectors, three PCR-generated fragments containing the entire sequences of the 0,6 kb murine phosphoglycerate kinase (PGK promoter), 1,5 kb CCYP46A1 cDNA and 0,6 kb regulatory element of woodchuck hepatitis virus post-transcriptional regulatory element were cloned to produce pAAV5/PGK-hCYP46A1 plasmids. These plasmids were used to produce AAV5/5-PGK-HACYP46A1 vectors (referred as AAV5-CYP46A1). The AAV vectors were produced by transient transfection of 293T cells and purified using caesium chloride (CsCl) ultracentrifugation gradient [123].

2. Histology

a. Tissue processing

The organs and tissues previously removed were fixed in a formaldehyde solution, divided into smaller sections, and positioned in tissue processing cassettes. Dehydration of tissues was achieved through 70% ethanol (v/v) (Fisher Chemical) for 1 hour, 95% ethanol (v/v) for 45 minutes, 95% ethanol (v/v) for 40 minutes and two series of 100% ethanol (v/v) for 1 hour each. After, the clearing was performed with two series of xylene (Fisher Chemical) for 1 hour each and wax infiltration with two series of paraffin (Luso Palex) in the incubator at 56°C for 1 hour each. The cassettes (Labor Spirit) were withdrawn from the incubator and set in embedding molds (Tebu-bio), to form a block, at a chosen orientation and filled with liquid paraffin at 56°C. The cassettes containing the tissues were placed on top of the mold, to cool down and solidify. The block was removed from the mold and stored at room temperature until use.

b. Sectioning

The paraffin blocks previously prepared, were cut in paraffin sections on a HM 325 Rotary Microtome (Thermo Fisher Scientific) at room temperature. The paraffin blocks that contained adipose tissue (white or brown) were cut in paraffin sections of 4 or 5 µm and the other organs, as liver and pancreas, were cut in sections of 3 to 4 µm. The resultant sections were set into microscopy slides and stored at room temperature.

c. Hematoxylin-eosin staining

This staining was performed according to the manufacturer guidelines (Merck Millipore). Briefly, the microscope slides were deparaffinized with two series of xylene for 3 and 2 minutes and rehydrate with 100% ethanol (v/v), 95% ethanol (v/v) for 4 and 2 minutes and finally two series of distilled water for 30 seconds each. The sections were stained with a hematoxylin solution modified (according to Gill III, Merck Millipore) for 30 seconds, washed in distilled water two times, for 2 and 1 minute, respectively. The sections were counterstained with 0,5% aqueous eosin Y solution for 1 minute and washed over again, in two series of distilled water for 1 minute each, and two series of xylene for 2 minutes. The sections dry out and set with Richard-Allan Scientific Mounting Medium (HM325, Thermo Fisher Scientific) and microscopy slide cover slips. With this type of staining, the cells nuclei will stain in dark purple, and the cells cytoplasm will stain in pink.

3. Microscopy image acquisition

The liver, pancreas and BAT sections from the different experimental groups that were previously processed were used to acquire images through the microscopy Zeiss Axio Imager Z2 (Microscopy Core Unit). The lenses used to accomplish the images were 10x and 20x with help of a camera AXIOCAM-ICC3, associated with the software AxioVision. The quantification of the images obtained were performed with Fiji. The BAT images were analyzed to calculate the adipocytes area and respective mean (both made with tools from Fiji). In the pancreas images was measured the pancreatic islets area and in the liver was measured the histological score, relative to the presence of steatosis or other hepatic anomalies. Ballooning was scored from 0-2: 0 for normal hepatocytes (cuboidal shape), 1

for presence of clusters of hepatocytes with a rounded shape and at last, 2 for the same grade as 1 but with enlarged hepatocytes (at least 2-fold) [124].

4. Protein extraction

The protocol for protein extraction was performed together with RNA extraction and begun with the addition of 1100 μL of QIAzol to the hypothalamus samples and then was homogenized with a syringe and needle. Then the samples were mixed for 30 seconds and set to homogenate for 5 minutes. Later, was added 200 μl of d-chloroform to the homogenate and mixed for 15 seconds. The homogenate rested for 2-3 minutes at room temperature and then was centrifugated at 12000 RPM for 15 minutes at 4ºC. The aqueous phase was removed (and used for the RNA extraction), while to the interphase and phenol phase was added 300 μl of 100% ethanol. The samples were mixed carefully and incubated at room temperature for about 2-3 minutes. After, the samples were centrifugated at 2000g for 2 minutes at 4ºC. The resulted supernatant with the protein fraction was divided into two 15 ml tubes, and then it was added 1,5 ml of isopropanol to precipitate the protein and mixed through inversion for 15 seconds. The total volume was separated into two 1,5 ml tubes and incubated at room temperature for 10 minutes. After that, the samples were centrifugated at 12000G for 10 minutes at 4ºC and the supernatant was removed. To each tube containing the pellet with the protein it was added 1ml of guanidine-ethanol solution 3M and let to incubate at room temperature for 20 minutes. After, the samples were centrifugated at 7500G for 5 minutes at room temperature and the supernatant was removed. The previous steps (addition of guanidine-ethanol, incubation, centrifugation, and removal of supernatant) were repeated 3 times. When these steps were completed, to each sample was added 1ml of 100% ethanol, then vortex and incubated at room temperature for 20 minutes. After, the samples were centrifugated at 7500G for 5 minutes at room temperature, the supernatant was removed, and the pellet were let air dried for 10 minutes. When dried, it was added 40 μl of Urea/DTT 10 M solution to each pellet and homogenized. At the end, the volume of duplicates was joined together and incubated at room temperature for 1 hour. Finally, the samples were incubated at 95ºC for 3 minutes, sonicated and stored at -80ºC.

5. RNA extraction

The aqueous phase resultant from the samples of protein extraction was used to extract the RNA. The RNA was extracted using the NZYTech™ RNA Isolation kit, according to the manufacturer's instructions. RNA concentration and purity were determined using Nanodrop Spectrophotometer (Thermo Fisher Scientific). RNA was stored at -80ºC until further analyses.

6. cDNA Synthesis

The cDNA was synthesized using 1µg of the previous extracted RNA, according to the kit iScriptTM cDNA Synthesis (Bio-Rad) instructions. In order to produce the cDNA, the mix containing the RNA, was inserted in the C1000 Touch Thermal Cycler (Bio-Rad) 5 minutes at 25ºC, 30 minutes at 42ºC, 5 minutes at 85ºC and infinite hold at 12ºC. cDNA was stored at 4ºC until further analyses.

7. RT-qPCR

The Real Time quantitative PCR was performed according to the kit SsoAdvancedTM SYBR® Green SuperMix (Bio-Rad). The protocol used to perform the quantitative PCR consisted in 95ºC for 30 seconds, then 40 cycles of two steps: 95ºC for 5 seconds and 60ºC for 5 seconds. After that, started the melt curve of 57ºC for 5 seconds with increase of 0,5ºC until 90ºC, and finally 12ºC forever. The results of every plate were analyzed through the BioRadCFX Software Management (Bio-Rad) where was able to see the efficiency of every primer.

The primers used were designed from the Invitrogen (ThermoFisher Scientific) and the dilutions used are described in Table 4.

Table 4. Primers and respective dilution used in the RT-qPCR.

The primers were diluted in DNAse free water, accordingly to the dilution that fitted better to the tissue that was analyzed (in this case hypothalamus). All primers were used to observe the differences in genic expression except the HPRT that was used as housekeeping gene. Sequences of the different primers are in Annex 2.

Primer	Dilution	Concentration
Human CYP	1:20	100 µM
Mouse CYP	1:20	
Mouse POMC	1:20	
Mouse AGRP	1:20	
Mouse TNF-α	1:10	
Mouse HPRT	1:20	

8. Statistical Analysis

To perform the statistical analysis and graphics it was used the GraphPad Prism 8 (GraphPad Software Prism) and the results obtained were expressed in mean ± SEM. To compare two groups was used Unpaired t-Student test with outliers identified by Grubb's test (alfa=0,05). The comparison of three groups or more was used the One-Way ANOVA with Bonferroni's multiple comparison test. Statistical significance defined as $p<0,05$ – [p-value <0,05 (*), p-value <0,01 (**), p-value <0,001 (***), p-value <0,0001 (****)].

RESULTS

1. Silencing Cyp46A1 in the hypothalamus leads to a reduction in its expression levels in C57BL/6J mice.

Cyp46A1 function in the hypothalamus and its impact in whole body-metabolism was the focus in this study. For that, it is essential to verify if the silencing or overexpression strategies were able to modify Cyp46A1 levels. To accomplish that, we realized RT-qPCR aiming to access the levels of Cyp46A1 in the hypothalamus in the different experimental groups. In the Non-Injected animals, it is possible to see a slight decrease in animals fed with HF diet when compared to the ones with CHOW diet, however it is not significant. **(Figure 19 A:** CHOW Non-Injected *n=6 vs* HF Non-Injected *n=6***)**. When compared the Non-Injected animals to the ones with silencing Cyp46A1, it is possible to see a decrease in the Non-Injected animals compared to the silenced Cyp46A1 animals, with no distinction of the diets. **(Figure 19 B:** CHOW Non-injected *n=6,* HFD Non-Injected *n=6 vs* CHOW AAV-shCyp46A1 *n=6,* HFD aav-shCyp46A1 *n=6*). It was also performed an semi-quantitative RT-PCR with AAVCyp46A1 animals, with no housekeeping gene. Since it was an exogenous gene (human form) that we were trying to express, we performed an electrophoresis gel to see if the overexpression was successful **(Annex 2)**. The result showed an effective overexpression of Cyp46A1.

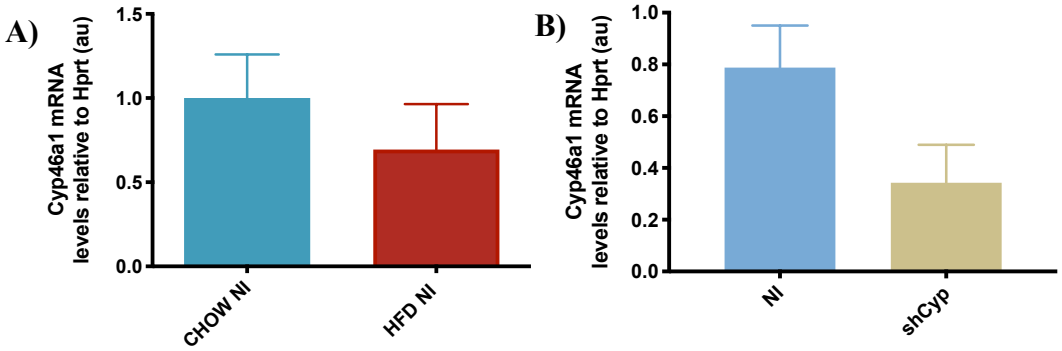

Figure 15. Cyp46A1 mRNA levels in the hypothalamus of the different experimental groups.

(A) Non-injected animals with an HF diet showed a small non-significant decrease in the Cyp46A1 mRNA levels compared to control group. [CHOW non-injected *n=6 vs* HFD non-injected *n=6* – p-value=0,4373] **(B)** The silencing of Cyp46a1 leads to a reduction (non-significant) in the Cyp46a1 mRNA levels compared to Non-injected AAV-shCyp46A1 animals. Both diets were grouped. [CHOW non-injected *n=6,* HFD non-injected *n=6 vs* CHOW AAV-shCyp46A1 *n=6,* HFD AAV-shCyp46A1 *n=6* – p-value=0,0899]. [Unpaired t-students Test].

2. Modulation of Cyp46A1 levels in the hypothalamus leads to alterations in the POMC mRNA levels in C57BL/6J mice.

POMC is one of the anorexigenic neuropeptides in the ARC and has a variety of functions. Among them all, the most important roles are on the whole-body metabolism, as POMC can control appetite since the stimulation of this neurons promotes satiety [125]. Therefore, alterations in their normal functioning could lead to obesity phenotype and some disorders, like type 2 diabetes. For that, we next accessed POMC levels in all experimental groups. In the group of Non-injected, animals with HF diet revealed higher POMC mRNA levels, when compared to the control group, although the difference was not significant **(Figure 20 A)**. In the AAVsh-Cyp46A1 groups, both diets revealed higher, but non-significant mRNA levels, when compared to the Non-Injected animals within the same diet **(Figure 20 B-C)**. In the animals with overexpressed Cyp46A1, it is possible to see a decrease in POMC mRNA levels with HF diet, compared to the ones with CHOW diet, although the difference did not reach statistical significance **(Figure 20 D)**.

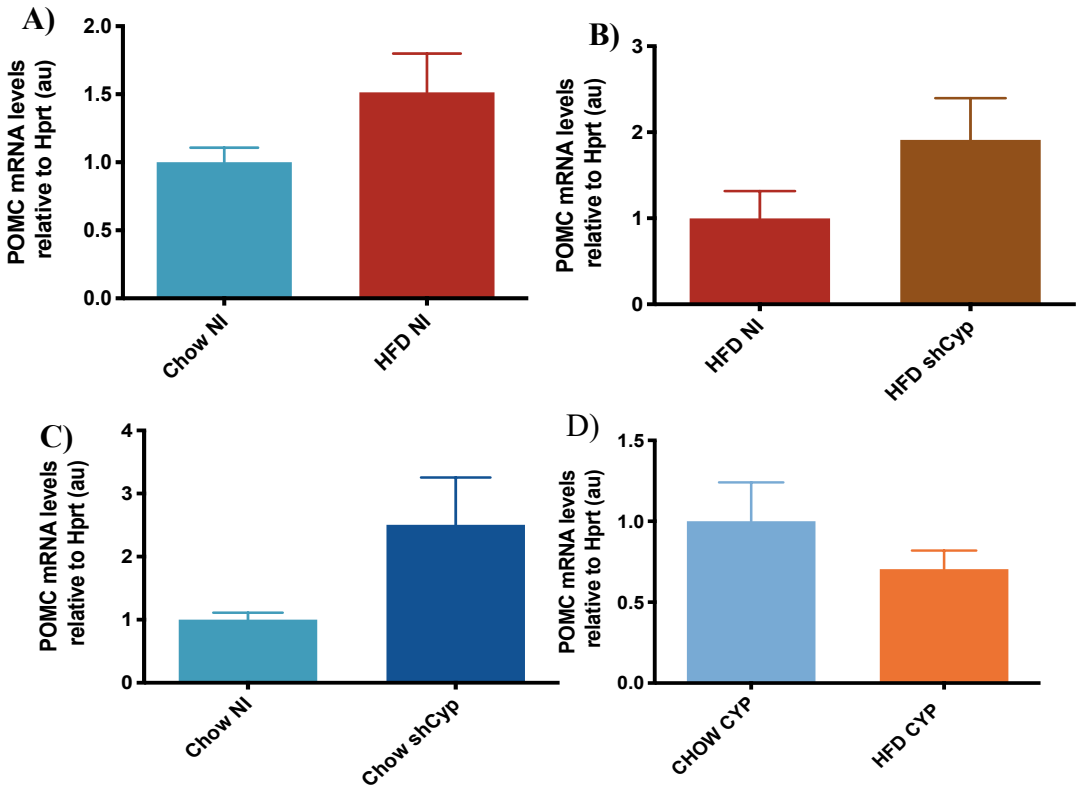

Figure 16. POMC mRNA levels in the hypothalamus of the different experimental groups.

(A) The HFD Non-injected animals showed higher non-significant levels of POMC mRNA levels compared to the CHOW Non-injected animals. [CHOW non-injected *n=6 vs* HFD non-injected *n=6* – p-value=0,0893] **(B)** In HFD animals, the silencing of Cyp46A1 also leads to a non-significant increase in the POMC levels. [HFD AAV-shCyp46A1 *n=6* – p-value=0,1670]**. (C)** The comparison between CHOW Non-injected *vs* CHOW AAV-shCyp46A1 revealed non-significant higher levels of POMC mRNA levels in the group with the silenced gene. [CHOW AAV-shCyp46A1 *n=6* – p-value=0,1066]. **(D)** The overexpression of Cyp46A1, revealed a decrease in the POMC mRNA levels in animals with HF diet, compared to the ones with CHOW. [CHOW AAVCyp46A1 *n=6 vs* HFD AAVCyp46A1 *n*=6 – p-value=0,6752] Data is expressed as mean ± SEM. Unpaired t-students Test.

3. Modulation of Cyp46A1 levels in the hypothalamus leads to alterations in the NPY mRNA levels in C57BL/6J mice.

NPY is an orexigenic neuropeptide produced in the ARC. Like POMC, it has the ability to control the whole-body metabolism, but instead of leading to satiety, it increases food intake and storage of energy as fat. NPY have also another functions, like lowering blood pressure, reducing stress and affects the circadian rhythm [126]. As it is situated in the ARC, alterations in the signaling pathway could lead to increasing food intake resulting in obesity. For that, we next analyzed the NPY levels in the different experimental groups. In the Non-Injected animals, the HF diet leads to a significant reduction in the NPY levels compared to the control group **(Figure 21 A:** CHOW Non-Injected *n=6 vs* HFD Non-Injected *n=6*, p-value=0,0409**)**. In the AAVsh-Cyp46A1 groups, the silencing of Cyp46A1 leads to a significant reduction in the NPY levels compared to Non injected animals **(Figure 21 B-C**; HFD Non-Injected *n=6 vs* HFD AAV-shCyp46A1 *n=6*, p-value=0,0275; CHOW Non-Injected *n=6 vs* CHOW AAV-shCyp46A1 *n=6*, p-value=0,0074**)**. Overexpression of Cyp46A1 resulted in the increase of NPY mRNA levels in animals with HF diet in comparison with CHOW diet **(Figure 21 D** – CHOW AAVCyp46A1 *n=6 vs* HFD AAVCyp46A1 *n=6*, p-value=0,0366**)**.

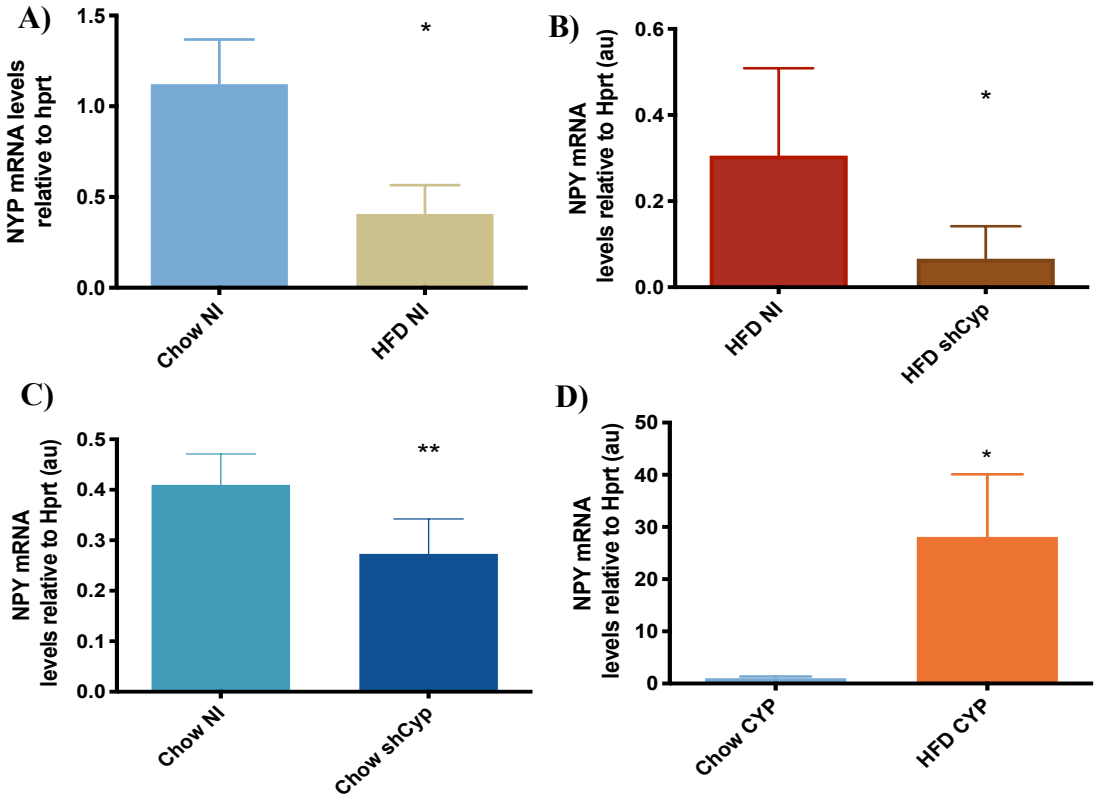

Figure 17. NPY mRNA levels in in the hypothalamus of the different experimental groups.

(A) In the Non-Injected animals, the HF diet showed a significant reduction in NPY levels, when compared to the animal fed with CHOW diet. [CHOW non-injected *n=6 vs* HFD non-injected *n=6*] – p-value=0,0409. **(B)** In the HF diet animals, the silencing of Cyp46A1 presented a significant reduction in the NPY levels when compared to the Non-Injected animals. [HFD AAV-shCyp46A1 *n=6*] – p-value=0,0275**. (C)** It is possible to see the same outcome in the CHOW diet compared to the HFD. The AAV-shCyp46A1 animals fed with CHOW diet showed a significant decrease in NPY levels in comparison to the control group. [CHOW AAV-shCyp46A1 *n=6*] – p-value=0,0074**. (D)** In the AAVCyp4A1 group it is possible to see a significant increase in NPY levels in animals fed with HF diet in comparison to CHOW diet. [CHOW AAVCyp46A1 *n=6 vs* HFD AAVCyp46A1 *n=6*] – p-value=0,0366. Data is expressed as mean ± SEM. [p-value <0,05 (*), p-value < 0,01 (**) – Unpaired t-students Test].

4. Modulation of Cyp46A1 levels in the hypothalamus leads to alterations in the TNF alpha mRNA levels in C57BL/6J mice.

Tumor Necrosis Factor alpha (TNF alpha) is a pro-inflammatory cytokine that is essential for the immune system during inflammation, abnormal cell proliferation, differentiation, and apoptosis [127]. Although macrophages and T-cells are the major producers of this cytokine, it can also be secreted by adipose tissue. Obesity is connected to type 2 diabetes, as adipose tissue secretes proteins (adipokines) that affect insulin sensitivity and for that, adipose tissue is now considered a contributor to insulin resistance. Recently, obesity was classified as a chronic state of low-intensity inflammation and so TNF alpha is the link between obesity and insulin resistance, due to the generality of type 2 diabetic person are obese and this cytokine is highly expressed in adipose tissue of obese subjects [128]. TNF alpha can also modulate the expression of orexigenic/anorexigenic neurotransmitters [129]. Therefore, we next assessed the levels of TNF alpha in the hypothalamus of the animals of the different experimental groups. In the control group, Non-injected animals with a HF diet presented higher mRNA levels of TNF alpha when compared to the CHOW diet animals, although the difference was not statistically significant **(Figure 22 A)**. Concerning the Cyp46A1 silencing, it was observed that animals in the HF diet group presented significantly higher levels that Non injected animals, whereas in the CHOW diet it was observed the contrary **(Figure 22 B-C** – HFD Non-Injected *n=6 vs* HFD AAV-shCyp46A1 *n*=6, p-value=0,0011; CHOW Non-Injected *n=6 vs* CHOW AAV-shCyp46A1 *n=6*, p-value=0,0469**)**. On the other hand, the overexpression of Cyp46A1 significantly increased the TNF alpha mRNA levels in the HF diet group, compared to CHOW diet animals **(Figure 22 D** – CHOW AAVCyp46A1 *n=6 vs* HFD AAVCyp46A1 *n=6*, p-value=0,0180**)**.

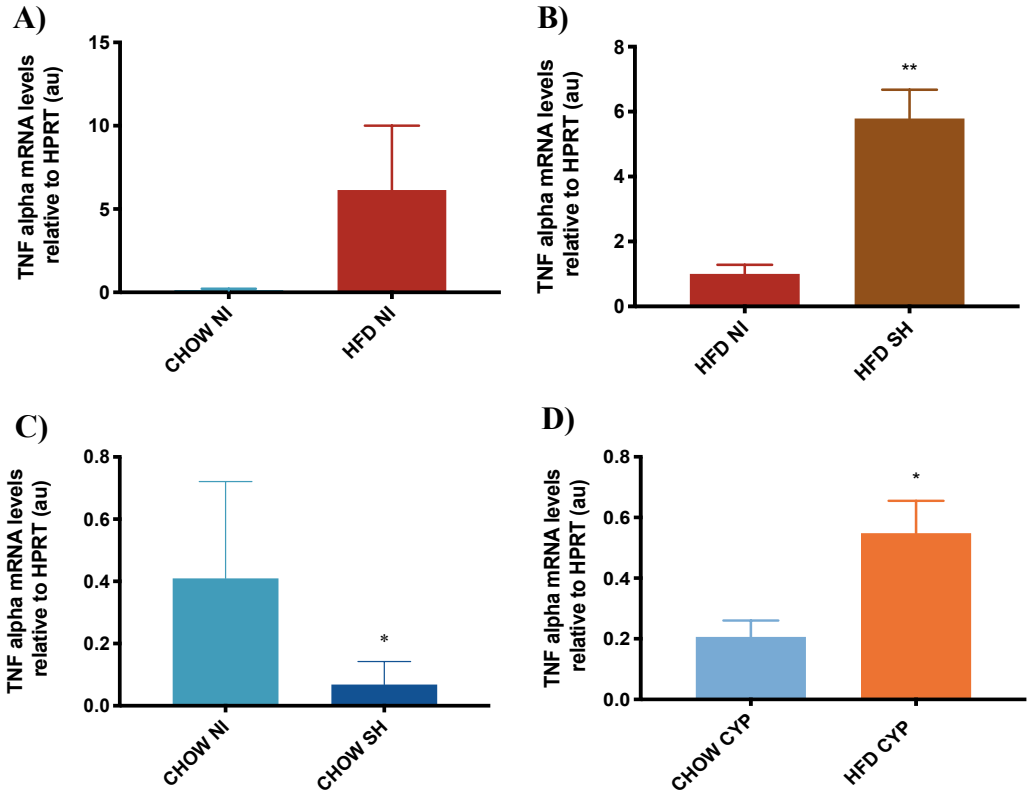

Figure 18. TNF-alpha mRNA levels in the hypothalamus of the different experimental groups.

(A) Although the difference is not statistically significant, it is possible to observe higher mRNA levels of TNF alpha in the animals with HF diet in comparison to the animals fed with CHOW diet, the control group. [CHOW non-injected *n=6 vs* HFD non-injected *n=6*] **(B)** In the Cyp46A1 silencing group, the animals with HF diet presented significantly higher levels of TNF alpha in comparison to the Non-injected. [CHOW AAV-shCyp46A1 *n=6*] - p-value=0,0011. **(C)** On the other hand, in the CHOW diet, animals with silenced Cyp46A1 present lower TNF alpha levels, compared to the Non-injected. [HFD AAVCyp46A1 *n=6*] - p-value=0,0469. **(D)** The overexpression of Cyp46A1 significantly increased the TNF alpha levels in the HF diet animals in comparison to CHOW diet animals. [CHOW AAVCyp46A1 *n=6 vs* HFD AAVCyp46A1 *n=6*] - p-value=0,0180. Data is expressed as mean ± SEM. [p-value <0,05 (*), p-value < 0,01 (**) - Unpaired t-students Test].

5. Silencing of Cyp46A1 gene in the hypothalamus induces the formation of lipid droplets in the liver of the C57BL/6J mice fed with CHOW and HF diet.

Cholesterol is fundamental for neuronal homeostasis and so its metabolism is tightly controlled. The brain uses a mechanism to export cholesterol, where it is converted to oxysterol (24OHS) by CYP46A1 and set in circulation. As 24OHS reaches the liver, is converted into intermediates to produce bile acids [130]. Some aspects of brain function depend on cholesterol turnover, like dendrites development, neuronal repair, and formation of axons. Neurons in hippocampus, after silencing Cyp46A1, presented higher cholesterol levels, leading to apoptotic death of neurons [131]. In this study, C57BL/6J wild type mice (n=69) were divided and subjected into two different diets, where 36 animals followed a low-fat control diet (CHOW, containing 10% of fat), and 33 animals had access to a high fat diet (HFD, containing 60% of fat). After 12 weeks following the respective diets (and 8 weeks after the stereotaxic injection), non-injected animals within CHOW diet presented normal hepatocytes with a single nucleus **(Figure 16 A)**. On the contrary, the Non-Injected animals with HF diet, it shows signs of hepatocyte ballooning, as its possible to see lipid droplets **(Figure 16 B, G** - CHOW Non-Injected *n=13 vs* HFD Non-Injected *n=10*, p-value=0,0128; CHOW Non-Injected *vs* CHOW AAV-shCyp46A1 *n=11*, p-value<0,0001; CHOW Non-Injected *vs* HFD AAV-shCyp46A1 *n=11*, p-value <0,0001**)**. Interestingly, the group of animals with silenced Cyp46A1 gene, presented an increase in lipid droplets, in both diets. In AAV-shCyp46A1-injected animals with CHOW diet, lipid droplets are present in greater higher number and size when compared to the HF diet animals **(Figure 16 C-D, H** – (CHOW Non-Injected *vs* CHOW AAV-shCyp46A1 *n=11*, p-value=0,0003)**)**, which could suggest steatosis (deposition of fat in the liver). In the AAV-Cyp46A1-injected animals, the hepatocytes showed a normal size, however, in the HF diet animals it is possible to observe some fat deposits, although in reduced number and comparable to the NI animals with CHOW diet **(Figure 16 E).**

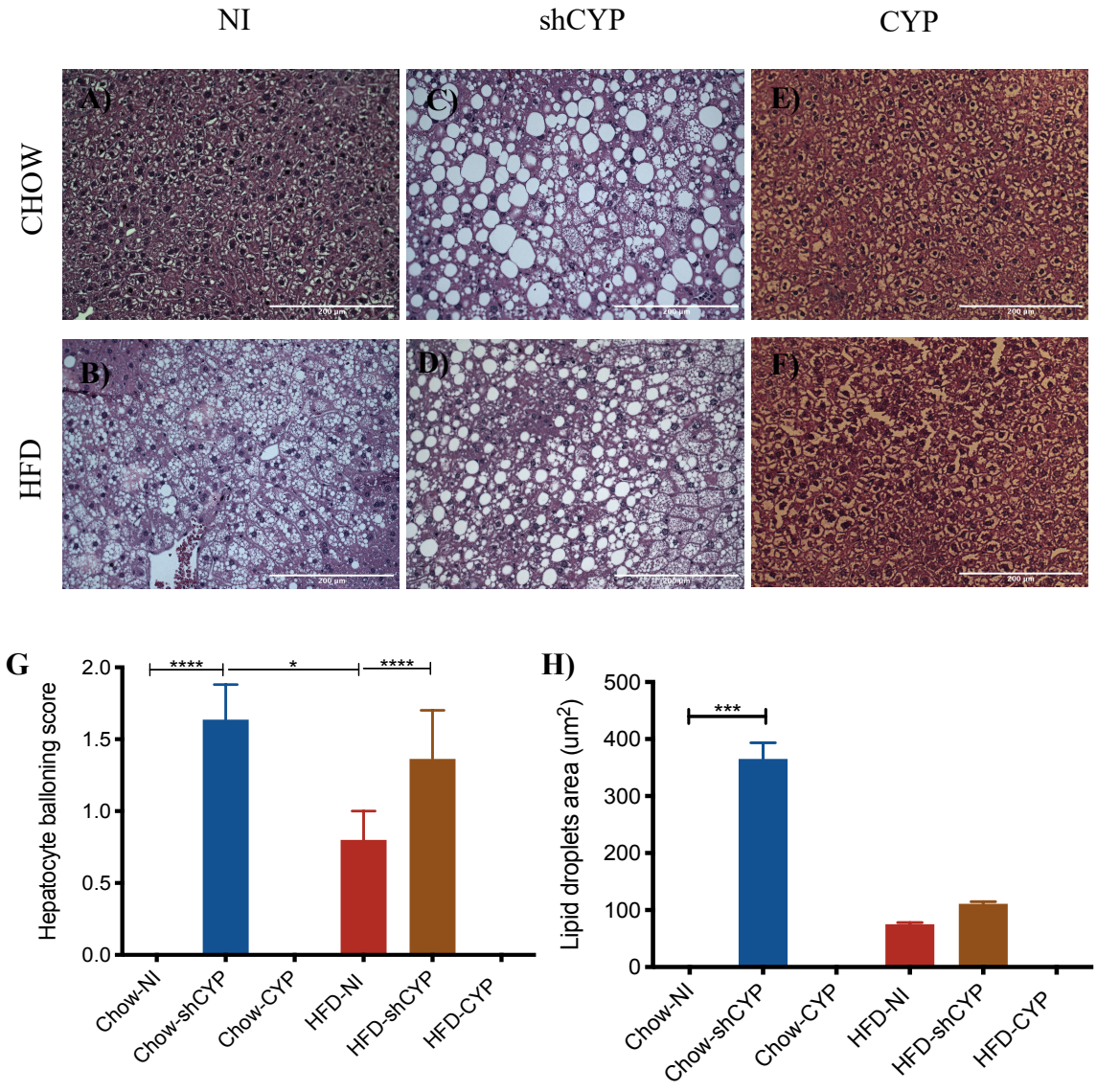

Figure 19. Silencing Cyp46A1 gene in the hypothalamus induces lipid droplets in the C57BL/6J mice fed with CHOW and HF diet.

The liver samples were histologically processed, and the hematoxylin-eosin (H&E) staining was performed previously. Representative images are shown in A-F panels. (**A and B)** In the non-injected animals, it is clear to observe a difference in both images, since the HFD animals show lipid droplets and hepatocyte ballooning when compared to CHOW animals. [CHOW non-injected *n=13* versus HFD non-injected *n=10*]. **(C and D)** The CHOW AVV-shCyp46A1 animals show more lipid droplets and larger, when compared to the HFD AAV-shCyp46A1 animals. [CHOW AVV-shCyp46A1 *n=11* versus HFD AAV-shCyp46A1 *n=11*]. **(E and F)** The AAV-Cyp46A1 injected animals resulted in small differences between them, showing no signals of lipid droplets or hepatocyte ballooning score, even in the HFD group [CHOW AAV-Cyp46A1 *n=12* versus HFD AAV-Cyp46A1 *n=11*]. **(G)**

Increase of hepatocyte ballooning score on the animals where the Cyp46A1 gene was silenced in both diets and in animals non-injected with HFD diet, as compared to AAV-Cyp46A1 injected animals. CHOW Non-Injected *n=13 vs* HFD Non-Injected *n=10*, p-value=0,0128; CHOW Non-Injected *vs* CHOW AAV-shCyp46A1 *n=11*, p-value<0,0001; CHOW Non-Injected *vs* HFD AAV-shCyp46A1 *n=11*, p-value <0,0001 **(H)** Significant increase in the lipid droplet area in the silenced animals with CHOW diet when compared to the other groups. No presence of lipid droplets was detected in non-injected animals with CHOW diet and overexpressed Cyp46A1 gene in both diets. (CHOW Non-Injected *n=13 vs* CHOW AAV-shCyp46A1 *n=11*, p-value=0,0003). **Scale bar: 200 μm**. Data is expressed as mean ± SEM. [p-value <0,05 (*), p-value < 0,001 (***), p-value <0,0001 (****) – One-Way ANOVA with Bonferroni's test].

6. Modulation of Cyp46A1 expression in the hypothalamus induces a diet dependent increase in the area of the Langerhans islets in the pancreas of C57BL/6J mice.

Alterations in diet, especially the one that leads to obesity, promotes insulin resistance. Insulin is secreted by islets present in the pancreas, called pancreatic islets (or Langerhans islets). Pancreatic islets are made up of endocrine cell types that have one function in common, maintain glucose homeostasis. Besides insulin, which is secreted by β-cells, these islets also include another hormone secreting cells: α-cells that produces glucagon, δ-cells that produce somatostatin, PP-cells that produce the pancreatic polypeptide and finally the ε-cells that produce ghrelin [132]. The conjunct of all these cells forms the Langerhans islets, and their size is related to function, as smaller islets secrete more insulin than larger islets [133]. For that reason, it is important to quantify the area of pancreatic islets. In Non-Injected animals, in both diets the pancreatic islets have a normal size, with an average diameter ranging from 100 μm to 500 μm [134] **(Figure 17 A-B)**. However, in Cyp46a1 silencing conditions, it is visible, in the animals with HF diet, an increase in the size of pancreatic islets **(Figure 17 G** – (CHOW Non-Injected *n=13 vs* HFD AAV-shCyp46A1 *n=11*, P-value=0,0022)**)**. This phenomenon is called hypertrophy, where the content of these islands increases, and so their area **(Figure 17 D)**. These signs are also visible in the AAV-Cyp46A1 animals with Chow diet **(Figure 17 H** – (CHOW Non-Injected *n=13 vs* CHOW AAVCyp46A1 *n=9*, P-value<0,0001)**)**, where it is clear to see an increase in the area of the islets and deformation (hypertrophy) **(Figure 17 E)**. In AAV-Cyp46A1 animals with HF diet, the pancreatic islets showed a normal size and form **(Figure 17 F)**.

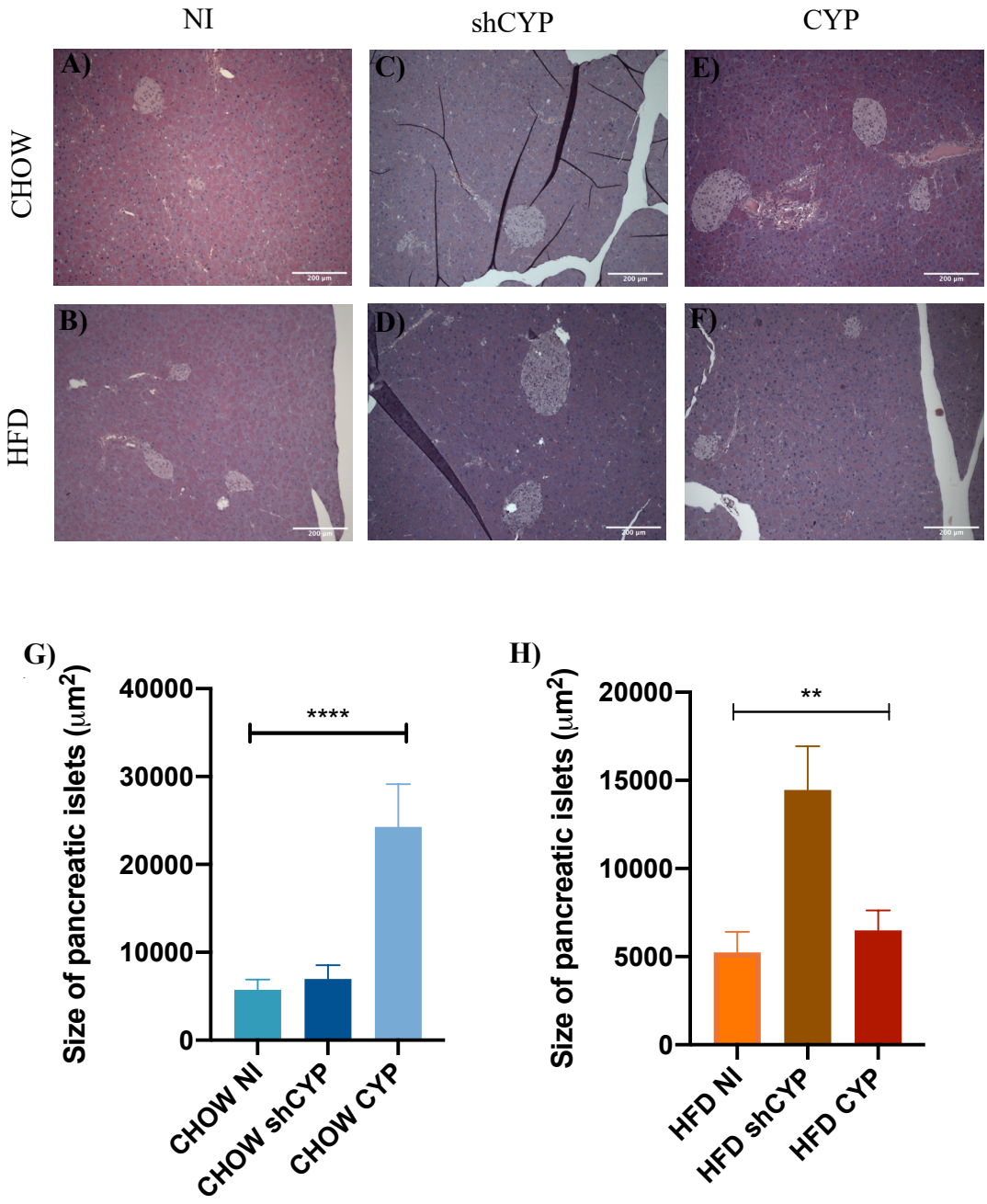

Figure 20. Modulation of Cyp46A1 in the hypothalamus induces an increase in the area of the Langerhans islets in the pancreas.

The pancreatic samples were histologically processed, and the hematoxylin-eosin (H&E) staining was performed previously. Representative images are shown in A-F panels. **(A and B)** The islet of Langerhans showed a normal sized in the non-injected animals both in CHOW diet and HF diet. [CHOW non-injected *n=13* versus HFD non-injected *n=10*]. **(C and D)** The HFD AAV-shCyp46A1 animals showed an increase in the size of pancreatic islets when compared to the CHOW AAV-shCyp46A1 animals, a phenomenon called islet hypertrophy. [CHOW AVV-shCyp46A1 *n=11* versus HFD AAV-shCyp46A1 *n=11*] **(E**

and F) The CHOW AAVCyp46A1 presented islet hypertrophy, an increase in the area of the islets of Langerhans and consequently β-pancreatic cells mass content, when compared to the HFD AAVCyp46A1 animals. [CHOW AAVCyp46A1 *n=12* versus HFD AAVCyp46A1 *n=11*]. **(G)** The CHOW AAVCyp46A1 animals showed a significant increase in the area of β islet cells comparatively to the other CHOW groups (CHOW Non-Injected *n=13 vs* CHOW AAVCyp46A1 *n=9*, p-value<0,0001). **(H)** Silencing of Cyp46A1 in animals fed with HF diet showed an increase in the area of pancreatic islands in comparison to the other groups with the same diet (HFD Non-Injected *n=13 vs* HFD AAV-shCyp46A1 *n=11*, p-value=0,0022). **Scale bar: 200 μm**. Data is expressed as mean ± SEM. [p-value < 0,01 (**), p-value <0,0001 (****) – One-Way ANOVA with Bonferroni's test].

7. Silencing Cyp46A1 gene in the hypothalamus induces hypertrophy of BAT adipocytes in CHOW and HF diets in C57BL/6J mice

Brown Adipose Tissue is formed by brown adipocytes that possess several small lipid droplets (multilocular) enriched with mitochondria. The principal function of this tissue is to transfer energy from food into heat (thermogenic capacity) [135]. The HF diet alters BAT normal structure, as Non-Injected animals showed an increase in lipid droplets, and therefore in the size of the adipocytes (**Figure 18 G** – Chow Non-injected *n=12* vs HFD Non-Injected *n=10*, p-value<0,0001**)**. In AAVsh-Cyp46A1 animals it is possible to observe BAT hypertrophy **(Figure 18 B)**. The lipid droplets appear to be unilocular and have an increase in size and deformation. This phenomenon is called "BAT whitening", as this aspect is seen in white adipose tissue **(Figure 18 C-D)**. In AAV-Cyp46A1 animals with CHOW diet, this phenomenon is also visible (**Figure 18 E**) although in the animals with HF diet the lipid droplets appear smaller, when compared to the AAV-shCyp46A1 group (**Figure 18 F and G** – CHOW Non-Injected *n=12* vs HFD AAVCyp46A1 *n=10*, p-value<0,0001).

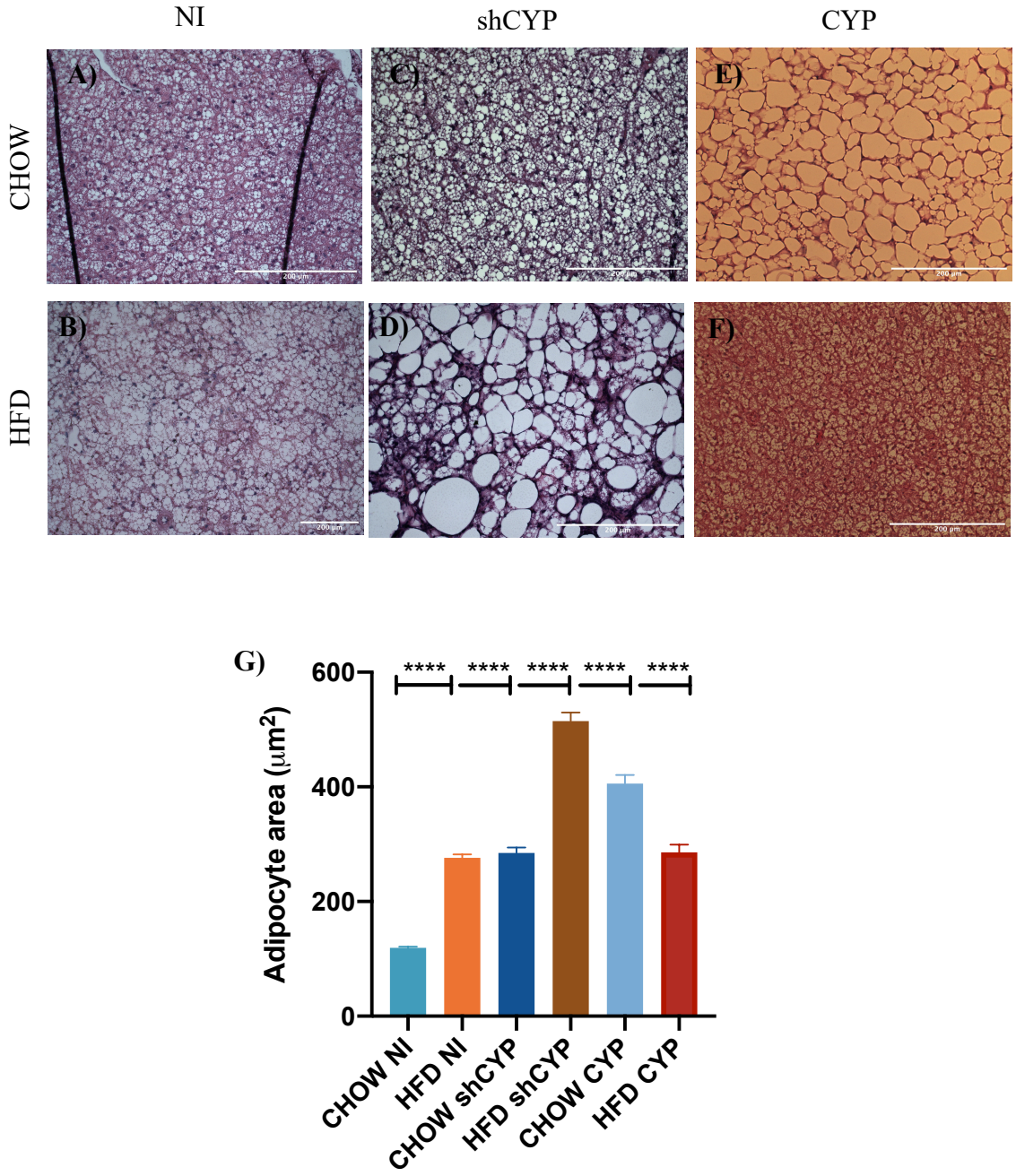

Figure 21. Silencing Cyp46A1 gene in the hypothalamus induces hypertrophy of BAT adipocytes in CHOW and HF diet.

The Brown Adipose Tissue samples were histologically processed, and the hematoxylin-eosin (H&E) staining was performed previously. Representative images are shown in A-F panels. **(A)** The CHOW Non-injected animals showed the characteristic phenotype of brown adipocytes, multiple small lipid droplets and large number of mitochondria. [CHOW non-injected n=12] **(B)** The HFD Non-injected animals, already show an increase of the lipid droplets size. [HFD non-injected n=10] **(C and D)** In both diets with the silencing of

Cyp46A1, leads to an increase in the size of lipid droplets and approaching the morphology of unilocular cells. [CHOW AAV-shCyp46A1 n=11 vs HFD AAV-shCyp46A1 n=9] **(E)** The CHOW AAVCyp46A1 animals also showed signals of "BAT whitening" although with smaller lipid droplets when compared to the AAV-shCyp46A1. [CHOW AAVCyp46A1 n=12] **(F)** The HFD AAVCyp46A1 animals presented the typical sings of Brown Adipose Tissue, very similar to the CHOW Non-injected animals. [HFD AAVCyp46A1 n=10]. **(G)** All groups presented statistically increases in adipocyte area when compared to the CHOW Non-Injected animals, where AAV-shCyp46A1 animals with HF diet showed the greatest increase. CHOW Non-Injected n=12 vs HFD Non-injected n=10, p-value <0,0001; CHOW Non-Injected vs CHOW AAV-shCyp46A1 n=11, p-value<0,0001; CHOW Non-Injected vs HFD AAV-shCyp46A1 n=9, p-value<0,0001; CHOW Non-Injected vs CHOW AAVCyp46A1 n=12, p-value<0,0001; CHOW Non-Injected vs HFD AAVCyp46A1 n=10, p-value <0,0001. **Scale bar: 200 μm.** Data is expressed as mean ± SEM. [p-value<0,0001 - One-Way ANOVA with Bonferroni's test].

DISCUSSION

Oxygen and nutrients are delivered to the entire body with the help of blood vessels. They travel the whole body, from the heart to every tissue and organ. However, the blood vessels that vascularize the brain have special properties, as they can regulate the movement of molecules, ions and cells between the blood and the brain, the so-called Blood Brain Barrier (BBB). This barrier protects neural tissue from toxins and pathogens and allows the proper neuronal function [136]. The brain is the organ that possess the highest content in cholesterol when compared to the rest of the organs, containing around of 20% of whole-body cholesterol. Due to the existence of the BBB, cholesterol cannot pass from blood circulation to the brain, neither from the brain to the blood. Therefore, the brain has its own cholesterol metabolism [137]. Cholesterol synthesis in the brain is only by *de novo* synthesis, and to maintain the homeostasis, it must also be excreted. As lipoproteins cannot enter the brain, they cannot also exit from it, so cholesterol must be converted to oxysterols, which consists in oxygenated derivates of cholesterol [138]. Cholesterol is converted to 24-S-hydroxycholesterol (24S-HC) by cholesterol 24-hydroxylase, encoded by Cyp46A1 [131]. Although Cyp46A1 can be expressed in a variety of locations, such as muscle tissues and endocrine tissues, the majority of its expression is in the brain, more specifically in the cerebral cortex, basal ganglia, and hypothalamus [139]. The arcuate nucleus, present in the hypothalamus, is responsible for regulating energy homeostasis and metabolism. It has the ability to sense the nutrients of the organism and incorporate hormonal signals in order to regulate food intake and energy expenditure. This nucleus is located near to the median eminence, an organ enriched with capillaries, leading to a permeable BBB [140]. Since the BBB near the arcuate nucleus is more permeable, it makes possible the expulsion of cholesterol from the brain to the liver, in order to be metabolized.

To get a better perception on the influences of silencing or overexpressing CYP46A1, we investigated the expression of several metabolic mediators in the hypothalamus. Cyp46A1 is fundamental for brain cholesterol metabolism and since its study was the main goal this project, we evaluated its mRNA levels in the different experimental conditions. The results showed a decrease in the mRNA levels of HF diet in Non-Injected animals in comparison to the control group. A study by Xiaorui Fan *et. al.* (2021) showed that diet influenced Cyp46A1 levels. Mouse fed with a diet rich in fats resulted in an increase in 24-OHC but a loss of the Cyp46A1 enzyme, which suggested that cholesterol synthesis might be improved or that elimination might be suppressed [141]. The results obtained agree. Since

the diet could indeed influence the Cyp46A1 levels, the next step was to observe the overall mRNA levels, without influence of the diet. As expected, the levels of AAV-shCyp46A1 are inferior compared to the Non-Injected animals. When both diets are combined, they do not influence the overall result, although this one is non-significant. This result proved that the silencing of Cyp46A1 gene was successful. The injection of an exogenous Cyp46A1 (human gene) was successfully accomplished, as it was possible to observe in an electrophoresis gel.

Next, and since the stereotaxic injections were conducted in the ARC, we evaluated the levels of neuropeptides expressed in this region. POMC is one of the anorexigenic neurons present in the ARC. Although this neuropeptide has a fundamental role in stress response and sexual function, the most important function is the control of food intake and appetite regulation. POMC has an important feature, the melanocortin system. The peptides resultant includes adrenocorticotrophic hormone (ACTH), and melanocyte-stimulating hormones (MSH) [142]. This system promotes anorexia and weight loss so overfeeding should be compensated. A study showed that food restriction reduced hypothalamic POMC mRNA expression while overfeeding increased hypothalamic POMC mRNA expression [143].

Although not significant, it is possible to observe a decrease in POMC mRNA levels in AAV-shCyp46A1 animals, in both diets. The opposite is showed at AAVCyp46A1 animals, with an increase in mRNA levels. On the other hand, when Cyp46A1 is overexpressed, resulted in a decrease in POMC mRNA levels, in contrast with silencing. These results suggest that the mainly reason is not diet, but the modulation of Cyp46A1 that increases or decreases POMC mRNA levels. Leptin activates POMC neurons, though their receptor LEPRB and this receptor is also expressed in other areas related to appetite, such as DMH, VMH and PVN [65]. This hormone, that regulates satiety, is released relatively to the levels of body fat storage. The inability of leptin to affect downstream pathways is called “leptin resistance” and is found mainly in obesity. A defective response to the leptin signal results in dysregulation of food intake and energy homeostasis [144]. Since Cyp46A1 can affect fat synthesis and storage, alteration in leptin pathways could be one of the reasons for some metabolic abnormalities.

On the other hand, NPY is an orexigenic neuropeptide produced in ARC neurons. NPY plays diverse roles in physiological functions, such as circadian rhythm, stress response and food intake [126]. Insulin activates NPY neurons, playing an inhibitory effect, as it promotes the feeding behavior [145]. NPY is also sensitive to diet composition, which means that it has a preferential ingestion of carbohydrates and fats. Long exposure to diet enriched with fats lead do adiposity development and a decrease in hypothalamic NPY expression. This is

a mechanism to reduce energy intake and stop the development of obesity [61]. The results obtained in this study showed a significant decrease in the AAV-shCyp46A1 animals when compared to the Non-Injected group. However, when Cyp46A1 is overexpressed, it is clear to see a significant increase in the HF diet compared to the CHOW diet animals. If diet does not influence NPY mRNA levels, the reason is modulation of Cyp46A1. Another interesting result, comparing POMC *vs* NPY, is that they have antagonist projections, as expected. Whenever one gets increases in mRNA levels, the other one gets decreased, which is in consistency and proved that diet did not influenced.

A diet enriched in fats leads to obesity and insulin resistance and therefore it starts a process called inflammation. A low-grade inflammatory state release certain pro-inflammatory cytokine, among them TNF-alpha [146]. Although there is not certain how obesity triggers inflammation, there are a few hypotheses. The first one is that a great amount of nutrients in adipocytes induces intracellular stress and consequently activates inflammatory cascades. The second hypothesis is that burden of adipocytes with fat raises infiltration of macrophages, and therefore inflammation [147]. The results obtained showed significant increases of hypothalamic TNF-alpha in both AAV-shCyp46A1 and AAVCyp46A1 animals with a diet enriched with fat. On the other hand, the opposite result is represented in the silencing of Cyp46A1 whit animals fed with CHOW diet. When the HF diet was present, it showed higher mRNA levels, as TNF-alpha primarily effect is to inhibit food intake. Contrarily, when CHOW diet is present, the mRNA levels decrease. These results are in agreement with a study that showed that this cytokine has the ability to influence anorexigenic/orexigenic neurotransmitters, producing an anorexigenic effect. It can modulate insulin and leptin signaling and therefore in the hypothalamus. TNF-alpha can mimic the effect of insulin/leptin in response to the need of the body [148].

The liver is an important organ controlling whole-body energy metabolism. It is essential to maintain homeostasis and is regulated by different metabolic hormones, like insulin and glucagon [149]. Many factors, such as lipids from diet, lipolysis of adipose tissue and *de novo* lipogenesis can alter liver homeostasis, resulting in an excessive pool of lipids stored in the liver [150]. This can be referred as Non-Alcoholic Fatty Liver Disease (NAFLD), characterized by the presence of hepatic steatosis [151], which can range from steatosis to steatohepatitis (Non-Alcoholic Steatohepatitis (NASH)), being characterized by inflammation and ballooning [152]. The results found in this study are aligned with these hallmarks, as the animals with HF diet showed increased hepatic lipid droplets formation

and hepatocytes ballooning, however they are not observed in the animal injected with the AAVCyp46A1 with HF diet. The formation of lipid droplets and hepatocyte ballooning are also visible in AAV-shCyp46A1 animals with CHOW diet. Ingestion of carbohydrates can be a major stimulant for synthesizing fatty acids (through hepatic *de novo* lipogenesis) when compared to dietary fat intake, resulting in the appearance of lipid droplets [153]. However, in this study, diet is not the reason for the appearance of lipid droplets in the liver. Since the signs are significantly visible in animals with silenced Cyp46A1, perhaps this is the reason for the appearance of lipid droplets and ballooning. Since Cyp46A1 is also present in the liver, the silencing in the hypothalamus can result in an accumulation of cholesterol in the liver. This can be related to mitochondrial dysfunction, which means that the lack of mitochondrial activity can trigger the formation of lipid droplets through switching metabolic pathways, from mitochondrial respiratory chain to glycolysis and fatty acid biosynthesis [154]. These results are not observed in the AAVCyp46A1 animals, as overexpression results in a reduction of cholesterol accumulation.

The NAFLD is related with some metabolic disorders, such as type 2 diabetes, hypertension, and obesity [152]. Obesity and type 2 diabetes are related to insulin resistance. Normally, the pancreatic islets β-cells secrete enough insulin to maintain normal glucose tolerance. However, when obesity occurs, β-cells are not able to compensate for the reduced insulin sensitivity leading to an insulin resistance phenotype. One of the reasons for β-cell dysfunction is non-esterified fatty acids (NEFAs) [155]. Although NEFAs are an important form of energy for a variety of organs, they are also precursors for the formation of triacylglycerols (TG) in adipose tissue, liver, and muscle, as a result of esterification [156]. NEFAs are fundamental for insulin release, but a high exposure of β-cell to NEFAs (*in vitro* and *in vivo*) leads to deterioration in glucose-stimulated insulin secretion and therefore insulin biosynthesis, leading to insulin resistance [155]. The accumulation of fat in the tissues and insulin resistance lead to hyperinsulinemia, which is related to an increase in islet mass and size [157]. Insulin secretion varies depending on the size of pancreatic islets. Small and large islets secrete the same amount of insulin when the glucose levels are low. However, in the presence of high glucose levels, small islets secrete two times more insulin than larger islets [133]. In this study, in the AAV-shCyp46A1 animals with HF diet, it was observed an increase of the size of Langerhans islets when compared to the Non-Injected animals, although the difference was not significant. When the CYP gene was overexpressed, the result was not the same. The increase in size of Langerhans islets is significant in the

AAVCyp46A1 animals with CHOW diet when compared to the Non-Injected group. These results also presented differences in phenotype, as the AAV-shCyp46A1 with HF diet and AAVCyp46A1 with CHOW diet presented larger islets with abnormal shape (hypertrophy). All together with a combination of a diet rich in fats, leads to an accumulation of fat content in the pancreas, provoking an enlargement of the Langerhans islets and consequently, lower release of insulin, which eventually leads to insulin resistance. On the other hand, when Cyp46A1 is overexpressed, there is an increased efflux of cholesterol content, and therefore metabolization in the liver. However, a diet rich in carbohydrates contributes to NAFLD more than fat itself. This diet can influence insulin action through alteration in plasma free fatty acids [158]. This can lead to an excessive insulin secretion, which reduces β-cell function, leading to insulin resistance. These results are consistent with the statements.

Obesity is typically defined as an excess of body weight for height, associated with excess adiposity that can not only be manifested as body size, but also metabolically [159]. The adipose tissue can be divided into White Adipose Tissue (WAT) and Brown Adipose Tissue (BAT). While WAT functions as energy storage, BAT oxidizes fat and generates heat via Uncoupling protein 1 (UCP1) [160]. The expansion of adipose tissue consists in an increase of adipocyte number (hyperplasia) and/or enlargement of adipocyte size (hypertrophy). Hyperplasia can be considered as “healthy” expansion, since it comes from progenitor cells, a phenomenon called adipogenesis. However, hypertrophy induces dysfunctional adipocytes with greater lipid content, contributing to adipose tissue inflammation and dysfunction [161] . Even though all groups showed significant increases in adipocyte area when compared to the control group, the AAV-shCyp46A1 with HF diet showed the highest increase in adipocyte area. In terms of phenotype, it is possible to observe differences in the AAVCyp46A1 animals fed with CHOW diet, as the adipocytes appear to look like unilocular lipids. This phenomenon is called “BAT Whitening” as typical BAT is converted to WAT phenotype, which means, it switches from multilocular lipid droplets to a unilocular [162]. Since Cyp46A1 is also present in adipose tissue, with the silencing in the hypothalamus, their presence can diminish. That accompanied with the process “BAT whitening”, and therefore loss of mitochondrial function can lead to inflammation of the adipose tissue.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this study, the main goal was to evaluate the impact of modulating Cyp46A1 in the hypothalamus on whole-body metabolism. The main conclusions of this work are:

- Silencing of Cyp46A1 revealed the appearance of hepatocyte ballooning and lipid droplets, in both diets. On the other hand, the overexpression of Cyp46A1 did not led to lipid droplets formation or hepatocyte ballooning.
- Overexpression of Cyp46A1 showed significant increases in the size of pancreatic islets, in both diets. Although it is possible to see an increase in islet area in the AAV-shCyp46A1 animals with HF diet, it is not statistically significant.
- Although there are significant increases of adipocyte area in all groups when compared to the control, it is possible to observe alterations in the phenotype of BAT in animals with CHOW diet (more visible in the AAVCyp46A1 animals).
- Cyp46A1 modulation altered the mRNA levels of NPY and TNF-alpha in the hypothalamus (also from POMC, however it is not significant).

These results altogether suggest an important role of the hypothalamic Cyp46A1 enzyme expression in the whole-body metabolism in partnership with diet, as the modulation of Cyp46A1 gene led to several modifications on the body. However, additional studies must be conducted to better comprehend these modifications.

It would be important to:

- Carry out protein and histological analysis in other organs, for example in muscle.
- Analyze the expression levels of additional markers of inflammation (JNK) and associated with cholesterol metabolism (SREBPs, HMGCR) in the liver and hypothalamus.
- Analyze the protein levels of different metabolic mediators in the hypothalamus, like insulin, leptin, AGRP and CART.
- Analyze the expression levels of different metabolic mediators in several organs and tissues, as pancreas and liver.
- Perform the quantification of cholesterol and oxysterols content in the hypothalamus through mass spectrometry.

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ANNEX

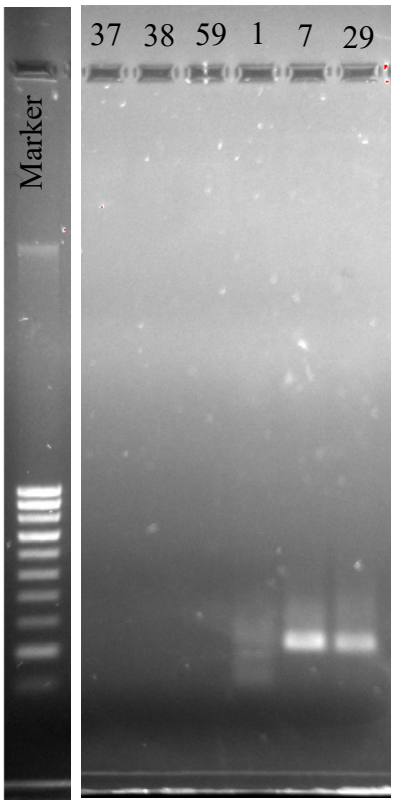
1. Primer sequence

Gene	Primer Sequence
Human CYP	Fwd: GCAGCGGAGTCATAGACC Rev: CAGCAGCATACTGGTCTCCA
Mouse Cyp	Fwd: TCCTCTCCTGTTCAGCACC Rev: CAGCTTGGCCATGACAACT
POMC	Fwd: CAACCTGCTGGCTTGCAT Rev: CGTACTTCCGGGGGTTTTCA
AGRP	Fwd: TCCCAGTTCCCAGGTCTAA Rev: CGCGGTTCTGTGGATCTAGC
TNF-α	Fwd: GCCTCTTCTCATTCCTGCTTG Rev: CTGATGAGAGGGAGGCCATT
HPRT	Fwd: GCTTACCTCACTGCTTTCCG Rev:CATCATCGCTAATCACGACGC

Annex 1. Primer sequences used in RT-qPCR.

2. Electrophoresis Gel

Electrophoresis gel in AAVCyp46A1 animals with human Cyp. It is possible to see that the overexpression in HFD diet is significant in comparison to the CHOW diet. The marker was on the first pit of the gel. For the marker, was used DNA V from NZYTech™.



Annex 2. Electrophoresis gel.

This gel is resultant from a RT-qPCR with overexpression with human Cyp46A1, with the purpose to see if the overexpression was succeeded. The gel is divided into CHOW diet and HF diet. HFD Non-injected: 37,38,59; HFD AAVCyp46A1: 1,7,29.