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EXPLORING THE PERIPHERAL CROSSTALK BETWEEN DOPAMINE AND NPY IN THE REGULATION OF ENERGY BALANCE

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular orientada pelo/a Professor Doutor Paulo Nuno Centeio Matafome e pelo Professor Doutor Carlos Manuel Marques Palmeira apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra, Departamento de Ciências da Vida

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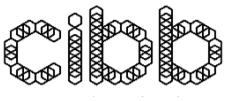
Master in Cellular and Molecular Biology



All the experimental work presented in this thesis was performed at the Institute of Physiology and Coimbra Institute for Clinical and Biomedical Research, Faculty of Medicine, University of Coimbra. The Human sample collection was performed at Centro Hospitalar e Universitário de Coimbra-Hospital Geral-Covões, Coimbra.

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INDEX

ABSTRACT

In the last years, there was a marked global rise in the prevalence of obesity, and their associated diseases. Obesity is believed to have two different phenotypes: metabolic healthy obesity and metabolic unhealthy obesity, which is the one associated with the development of metabolic syndrome and type 2 diabetes. The metabolically unhealthy one is associated with adipose tissue dysfunction and dysregulation of the systems that control energy homeostasis. NPY and dopamine system are two important systems that regulate energy balance at several levels. Centrally, they are extensively study, and they are known to interact and regulate each other, in an important crosstalk for the regulation of energy balance. In the last years, these systems have been acknowledged by their important peripheral functions, participating in lipid and glucose metabolism, adipogenesis and angiogenesis (etc.). However, the existence of a peripheral crosstalk between these systems and its importance for the regulation of energy balance has not been demonstrated yet.

The aim of this work was to unravel this peripheral crosstalk between NPY and dopaminergic systems in the adipose tissue, to understand how they modulate each other, and to investigate the importance of this interaction for the regulation of peripheral energy balance and adipose tissue function.

To achieve this, genetic expression profile of the NPY and dopaminergic system in the visceral adipose tissue of obese subjects with metabolic dysregulation was performed by a high-throughput real-time polymerase chain reaction. Additionally, we assessed the effect of dopaminergic system modulation through bromocriptine in the NPY system of Goto-Kakizaki rats fed a high-caloric diet. The acute effects off stimulation with dopamine and NPY were investigated in *ex-vivo* samples of epididymal and mesenteric adipose tissue in the presence of specific dopamine receptors' inhibitors. The direct effect of dopamine on lipid accumulation in the adipocytes were investigated in a 3T3-L1 fibroblasts cell line. The NPY modulation of dopaminergic system was also observed in these cells.

Our group showed that the progression of metabolic dysregulation in obese individuals leads to a deep alteration of NPY and dopaminergic machinery in visceral adipose tissue. We also demonstrated that these two systems are correlated between each other in human visceral tissue and are also correlated with important genes involved in adipose tissue metabolism and plasticity. Furthermore, we also demonstrated that this crosstalk existed in the adipose tissue of a diabetic obese rat model. Bromocriptine induces a remodeling in NPY system in peri-epididymal adipose tissue, with significant increases in NPY1R, NPY5R and NPY protein levels. Regarding the acute effects of this modulation, the condition where AT samples were incubated *ex vivo* with dopamine and NPY_{3-36} in the present of domperidone, resulted in marked increases of catabolic activity in both epididymal, with increase in phospho-ATGL and phospho-AMPK levels, and mesenteric, with increase only in phospho-ATGL levels. Contrarily, dopamine induces lipid accumulation in adipocytes, in an effect that appears to be induced by D2R. Lastly, stimulation with NPY_{3-36} failed to modulate dopaminergic system in the adipocytes.

The NPY and the dopaminergic system are indeed correlated in the adipose tissue, and they seem to regulate several important metabolic functions. With the aggravation of metabolic dysregulation these systems are profoundly altered, which could lead to the dysregulation of such AT metabolic functions. Pharmacological modulation of these systems, as we observed in the animal study, could be an interesting therapeutic strategy to restore these systems in the adipose tissue, improving overall metabolic health.

Key words: metabolic diseases; energy balance; neuropeptide Y; dopamine; adipose tissue.

RESUMO

Nos últimos anos, houve um aumento global marcante na prevalência da obesidade, elevando o estado desta doença a uma epidemia. Atualmente, a obesidade é conhecida por apresentar dois diferentes fenótipos, a obesidade metabolicamente saudável e a obesidade metabolicamente não saudável, esta última associada ao desenvolvimento da síndrome metabólica e da diabetes tipo 2. A forma não saudável está relacionada à disfunção do tecido adiposo e à desregulação dos sistemas que controlam a homeostase energética. O sistema NPY e o sistema dopaminérgico são importantes na regulação do equilíbrio energético a vários níveis. Centralmente, são dois sistemas extensivamente estudados e conhecidos por interagirem e se regularem um ao outro, em um importante "crosstalk" para a regulação do equilíbrio energético. Nos últimos anos, esses sistemas têm sido reconhecidos pelas suas funções a nível periférico, tendo um papel importante no metabolismo lipídico e glicolítico, na adipogênese e angiogênese, entre outras funções. No entanto, a existência de um "crosstalk" periférica entre estes sistemas e a sua importância para a regulação do equilíbrio energético ainda não foi demonstrado.

O objetivo deste trabalho foi investigar essa comunicação periférica entre os sistemas NPYérgico e dopaminérgico, entender como um pode modular a ação do outro e desvendar a importância dessas interações para a regulação do equilíbrio energético periférico.

Para alcançar este objetivo, foi realizada uma análise genética da expressão dos genes envolvidos no sistema do NPY e da dopamina, no tecido adiposo visceral de indivíduos obesos com desregulação metabólica, através de "high-throughput real-time polymerase chain reaction". Além disso, avaliamos qual o efeito da modulação do sistema dopaminérgico, utilizando a bromocriptina, no sistema NPY de ratos Goto-Kakizaki alimentados com uma dieta rica em calorias. Os efeitos agudos da estimulação com dopamina e NPY foram investigados em amostras *ex vivo* de tecido adiposo epididimal e mesentérico. Seguidamente, investigámos o efeito da dopamina na acumulação lipídica nos adipócitos, utilizando uma linhagem celular de fibroblastos 3T3-

L1. Por último, averiguamos qual o efeito da estimulação de adipócitos com NPY, na modulação do sistema dopaminérgico.

O nosso grupo demonstrou que a progressão da desregulação metabólica em indivíduos obesos leva a uma profunda alteração da maquinaria NPYérgica e dopaminérgica no tecido adiposo visceral. Também observámos que esses dois sistemas estão correlacionados entre si no tecido adiposo humano e que também se encontrão relacionados com genes importantes envolvidos no metabolismo e plasticidade do tecido adiposo, sugerindo um possível envolvimento destes sistemas na regulação de funções importantes. Além disso, também demonstramos que essa comunicação existe no tecido adiposo de um modelo animal de rato obeso e diabético. A bromocriptina induziu uma remodelação no sistema NPY no tecido adiposo peri-epididimal, com aumentos significativos nos níveis das proteínas NPY1R, NPY5R e NPY. Em relação aos efeitos agudos dessa modulação, a condição em que amostras ex vivo foram incubadas com dopamina e NPY_{3-36} na presença de domperidona, resultou em aumentos acentuados da atividade catabólica tanto no tecido epididimal, com aumento significativos nos níveis de fosfo-ATGL e fosfo-AMPK, quanto no tecido mesentérico, com aumento apenas nos níveis de fosfo-ATGL. Seguidamente, a dopamina mostrou ser capaz de induzir acumulação lipídica nos adipócitos, em um efeito que parece estar relacionado com a sinalização via recetor D2R. Por fim, a estimulação com NPY_{3-36} não mostrou ser capaz de modular o sistema dopaminérgico nos adipócitos.

O sistema do NPY e o sistema da dopamina estão correlacionados no tecido adiposo e parecem regular diversas funções metabólicas importantes. Com o agravamento da desregulação metabólica, esses sistemas são profundamente alterados, o que pode levar à desregulação dos processos que estes controlam. A modulação farmacológica desses sistemas, como observado no estudo animal, poderia ser uma estratégia terapêutica interessante para restaurar esses sistemas no tecido adiposo, melhorando a função correta do tecido e a saúde metabólica geral.

Palavras-chave: doenças metabólicas; balanço energético; neuropéptido Y; dopamina; tecido adiposo.

MSC-PUBLICATIONS AND SCIENTIFIC COMMUNICATIONS

Oral Communication

Some of the work presented in this thesis has been already presented as an oral communication in national and international scientific.

March 2023- Lopes E., Rosendo-Silva D., Matafome P. *O crosstalk periférico entre a Dopamina e o NPY*, March 2023, 19º Congresso da Sociedade Portuguesa de Diabetologia, Vilamoura, Portugal.

June 2023- Lopes E., Rosendo-Silva D., Matafome P. *Peripheral crosstalk between Dopamine, Neuropeptide Y and Melanocortin systems*, June 2023, 57º Annual Scientific Meeting of European Society for Clinical Investigation, Prague, Czech Republic.

This work was also selected to be presented in the form of short oral communication in the 59º European Association for the Study of Diabetes Annual Meeting, that this year is going to be in Hamburg, Germany, 2-6 October 2023.

Scientific Publication

February 2023- Sousa D., Lopes E., Rosendo-Silva D., Matafome P. *The Bidirectional Relationship of NPY and Mitochondria in Energy Balance Regulation*, *Biomedicines* **2023**, 11, 446. https://doi.org/10.3390/ biomedicines11020446.

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ACL	ATP citrate lyase
AgRP	Agouti-related peptide
AMPc	cyclic adenosine monophosphate
ANS	Autonomic nervous system
ARC	Arcuate Nucleus
AT	Adipose tissue
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
BMI	Body Max Index
cAMP	cyclic AMP
CNS	Central Nervous System
CPT-1	Carnitine palmitoyltransferase
CVD	Cardiovascular diases
D1R	Dopamine receptor 1
D2R	Dopamine receptor 2
D3R	Dopamine receptor 3
D4R	Dopamine receptor 4
D5R	Dopamine receptor 5
DA	Dopamine
DMEM	Dulbecco's Modiefied Eagles Medium
DPP4	Dipeptidyl peptidase 4
DRs	Dopamine receptors
EAT	Epididymal adipose tissue
EWAT	Epididymal white adipose tissue
FDA	Food and Drug Administration
FFAs	Free fatty acids
GABA	γ-aminobutyric acid
GK	Goto Kakizaki

GLP-1	Glucagon-like Peptide 1
GLP1R	GLP-1 receptor
GLUT-4	Translocation of glucose transporter type 4
HbA1c	Glycated haemoglobin
HCD	High-caloric diet
HDL	High-density lipoprotein
HSL	Hormone sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
ICV	Intracerebroventricular
IKKβ	Nuclear factor kappa-B kinase subunit beta
IL-6	Interleukin-6
InsR	Insulin receptor
JNK	c-Jun N-terminal kinase
MC3R	Melanocortin receptor 3
MC4R	Melanocortin receptor 4
MCRs	Melanocortin receptors
MetS	Metabolic syndrome
MHO	Metabolic healthy obesity
MUO	Metabolic unhealthy obesity
Nac	Mesolimbic pathway
NF-Kb	Nuclear factor kappa light chain enhancer of activated B cells
NPY	Neuropeptide Y
NPY1R	Neuropeptide Y receptor 1
NPY2R	Neuropeptide Y receptor 2
NPY5R	Neuropeptide Y receptor 5
NPYRs	NPY receptors
PBS	Phosphate-buffered saline
pEAT	Peri-epididymal adipose tissue
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator-1alpha
pHSL	Phosphorylated hormone-sensitive lipase
PKA	Protein kinase A

PKC	Protein kinase C
PNS	Parasympathetic nervous system
POMC	Proopiomelanocortin
PP	Pancreatic polypeptide
PPAR-α	Peroxisome proliferation activated receptor-alfa
PPARγ	Peroxisome proliferation activated receptor-gamma
PPYR1	Pancreatic polypeptide receptor 1
PTMs	Post-translational modifications
PYY	Peptide YY
SAT	Subcutaneous adipose tissue
SD	Standart Diet
SNS	Sympathetic nervous system
T2DM	Type 2 Diabetes Mellitus
TH	Tyrosine hydroxylase
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
UCP1	uncoupling protein 1
VAT	Visceral adipose tissue
WAT	White adipose tissue
WB	Western Blot
α-MSH	α-melanocyte stimulating hormone

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1- INTRODUCTION

I. Epidemiology of Metabolic Diseases: Obesity, Type 2 Diabetes and Metabolic Syndrome

Our current society is characterized by the increase in sedentary habits and the facility to obtain food, which leads to more high caloric diets. Nowadays, obesity is one of the most common diseases with a great prevalence in our modern society. According to the World Health Organization European Region Obesity Report, 60% of the adult population in Europe and nearly one in three children are either overweight or obese (1). Several authors consider this disease a worldwide pandemic, being the fourth greatest cause of death (1). In 2018, 22% of the Portuguese population had obesity, being more prevalent with the increase of age (2). Moreover, in this 22% of the population, 51% of the adults and 80% of the elderly have abdominal obesity (2). Associated with this massive prevalence, this comorbidity remains an increased risk factor for the development of several diseases such as cardiovascular diseases (CVDs), Type 2 Diabetes Mellitus (T2DM), and even cancer (3).

Obesity is characterized mainly by an excessive accumulation of body fat that happens when there's a long-term positive energy balance, with the energy intake exceeding energy expenditure, which favors excessive nutrient accumulation. In clinical practice, this condition is traditionally assessed by Body Max Index (BMI), which reflects the quotient between body weight, in kilograms, and the height of the individual, in square meters (kg/m^2) (4). Individuals can distinguish in different categories according to BMI: normal range (18.5-24.9 kg/m^2); overweight (25.0-29.9 kg/m^2); class 1-obesity (30.0-34.9 kg/m^2); class 2-obesity (35.0-39.9 kg/m^2) and class 3-obesity (≥40 kg/m^2) (3). Therefore, an individual with a BMI above 30 kg/m^2 is classified as having obesity (5). Thus, this index is a quick and simple clinical tool to classify patients into different categories and evaluate the associated risk. However, this measurement has great limitations, such as the incapacity to characterize body fat distribution (6). One of the first reports of BMI limitations was a study developed by Ahmed et al. where they evaluated the ratio of waist-to-hip circumferences and tried to correlate with the risk of insulin resistance and glucose intolerance. They conclude that higher proportion of abdominal fat was associated with an increased health risk independently of the BMI of the patients (7). So, they showed the importance of the location where the fat is

accumulated over the quantity of accumulated fat, to the development of metabolic comorbidities related with obesity. The advancement of technology allowed the development of imaging techniques that let us evaluate body fat distribution and understand how this is involved with the development of cardiometabolic complications (8). Several studies using this imaging techniques were able to confirm that excess visceral adiposity, over general excess fatness, is strongly correlated with the development of metabolic abnormalities, such as insulin resistance, hyperinsulinemia and glucose intolerance (8). Moreover, excess visceral adiposity can be found in non-obese patients and a percentage of patients with obesity don't have abnormal levels of visceral adiposity, which shows, one more time, the limitations of BMI to evaluate the metabolic status and the risk of developing cardiometabolic complications.

Thus, obesity is a multifactorial disease that varies according to individual's biological, genetical and epigenetic characteristics, but also to social and economic environment. Due to this diversity, several studies emphasize that individuals with obesity show different predisposition to the development of T2DM and CVD's, with a subgroup with significant lower risk than should be expected from their BMI. Consequently, in obesity it's possibly to distinguish two different conditions: metabolically healthy obesity (MHO) and metabolically unhealthy obesity (MUO). Stefan et al. performed a study with a well-constituted cohort of patients and was able to link higher ectopic liver fat content and visceral adiposity to MUO phenotype (9). In contrast, better cardiorespiratory fitness, insulin secretion and sensitivity, and lower body subcutaneous fat mass were correlated with individuals that were MHO (10). One factor that might explain this distinct fat distribution is the loss of subcutaneous adipose tissue capacity to expand, which results from a state of permanent positive energy balance. Indeed, when comparing pairs of individuals with matched age, gender, and BMI, but distinct metabolic health, in the MHO individuals is observed lower amounts of visceral and liver fat, less immune cell infiltration in visceral adipose tissue depots, a better pattern of adipokines secretion and lower adipocyte size (11). Moreover, MHO prevalence studies show a large variation, because the criteria to define this phenotype differs across the different studies (12). Besides that, it hasn't been confirmed if MHO is a steady state or if is a state that, if nothing's done, will eventually transit to MUO, with

the loss of metabolic health and the onset of metabolic syndrome (MetS) (12). In a meta-analysis of several studies where they followed-up nearly 6000 individuals during 3 to 10 years, they verified that almost half of the individuals that were classified as MHO ended up developing one or more cardiometabolic abnormality (13). So, MHO doesn't seem to be a permanent state and individuals in this phase have a high probability to transit to MUO and develop MetS. Therefore, some kind of treatment should be applied to the individuals that demonstrate an MHO phenotype even if they don't demonstrate cardiometabolic alterations. The understanding of the molecular mechanisms that are involved in the two different phenotypes might be helpful to delay or even prevent the transition to the MUO and the development of MetS. As described before, fat distribution seems to be an important feature to distinguish between the two phenotypes. Therefore, understanding adipose tissue function and how obesity alters it, is important to the onset of metabolic abnormalities in obesity.

The process of metabolic dysregulation in MUO is multifactorial and complex. Thus, the definition of MetS has been severely discussed across the recent years and despite all that, this topic still brings some controversy. The most accepted and used definition, that allows a most confident clinical diagnosis is the one that was presented by the National Cholesterol Education Program/Adult Treatment Panel III. According to this criteria, metabolic syndrome is present if three or more of the following conditions are attended: high abdominal adiposity, hypertension, hypertriglyceridemia, increased levels of fasting glucose and hypercholesterolemia (14). So, metabolic syndrome permits an intercorrelated view between several metabolic features such as insulin resistance, visceral adiposity, atherogenic dyslipidemia and endothelial dysfunction, and allow to identify patients with higher risk to develop CVDS and T2M, and also to associate patients with similar pathophysiology (15).

II. Adipose Tissue

In the past, adipose tissue (AT) was only acknowledged by the storing capacity and was seen as an organ which the only function was the storage of nutrients, mainly fat. However, nowadays, the AT is known to act as an active endocrine and paracrine

organ, that through the secretion of several factors or the stimulation of the sympathetic nervous system and the brain, can influence several important processes in the organism such as blood pressure, energy balance, appetite, lipid metabolism in other tissues, immunity, insulin sensitivity and angiogenesis (16). Regarding nutrient storage, in order to accomplish the role as the major energy-storage tissue, adipose tissue has important mechanisms that allow a dynamic and rapid remodeling of the tissue to respond to alterations in nutrient availability, either by expanding in an almost unlimited capacity, or, in contrast, by the stimulation of catabolic processes and release of nutrients when at times of nutrient deprivation (17). Obesity pathologically accelerates this remodeling, leading to deficient tissue expansion (17). This expansion can be achieved by two different mechanisms: hypertrophy, that is when the adipocyte grows in volume, and hyperplasia, that is when there is an increase in the number of adipocytes due to preadipocytes proliferation and differentiation. Healthy expansion is more associated with a recruitment of adipocyte precursor cells and stromal cell types, which ensure a correct vascularization of the new adipocytes. In contrast, pathological expansion is associated with a need to a rapid growth of the tissue, which leads to an enlargement of the existing adipocytes and to alterations in inflammatory profile and tissue function (17).

II.a. Characterization of the tissue and normal function

AT is heterogeneous and complex tissue composed by different type of cells which interaction is essential for the homeostasis and function of the tissue. Besides the cells with lipid storage capacity called adipocytes, that normally only accounts for 50% of the tissue cellular content, several immune cells, such as macrophages and lymphocytes, endothelial cells, mesenchymal stem-cells, preadipocytes, and neuronal cells are part of the adipose tissue composition (18,19). The interactions between all these cells are fundamental to maintain organization, health, and function of the tissue. For instance, it was demonstrated that the vascular network and the extracellular matrix that supports these blood vessels develop before adipocytes, during embryonic development (20), and that mature adipocytes are surrounded by one or more capillaries (21), showing the importance of non-adipocyte cells in the AT function.

According to morphology and function, it is possible to distinguish two types of AT: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT represent the most common type of AT in the human body and the main function of these depots is to store lipids in the form of triglycerides. White adipocytes are typically spherical cells with low mitochondrial mass, which size mainly depends on the size of a single lipid droplet where the triglycerides are stored (22). Contrarily, brown adipocytes contain triglycerides coupled in multiple small vacuoles, having a lower storage capacity. Moreover, the most important organelles in these cells are the mitochondria, which are large, numerous and essential for BAT main function as a dissipator of energy through the production of heat, responsible for maintaining core body temperature (22). Normally, mitochondria store energy as a proton gradient across the inner membrane, which is used to produce ATP. However, protons can run back along the membrane without producing ATP, dissipating this energy as heat in a process called non-shivering thermogenesis. This is possible due to uncoupling protein 1 (UCP1) in the inner mitochondria membrane, which creates a short-circuit route for the protons, dissipating the gradient before the production of ATP (22). Hence, UCP1 is a protein marker of BAT and its expression is increased by cold exposure and feeding, via norepinephrine release from sympathetic nervous system (22). Furthermore, due to the higher mitochondrial activity and oxygen demand, BAT contains more capillaries than WAT, as well as more nerve endings, which points to a greater regulation of BAT activity by central nervous system (23). In the recent years, it has been observed that cold exposure and activation of β-adrenergic receptors stimulate the appearance of a new type of adipocytes in WAT depots named beige adipocyte (24). The origin of this type of adipocytes is still controversial, some defend that these cells are derived from the transformation of mature white adipocytes, in a process called “browning” of white adipocytes, others support that a distinct precursor for beige adipocytes exists (24). However, beige adipocytes present a more brown-like phenotype, with a higher thermogenic capacity than white adipocytes. Indeed, it has been shown that beige adipocytes recruited by WAT under stimulus can enhance adipose tissue capacity for absorption of glucose and lipids and increase energy expenditure processes, such as thermogenesis (25). Because of this, the interest of study beige adipocyte’s role in processes such as weight control, energy balance regulation, glucose tolerance and insulin sensitivity has been growing,

with several studies pointing these cells as possible therapeutic targets to treat or prevent obesity and T2DM. As mentioned before, the cells in adipose tissue with the major storage capacity are the adipocytes. These cells accumulate lipids in cellular structures named lipid droplets, protecting the cell against direct lipid contact with the cytoplasm. Perilipin A is an important protein in the coating of lipids at the lipid droplet, enhancing their storage capacity (19). Fatty acids are accumulated in the adipocytes in the form of triglycerides. After their uptake from the circulation, fatty acids are transformed in triglycerides through a process called esterification, where one molecule of glycerol interacts with three different fatty acyl chains to form a triglyceride (26). Insulin can stimulate this process either by: 1) inducing the translocation of glucose transporter type 4 (GLUT-4) to the membrane, enhancing glucose uptake and, in consequence, the production of glycerol, or 2) by inhibiting lipolysis, the process of breakdown of triglycerides, through the inhibition of adenylate cyclase. This leads to a decrease in the levels of cyclic adenosine monophosphate (AMPc), which in turn inhibits protein kinase A (PKA) ability to phosphorylate and activate hormone sensitive lipase (HSL) (19). The fatty acids are transported from the extracellular medium to the cytoplasm by fatty acid transporter (FATP/CD36). The expression of this transporter and of other proteins important for lipid storage, metabolism and oxidation, are controlled by the peroxisome proliferation activated receptor-gamma (PPARγ) (19). This nuclear receptor, besides controlling the lipogenesis mechanism, has also an important function in the differentiation of preadipocytes into mature adipocytes. Dysregulation of PPARγ can lead to expansion of the tissue in a hypertrophic manner, which is associated with obesity (27,28). The process that originates fatty acids from a triglyceride is called lipolysis. Hormones such as glucagon, cortisol, and adrenaline, when acting on adipose tissue, activate adenylate cyclase, which leads to an increase in production of AMPc. In consequence, an increase in PKA and HSL activation will follow, being responsible for the catabolic reaction. This process can also be activated by pro-inflammatory cytokines (29,30). Peroxisome proliferation activated receptor-alfa (PPAR-α) is expressed in catabolic tissues, such as the skeletal muscle, and regulates the expression of several proteins important in the oxidation of this lipids (19).

According to the distribution around the body, WAT can be divided as subcutaneous (SAT) and visceral adipose tissue (VAT). Besides location, these WAT depots differ in their endocrine function, lipolytic activity and the sensitivity to several hormones such as insulin (16). SAT is where 80% of body fat is accumulated and represents the normal physiological buffer, absorbing and storing the excess of free fatty acids (FFAs) and glycerol in the form of triglycerides (31). In contrast, VAT only accounts for 10-20% of body fat in men and 5-8% in women, being characterized by having higher vascularization and innervation compared with SAT (16). VAT adipocytes are larger and more insulin-resistant than the small adipocytes present in the SAT, more prone to the uptake of FFAs. However, since inflammatory cells are more prevalent in VAT, this tissue can secrete higher levels of important adipokines and pro-inflammatory proteins, important in the regulation of whole-body inflammatory and metabolic state (32). As show above, VAT is more related with the develop of MetS and T2DM, than general BMI. Indeed, the dysregulation of these cells associated with obesity can lead to alterations in the secretion levels, which influences the development of insulin resistance and other metabolic complications. On another hand, SAT is the major producer of leptin (31). Leptin is one of the main hormones produced by WAT, being fundamental in correct and healthy tissue growth, by influencing angiogenesis and other important processes(19).

II.b. Dysfunction of the adipose tissue: a route to metabolic disease

Obesity is associated with adipose tissue dysfunction. In this case, the tissue growths in an unorganized way, that can affect the development of blood vessels and the correct nutrient and oxygen supply to some regions. This originates hypoxic regions that mainly affect the proliferation and differentiation of preadipocytes (33). The overall loss of proliferation will conduct to lipid accumulation in existing adipocytes and, consequently, the growth of the tissue will happen in an unhealthy hypertrophic way, instead of a healthy hyperplasic way (34). Adipose tissue dysfunction is also characterized by a development of a more sustained inflammatory state. Inflammation in normal states is a protective mechanism for uncontrolled tissue expansion, which objective is to block the nutrient uptake and to stop unlimited tissue growth. The dysfunction leads to an increase in production of pro-inflammatory cytokines and to the

secretion of chemokines to recruit monocytes from the circulation (34,35). Adipose tissue dysfunction and related inflammation can stimulate monocytes' differentiation into macrophages. The most common population of macrophages in dysfunctional tissue is the M1- like, which have a more aggressive and pronounced proinflammatory secretome (34). Tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) are two of the most produced cytokines. TNF-α has been shown to downregulate proteins that participate in lipid storage and esterification, by interfering with the PPARγ, while stimulating lipolysis (36). This leads to a decrease in storage capacity and to worsening of lipolysis dysregulation, which is typical of dysfunctional adipose tissue.

Obesity is also highly related with type 2 diabetics and insulin resistance (37). Overnutrition and long-term positive energy balance will lead to fat accumulation. The adipocytes react to this by upregulating the expression of proteins involved in fatty acid esterification and triglycerides storage. However, when they achieve the maximum capacity, fatty acids will start to accumulate in other tissues, such as the liver and the skeletal muscle, and form ectopic fat depots (38,39). These tissues have limited capacity to fatty acid oxidation, so they will start to accumulate intermediates from lipid metabolism, such as ceramides and diacylglycerols. There are several protein kinase C (PKC) isoforms that can be activated by increased levels of these intermediates, mainly by diacylglycerols (34). PKC can act directly on the insulin receptor or indirectly, by activating other stress kinases, such as c-Jun N-terminal kinase (JNK), culminating in the phosphorylation of the insulin receptor in serine residues (40). This will lead to an incapacity of the proteins necessary for insulin signaling to interact with the receptor, leading to the blockage of the signaling pathway and to insulin resistance development. Besides this, PKC can also activate inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), permitting the translocation of the nuclear factor kappa light chain enhancer of activated B cells (NF-Kb) to the nucleus and the expression of several proinflammatory molecules such as TNF-α and IL-6 (35,41). These cytokines are highly produced and secreted by adipocytes and macrophages in the adipose tissue and the activation of their receptors in other cells and tissues leads to activation of NF-kB and JNK, inducing insulin signaling blocking (35). The high levels of circulating fatty acids related with adipose

tissue dysfunction, also have been showed to be able to induce insulin resistance by activating toll-like receptor (TLR4), contributing to JNK activation (34,35).

So, as can be seen, adipose tissue dysfunction is a complex and heterogeneous process that can lead to severe consequences.

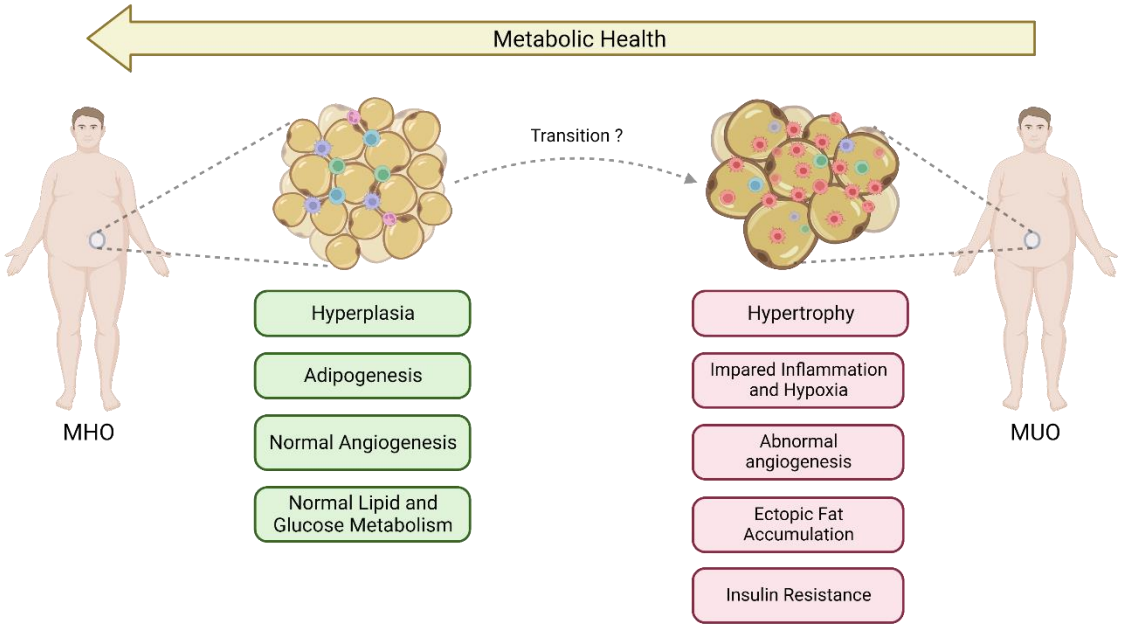

Figure 1. MHO vs MUO: Adipose Tissue and Metabolic Health*. MHO despite being obese maintain metabolic health and correct adipose tissue function, maintaining hyperplasic and adipogenesis capacity coupled with a normal angiogenic process and lipid and glucose metabolism. However, MUO are characterize by the disruption of AT, with the loss of hyperplastic capacity, impaired inflammation, hypoxia and angiogenic process. Consequently, there is an increase in ectopic fat accumulation and the development of insulin resistance. Created with BioRender.com.*

III. Central and Peripheral Crosstalk: Gut-AT-Brain in the regulation of energy balance

Energy balance is composed and affected by energy intake, energy expenditure and also by the capacity of energy storage. Energy intake depends on the energy obtained from food intake, that can come mainly from proteins, carbohydrates, and fats. In contrast, energy expenditure involves not only the energy spent in the physical activity, but also the energy required to maintain body function and temperature (42). Humans and the most part of animals can maintain body weight relatively constant throughout prolonged periods of time, despite short-

term alterations in food intake or energy expenditure. However, long periods of positive energy balance, when the energy intake is higher than the energy expenditure, can lead to an increase in body weight and adiposity. Obesity can be seen as a chronic positive energy balance state, where the great amount of nutrients ingested is not in agreement with the energy spent by the organism, which leads to an abnormal accumulation of nutrients. To prevent this from happening, there is a complex homeostatic mechanism involving communication between Central Nervous System (CNS) and several peripheral tissues to regulate body weight and important processes of energy balance, such as food intake/satiety and energy expenditure (43).

The gastrointestinal tract, or gut is one of the most active endocrine organs, being capable of producing and secreting more than 30 hormones. Upon a meal, the enteroendocrine cells can sense the nutrients, stimulating the release of these hormones that will either act through stimulation of the vagal sensory nerves present in the gut, giving important information to the CNS to regulate appetite, or by the systemic circulation and act in several organs affecting important processes such as glucose and lipid metabolism. The pattern and quantity of hormone release by the enteroendocrine cells is heavily affected by oscillations in lumen nutrients (44). One example of these hormones, is Glucagon-like Peptide 1(GLP-1), mainly produced in the L-cells in the intestine, where is the result of the cleavage of proglucagon by the prohormone convertase 1 and 3 (PC1 and PC3) (45). GLP-1 belongs to the incretin's family, so it has a major role in inducing an increase in glucose-stimulated insulin release from β-cells in the pancreas. As a result of this effect, insulin secretion induced by oral glucose is 71% higher than the one obtained from intravenous administration, with equal glucose plasma levels (46). So, this effect is very important to have a better and higher secretion of insulin upon a meal, enhancing glucose uptake, insulin sensibility and preventing hyperglycemia. Besides this short-term effect in boosting insulin release, GLP-1 can also promote insulin gene transcription and biosynthesis, preventing β-cell exhaustion and apoptosis, while stimulating β-cell proliferation, showing a long-term protective role in β-cells (47). Moreover, certain neuron can produce GLP-1, that either by this local production or by systemic circulation can interact with certain brain areas, namely the hypothalamus, and induce satiety through the activation of GLP-1 receptor (GLP1R) (48). So, the gut hormones are important and can modulate the actions of several tissues, such as the brain, pancreas and adipose tissue, not only in a short-term way, preparing them for meal ingestion, but also in a long-term way, affecting the metabolic function of these tissues.

As discussed before, AT has the important function of being the main energy buffer in the organism, so its function must be tightly regulated so that it can adjust to the different states of energy balance. Gut-derived hormones are main regulators of this function, which inform the

AT about the pattern of nutrient absorption, favoring the correct nutrient uptake and storage. This points to an important dynamic crosstalk between the gut and the AT. Indeed, gut hormones have been shown to influence glucose uptake, lipid metabolism, insulin sensibility, endocrine function, and the plasticity of AT (49). For instance, GLP-1 has been demonstrated to have a lipolytic effect in 3T3-L1 adipocytes and in human AT explants (50). Moreover, GLP-1 has also been shown to prevent AT dysfunction, either by enhancing insulin sensibility in the AT, through the upregulation of GLUT4, insulin receptor subunit β and substrate 1 expression (51), or by inducing a favorable remodeling of the AT, promoting adipocyte hyperplasia over hypertrophy, through the increase of PPARγ (52). Due to these beneficial effects, current therapies to treat obesity and T2DM involve GLP-1R agonists, such as semaglutide and liraglutide, which promote a significant weigh lost complementary to an enhancement of glycolytic, lipid and inflammatory profile (53). So, AT function is highly controlled by these factors secreted by the gut, modulating energy needs and maintaining energy homeostasis. However, as discussed before, AT is the largest endocrine organ of the body and some of the AT's secreted hormones and factors have an important role in the control of appetite, energy expenditure and whole-body metabolism. Such as the gut, these factors can act in different organs. One of the predominant signals derived from the AT is leptin, which secretion is proportional to fat mass. However, an increase of leptin levels is observed in times of nutrient availability and a decrease in fasting times. This variation in leptin levels is in line with the ability to centrally suppress appetite and increase energy expenditure (54). So, these peripheral signals are capable of interacting with brain regions, giving information about the current energy status of the body, in order to modulate either energy intake or energy expenditure processes to maintain homeostasis. Alterations in this crosstalk between central and peripheral tissues can lead to the disruption of this homeostasis.

Regarding the central control of energy balance, homeostatic control involves a complex network of brain centers capable of sensing peripheral signals, either through circulation or by the afferent nerves, which can inhibit or activate specific neurons, activating short- and long-term energy balance pathways (19). The main brain region involved in homeostatic control of energy balance is the hypothalamus. This region is formed by several nuclei that are linked between each other and can receive inputs from other hypothalamic or non-hypothalamic brain areas (55). A particular important nucleus in the hypothalamus, is the Arcuate Nucleus (ARC). ARC is the region located near to the semipermeable blood-brain barrier, which allows a direct interaction with nutritional, metabolic, hormonal and other signals from the circulation, being the first region that integrates peripheral signals into the hypothalamus (19). This region has a

higher expression of hormone receptors, critical to the capacity of signal integration. ARC is constituted by two distinct and antagonistic types of neurons: the orexigenic Neuropeptide Y (NPY) and Agouti-related peptide (AgRP)-expressing neurons and the anorexigenic proopiomelanocortin (POMC)-expressing neurons (56). A higher activation of NPY/AgRP neurons is seen in fasting times, with higher secretion of NPY and AgRP. NPY, as the most powerful orexigenic peptide, will directly stimulate food intake, while decreasing energy expenditure through the interaction with second order neurons in the hypothalamus (57). Moreover, AgRP is an antagonist of α-melanocyte stimulating hormone (α-MSH), so it will compete with this peptide for binding to melanocortin receptors (MCRs) in second order neurons, blocking their anorexigenic functions (58). Furthermore, in the ARC, NPY/AgRP neurons are capable of directly inhibiting POMC neurons via inhibitory γ-aminobutyric acid (GABA) production, potentiating food intake (59). On the other hand, POMC neurons are activated upon nutrient ingestion. These neurons can produce POMC that is cleaved and originates α-MSH, which is released to activate melanocortin receptor 3(MC3R) and 4 (MC4R) anorexigenic effects. The activation of these receptors in second order neurons, either in other hypothalamic nuclei or in other brain regions, leads to a reduction in food intake and a stimulation of energy expenditure(60). The hypothalamus after sensing the different nutrient and hormonal signals can coordinate a response through the parasympathetic nervous system (PNS) and the sympathetic nervous system (SNS) in order to regulate energy expenditure and peripheral metabolism (19). An increase in α-MSH production in the ARC, leads to activation of central SNS outflow which results in an increase BAT's energy expenditure, through non-shivering thermogenesis. On the other hand, NPY/AgRP neurons activation in the ARC is correlated with the blockage of SNS outflow, which promotes inhibition of BAT thermogenic activity (61). As mentioned before, several peripheral signals can modulate ARC neurons' activation. For instance, leptin, can act as an anorexigenic factor by exerting a direct inhibitory effect on NPY/AgRP neurons, decreasing the expression of AgRP, and by directly exciting POMC neurons, inducing the expression of POMC (43). The net effect of leptin actions upon ARC neurons stimulation results in the inhibition of food intake and in the increase of energy expenditure. Similarly, insulin, is another example of a peripheral signal that exerts a similar effect in ARC neurons. However, insulin seems to act in a different subpopulation of POMC neurons being related with regulation of AT lipolysis (43) and gluconeogenesis in the liver (62). Regarding orexigenic peripheral factors, ghrelin, the hormone predominantly secreted by the stomach during starvation, robustly stimulates food intake through the direct activation of NPY/AgRP neurons and can also promote body weight gain and adiposity via interactions with other hypothalamic areas (63). Besides this, Ghrelin can also inhibit POMC neurons, blocking the

release of α-MSH (64). NPY/AgRP neurons are also activated by mechanical signals that are emitted by the gut through the vagus nerve, in times of starvation (65). So, this crosstalk and communication between peripheral and central tissues is fundamental to maintain energy homeostasis and control body weight.

The central homeostatic control of energy balance is dysregulated with the development of obesity and T2DM. For instance, there are evidence that point to the development of resistance to the hormones that act on brain homeostatic regions. Indeed, for example, leptin levels are higher in most cases of obesity (66). However, leptin's effect on reducing food intake and increasing energy expenditure is not observed in obese patients, which points to a possible leptin resistance. In fact, in diet-induced obese mice (DIO mice), leptin is unable to decrease food intake, although its high circulating levels. This incapacity is due to an insensitivity of melanocortin neurons in the ARC to leptin (43). Insulin resistance development is another common characteristic present in patients with obesity or T2DM. DIO mice also present insulin resistance at the hypothalamic level, contributing to the dysregulation of these brain centers, observed in obesity (43). At last, NPY/AgRP neurons of DIO mice have shown to develop resistance to ghrelin, with a lower capacity to induce secretion of NPY and AgRP (67). Hyperleptinemia seems to contribute to this outcome. So, obesity seems to affect this important crosstalk, leading to the dysregulation of energy homeostasis. The restoration of this crosstalk between the periphery and the brain, through the modulation of these hormones effects in whole body, could be a possible good therapeutic target to treat obesity, T2DM and MetS.

IV. NPY system: central and peripheral control of energy balance

NPY is not just an appetite regulator since it also exerts important actions in the regulation of energy balance either through the modulation of autonomic nervous system (ANS) output or by direct interaction with different peripheral tissues through NPY receptors (NPYRs) (68). This peptide is primarily expressed in the central and peripheral nervous system, with the highest concentrations being present in hypothalamic ARC. Peripheral tissues, such as the adipose tissue and bone, also have demonstrated to express NPY (69). NPY orexigenic effect is sustained by the increased NPY levels observed in the hypothalamus in periods of long fasting conditions (69). Specific administration of NPY in the hypothalamus promotes the development of multiple characteristics of obesity, such as hyperphagia, hyperinsulinemia and lipogenesis in the adipose tissue, in normal rodents (70,71). In the other hand, deletion of NPY gene protects

mice fed a high fat diet from weight gain, showing the importance of NPY for obesity development (72)

NPY acts through six receptors that associate with Gi protein. These receptors are divided in two subfamilies based on sequence alignment. The association with Gi leads to a reduction in cAMP levels and modulation of Ca2+ and K+ channels, which triggers several downstream responses (69). The NPY system is formed by three different native forms of peptides including NPY_{1-36}, pancreatic polypeptide (PP) and peptide YY (PYY). These peptides can suffer post-translational modifications (PTMs) by different enzymes, such as dipeptidyl peptidase 4 (DPP4), originating different NPY fragments that have distinct affinities for the NPY receptors. For instance, DPP4 cleaves NPY, originating NPY_{3-36}, a cleaved form with a higher affinity for the NPY receptor-2 (NPY2R) (73). NPY neurons in the ARC have projections to several hypothalamic areas and extrahypothalamic areas, such as the reward system (74,75). These projections suggest a complex network that regulates and is responsible for the effects of NPY on appetite and energy homeostasis. The effect of NPY on the hypothalamus depends on the receptor that is activated. Activation of central NPY1R receptors leads to a significant increase in appetite. Several studies associate hypothalamic NPY1R receptors with anabolic effects in conditions of high levels of hypothalamic NPY (76). NPY receptor 5 (NPY5R) activation in the hypothalamus has a similar outcome than NPY1R, stimulating food intake (77). NPY was also demonstrated to inhibit MC4R expression in hypothalamus, contributing to the orexigenic effect of NPY, through the decrease of anorexigenic stimulus (78). Contrarily, the NPY2R receptor has been indicated as an autoinhibitory receptor, that leads to a reduction in NPY expression in NPY expressing neurons, being fundamental in the postprandial state. Peripheral administration of PYY3-36, a potent NPY2R agonist, leads to a significant reduction in food intake (79,80). Therapies involving the potentiation of NPY2R function or the blockage of NPY1R effects at the hypothalamus levels can be potential good approaches to treat obesity (76). The functions of the other NPYRs at central level are still not clear. Other peripheral tissues also express NPYRs, so the effects in these tissues must be also consider.

Several NPY receptors have been found in peripheral tissues, however the exact functions of all these receptors in these tissues are not completely clear. For instance, NPY can act in these tissues by the action of the SNS or by direct interaction with the tissue, either by circulation or by an autocrine or paracrine way, when the NPY is expressed in the tissue. NPY-mediated modulation of SNS activity is an important factor in the control of NPY's effect on energy expenditure. Indeed, higher levels of NPY in the hypothalamus correlate with central inhibition of the sympathetic peripheric innervation, which leads to alterations upon energy

balance processes in the innervated tissues, such as the BAT and WAT. It was demonstrated that, in BAT, intracerebroventricular (ICV) injection of NPY resulted in the decrease of thermogenesis, through a reduction in mitochondria's levels of UCP1, resulting in an overall decrease of energy expenditure (81). Moreover, in WAT the NPY action mediated by the SNS results in a stimulation of fat accretion (81). So, NPY-mediated effects through the SNS seem to be a positive energy balance stimulus towards nutrient storage that potentiates fat accumulation and reduces energy expenditure.

Regarding direct action upon the tissue, NPY1R and NPY receptor 5 (NPY5R) activation through circulating NPY levels doesn't seem to affect BAT thermogenesis (82). However, in WAT, direct interaction with the adipocytes seem to potentiate the SNS-induced positive energy balance. 3T3-L1 adipocytes cultured with NPY showed an increase in lipogenesis markers, such as PPARγ, complementary to a decrease in lipolysis markers, such as phosphorylated hormone-sensitive lipase (pHSL). This effect was mediated via NPY1R activation (68). Moreover, NPY2R activation in WAT seems to potentiate the proliferation of preadipocytes and endothelial cells, being related with adipogenesis and angiogenesis (83). So, the two receptors seem to be important to stimulate fat accumulation at WAT, however, NPY2R action appears to potentiate healthy fat accumulation, through the stimulation of adipogenesis and angiogenesis, crucial process in hyperplasic expansion of WAT.

NPY system is also present in other peripheral tissues. For instance, in the liver NPY appears to regulate hepatic lipid oxidation. NPY seems to downregulate carnitine palmitoyltransferase (CPT-1), a protein fundamental to mitochondrial β-oxidation through the activation of NPY1R (84). A similar process is observed at the muscle level, with the same outcome regarding CPT-1 (84). Pancreatic β-cells, in the pancreas, have also been shown to express NPY receptors. The NP1R activation on these cells lead to an inhibition of insulin secretion (85). Thus, NPY peripheral actions appear to be important to the regulation of overall lipid and glucose metabolism. This peripheral modulation potentiates energy storage and accumulation, either by stimulating WAT capacity for lipid accumulation and by decreasing lipid oxidation not only by direct inhibition in the liver and the muscle, but also via SNS inhibition in the BAT. This peripheral modulation is in concordance with the central actions of NPY, mainly regarding food intake. While central modulation stimulates nutrient ingestion, peripheral modulation prepares the organism to receive and store those nutrients, while decreasing the expend of energy. The dysregulation of this system is observed in obesity. Several studies point to higher NPY serum levels in individuals with obesity, compared with lean subjects (86). This could lead to severe consequences involving a higher energy uptake, through increases in food

intake stimulation, with a coupled increase in adiposity and WAT expansion, and a decrease in energy expenditure. The conjugation of these factors could lead to an overall chronic positive energy balance. However, the total actions of NPY and the alterations upon obesity establishment are not fully understood.

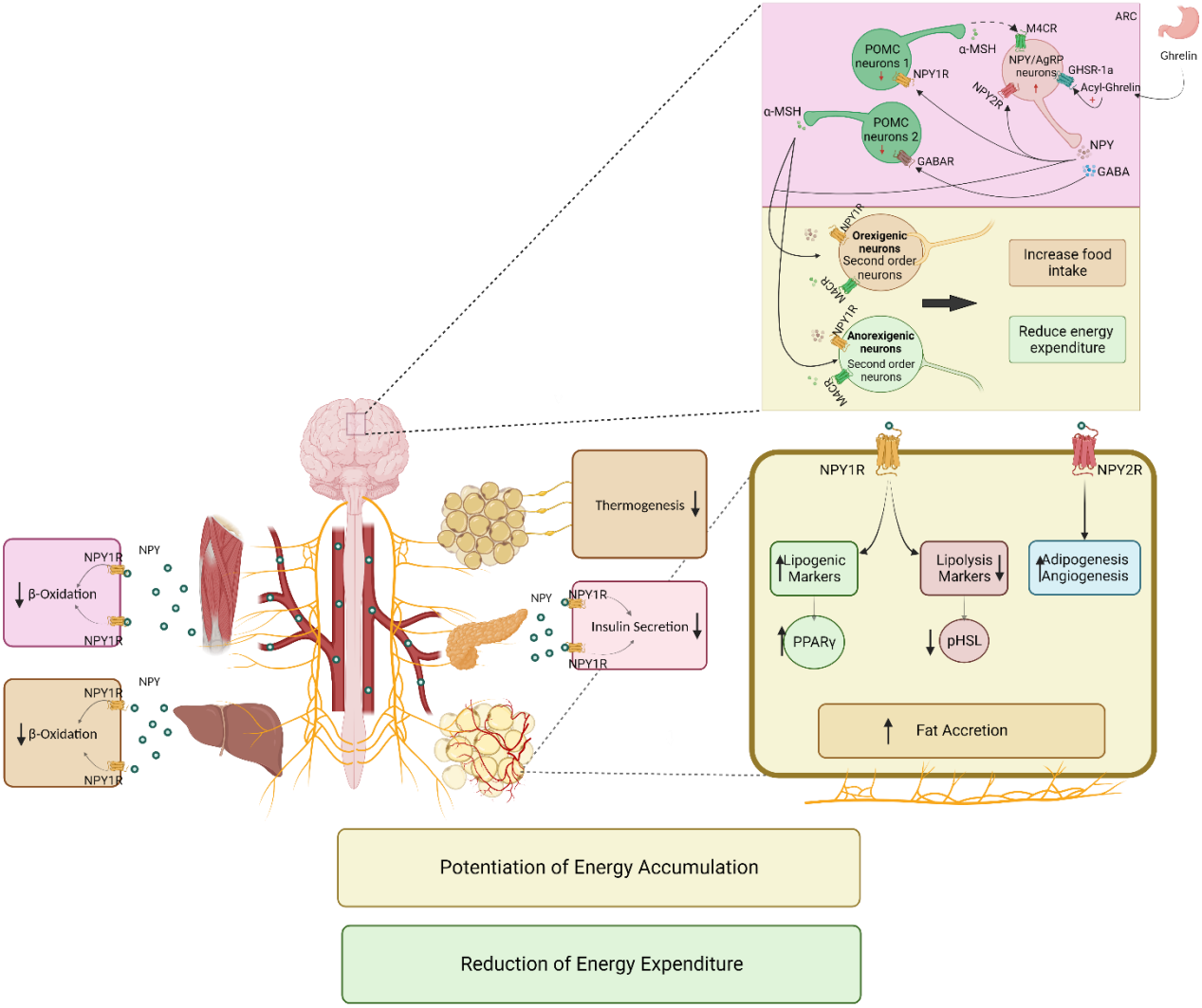

Figure 2. NPY regulation of Energy Balance. *NPY regulates energy balance through central and peripheric actions. In the ARC, during fasting acyl-ghrelin stimulates orexigenic neurons firing, releasing NPY and GABA, inhibiting POMC neurons, which leads to the activation of second order neurons, activating circuits that ultimately conduct to the stimulation of food intake and the reduction of energy expenditure via SNS, leading, for example to reduction of thermogenesis in BAT. NPY action on the liver and muscle decreases β-oxidation via NPY1R. In the pancreas, NPY increases insulin secretion via NPY1R. In WAT, NPY potentiates fat accretion by reducing lipolysis and increasing lipogenesis, via NPY1R, and stimulating adipogenesis and angiogenesis, via NPY2R. Created with BioRender.com.*

V. Dopamine system: central and peripheral control of energy balance

Dopamine is an important neurotransmitter in the central nervous system, responsible for the regulation of neuronal functions such as emotion, food intake, endocrine homeostasis, cognition, among others. The dopaminergic neurons are organized in the midbrain substancia nigra and VTA (87). These dopaminergic neurons in the VTA form an important circuit by projections to the NAc, named mesolimbic pathway, also known as the reward system. This pathway is important in the hedonic behavior of eating and can also substantially affect energy homeostasis and feeding. Dopaminergic neurons are also present in the ARC (88). Indeed, it was demonstrated that whole-body dopamine deficiency is not lethal in the embryonic stage, but in the postnatal stage leads to death by starvation(89). Moreover, starvation intensifies mice's dopaminergic signaling in reward system, an effect that is blunt by leptin and insulin action (90).

There are a total of 5 dopamine receptors (DRs) and all of them are members of the G-protein coupled receptors family. These are divided into two subfamilies: dopamine receptor 1 (D1R) and dopamine receptor 5 (D5R) are D1-like receptors and they are coupled with Gs proteins leading to an increase of intracellular cAMP level; dopamine receptor 2 (D2R), dopamine receptor 3 (D3R) and dopamine receptor 4 (D4R) are D2-like receptors, being coupled with a Gi protein and leading to inhibition of adenyl cyclase and to a decrease in cAMP levels (91). D1-like receptors also can be coupled to Gq protein that can activate PKC and lead to an increase in intracellular Ca2+ (91). The most common receptors in the central nervous system are the D1R and D2R. At the WAT level, mature adipocytes present higher levels of D1R expression than D2R (92,93). Several studies show that dopamine receptors can form heterodimers between each other and even with other receptors, such as adenosine receptors (94). The outcome of dopamine action depends on the receptors and different heterodimers that are activated, leading to distinct physiological roles.

Dopamine can induce food intake by the reward system. Ingestion of palatable food leads to the release of Dopamine in VTA neurons into NAc, inducing food craving (95). D2R has an inhibitory effect in this pathway, decreasing dopamine release. Individuals with decreased central D2Rs develop more frequently and more easily compulsive behaviors (18). In patients with obesity, D2R levels are decreased, which may be one of the reasons for compulsive eating behaviors (18). In the ARC neurons, both orexigenic and anorexigenic neurons have been shown to express dopamine receptors, especially D1R and D2R. However, while the proportion of *Drd1*-expressing neurons was similar between orexigenic and anorexigenic neurons, POMC neurons show a much larger proportion of *Drd2*-expressing neurons, compared with NPY/AgRP neurons

(90). Higher expression of *Drd2* correlated with *Glp1r* showing that the subpopulation of POMC neurons with higher GLP-1 receptor also expressed higher levels of D2R (90). In this study, was also observed that the largest proportion of NPY-expressing neurons in the ARC were excited by dopamine in a concentration-dependent manner, contrarily, to what was observed in POMC neurons, where the largest proportion of neurons were inhibited by dopamine. This inhibition was mediated by D2R, pointing to an inhibitory effect on neuron firing frequency (90). Regarding D1R, activation of this receptor in orexigenic neurons leads to increased expressing of orexigenic neuropeptides, being associated with a stimulation of food intake (96). However, NPY/AgRP neurons also express D2R that is activated in low levels of dopamine. So, it seems that in low levels conditions, D2R inhibitory effect is activated, followed by an activation of D1R orexigenic effect, upon the increase in dopamine levels (96). Therefore, dopamine appears to be able to control both sides of energy balance at a central level. Dopamine is capable of inhibiting food intake, through D2R activation in NPY/AgRP neurons in conditions of low dopamine levels, characteristic of satiety periods. On the other side, dopamine stimulates feeding, not only by the action on the reward system, but also through the stimulation of NPY/AgRP neurons via D1R and the blockage of anorexigenic POMC neurons via D2R. Moreover, POMC neurons positive to D2R reduce food intake and increase temperature, showing a role in the mediation of energy expenditure (90). The dysregulation of dopamine system observed in obesity, either by increased hypothalamic D1R and D2R levels and by alterations of dopamine circulating levels, can potentiate the orexigenic effect of dopamine, leading to an imbalance in energy homeostasis (96)

Outside of the central nervous system, dopamine can regulate processes such as hormone secretion, gastrointestinal motility and can also play an important role in energy homeostasis and balance, by regulating glucose metabolism. At the peripheral level, dopamine plasma levels can originate from adrenal medulla, sympathetic nerves, and local production in non-neuronal cells, such as intestinal cells and WAT adipocytes (91). Regarding WAT, dopamine can act via direct interaction through circulation, via sympathetic nerves release or by the infiltration of macrophages and lymphocytes (97).Such as in the brain, D1R and D2R also have opposite effects in this tissue regarding lipid metabolism. D1R is associated with catabolic processes. Yu Jing et.al demonstrated that incubation of 3T3-L1 adipocytes with Fenoldopam, a specific D1R agonist, drastically reduced lipid content, while incubation with SCH23390, a D1R antagonist, promote the accumulation of lipid, pointing to an increase in lipolysis via D1R (98). This was confirmed by further measurement of key lipolytic enzymes HSL and adipose triglyceride lipase (ATGL), and FFAs levels, which were all upregulated upon Fenoldopam

treatment and downregulated after SCH23390 incubation(98). D1R activation is followed by an increase in cyclic AMP (cAMP), which will activate protein kinase A (PKA) and consequently phosphorylate several downstream proteins (99). One of the downstream effects is the increased expression of peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α), an important protein for mitochondria biogenesis. Indeed, D1R activation led to a higher expression of PGC-1 α, with the consequent increase in mitochondrial content. Coupled with this, an increase in UCP1was observed, suggesting that D1R not only promotes energy expenditure in the WAT, but also stimulates the browning of white adipocytes (98). This effect was demonstrated in vivo, where Fenoldopam treatment prevented body weight gain and improve glucose tolerance in high fat diet- fed mice, pointing to an enhanced energy expenditure in WAT via D1R activation (98). In contrast, in mesenteric WAT and epididymal WAT, D2R inhibition led to an increase phosphorylation of HSL, AMPK, ATP citrate lyase (ACL) and acetyl-CoA carboxylase (ACC), leading to a promotion of lipid oxidation (100). So, D2R activation in WAT seems to be more related with energy accumulation, by the inhibition of lipolysis and the stimulation of adipogenesis and lipogenesis. D2R also potentiates insulin-mediated glucose uptake (100).

In the pancreas, β-cells express D2R, and the activation of these receptors leads to an inhibition in glucose-stimulated insulin secretion (100). The whole-body deletion of D2R in mice, lead to the development of glucose intolerance and abnormalities at the level of insulin secretion. Also, in humans that were taking neuroleptic drugs, which can block dopamine receptors, it was observed hyperinsulinemia and adverse effects on weight gain and impaired glucose tolerance (101). Dopamine also regulates glucose uptake in the liver and skeletal muscle. For instance, D1R activation in skeletal muscle and D2R in liver, increases glucose uptake (100).

As already mentioned, dopamine system is dysregulated in obesity and T2DM, either centrally or peripherally. The modulation of the dopaminergic system has been gaining interest as a therapeutic target to treat these diseases. Bromocriptine is a drug that functions as an agonist for the D2R and has been approved by the Food and Drug Administration (FDA) to treat T2DM in the USA (102). This drug has been acknowledged by its effect on the increase of insulin sensitivity, through its protective effect against beta cell exhaustion, and through the positive regulation of the hypothalamic sympathetic output (102). However, the action on other peripheral tissues is not fully understood. In a model of diabetic mice fed a high caloric diet, chronic bromocriptine treatment led to an increase of D1R expression in epididymal WAT, which upregulated catabolic processes, promoting lipid oxidation, also improving insulin sensibility, with increased levels of insulin receptor (InsR) and GLUT4. The bromocriptine effect in

enhancing lipid metabolism and metabolic activity in WAT resulted in a protective effect against lipotoxicity and associated insulin resistance development in the liver, which demonstrated lower levels of triglyceride content (102). This upregulation was associated with an improvement in metabolic profile, with decreased weight gain, plasma triglycerides, fatty acids and fasting glycemia (102). So, the action of bromocriptine on peripheral tissues, such as the adipose tissue, can contribute to the positive outcome that can be observed in patients that undergo bromocriptine treatment, showing the importance of studying traditional central systems, as the dopaminergic system and the NPYergic system, in peripheral tissues.

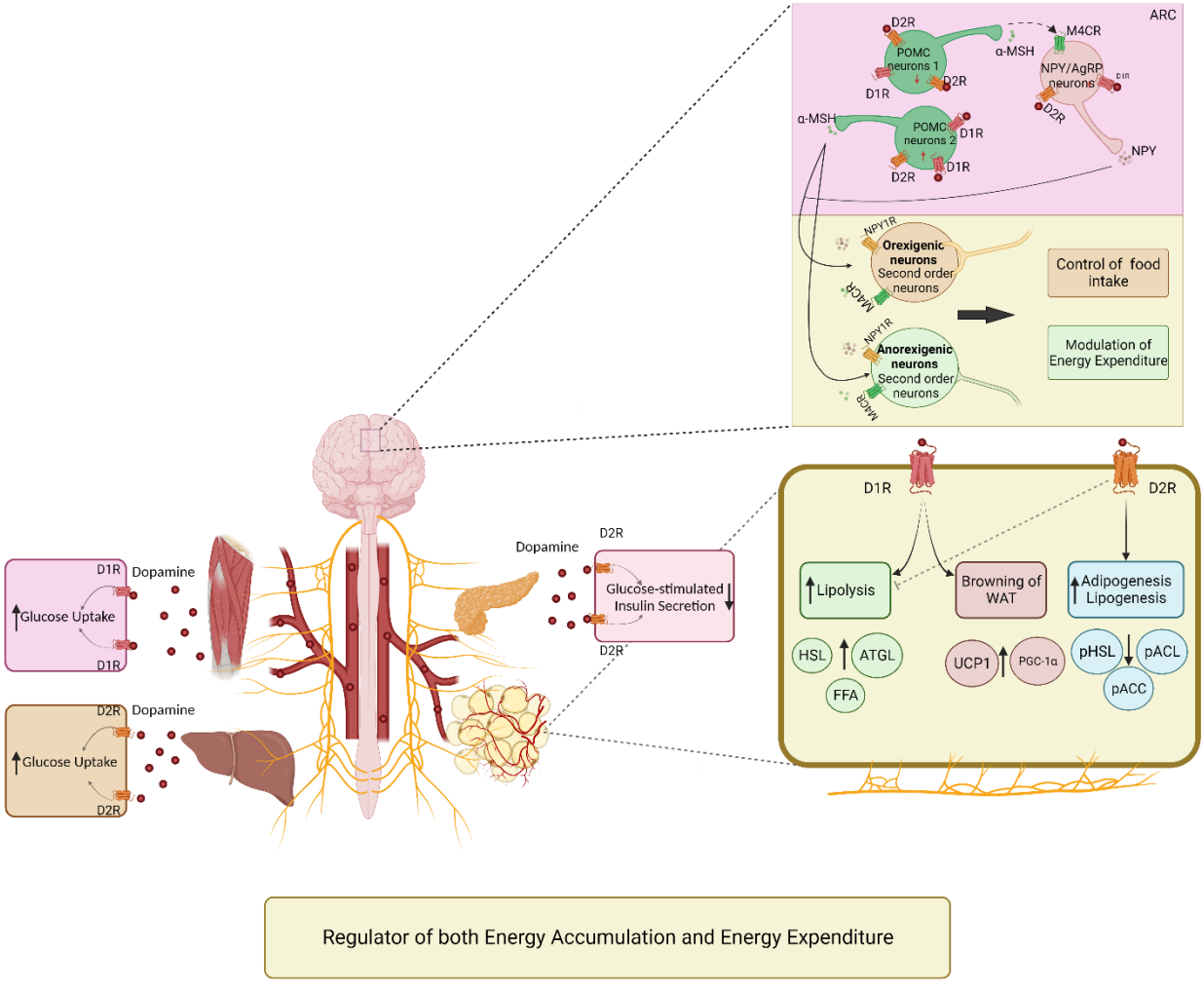

Figure 3. Dopamine regulation of Energy Balance. *Dopamine regulates energy balance through central and peripheric actions. In the ARC, depending on the receptor that is activated there are different outcomes regarding food intake and energy expenditure. Peripherally, dopamine increases glucose uptake in the muscle, via D1R, and in the liver, via D2R, and also stimulates glucose-stimulated insulin secretion in the pancreas, via D2R. In WAT, D1R activation leads to stimulation of lipolysis and browning of WAT. Contrarily, D2R activation potentiates adipogenesis and lipogenesis, while inhibiting lipolysis. Created with BioRender.com*

VI. Crosstalk between Dopamine and NPY: a new potential therapeutic target

Besides their individual and straight functions, these hormones also appear to have some effects in the modulation of each other, affecting their signaling and production. For example, it has been suggested a possible crosstalk between dopamine and NPY signaling at a central level. Dopamine can exert several functions in the hypothalamus influencing energy balance. Zhang et al. demonstrated tyrosine hydroxylase (TH)-positive neurons constitute a unique dopamine-releasing population in the ARC. Neither POMC nor NPY/AgRP show expression of TH (103). In another study, they also observed that activation of a specific population of NPY/AgRP neurons expressing D1R leads, per se, to an increase in food intake. Genetic ablation of *Drd1* in these neurons attenuates high fat diet associated behaviors. (104). These results suggested that part of the orexigenic effect of dopamine is possible thanks to the modulation of NPY production and signaling, and points to the crosstalk between the two peptides. However, in obesogenic cases, D1/D2 agonists were shown to normalize NPY levels at the hypothalamus, leading to an anorectic response translated by a changing in feeding behaviors and a decreasing in hyperphagia (105). One possible explanation for these contradictory effects is that obesity promotes changes in dopaminergic signaling at the hypothalamus level, altering D1R and D2R expression in the neurons, which leads to different effects.

This crosstalk is also verified by the existence of NPY neurons and receptors in the NAc and VTA, that are regions involved in the dopaminergic reward circuit, and by the fact that NPY/AgRP neurons in the hypothalamus present projections to these reward areas. Intra-NAc and Intra-VTA NPY increases dopamine efflux, associating with food-motivated behavior(106). The study by Roseberry et al. demonstrated that NPY can inhibit a subpopulation of dopaminergic neurons in the VTA, either by direct NPY1R-mediated activation of GIRK channel current or by indirect reduction in glutamate release. They also showed to have excitatory effects in another subpopulation of VTA dopamine neurons. Although this seems to be contradictory, the net effect of the NPY leads to an increase food motivation and intake (106).

There are several evidence that point to this crosstalk at a central level, although the interactions between NPY and dopamine are not fully understood. Even less is known about how these hormones modulate each other effects in peripheral tissues and organs. Several studies point to the individual effects of each of these hormones in the periphery. However, studies aiming to understand how the modulation of one of the molecules can affect the other's signaling and effects are very scarce. For instance, a peripheral crosstalk between dopamine and

GLP-1, two important energy balance regulator hormones, was demonstrated (107). In this study, our group demonstrated that postprandial dopamine levels are highly affected by meal constitution and nutrient sensing in the gut and that gut remodeling, through sleeve gastrectomy, modulates dopamine machinery in WAT, with an increase in D1R, D2R and TH protein levels. Moreover, they also observed that bromocriptine treatment in an obese and diabetic rat model, upregulated GLP-1 signaling in both WAT and BAT, but liraglutide treatment failed to modulate Dopamine signaling, suggesting that in adipose depots this crosstalk could have unilateral effects (107). So, it can be hypothesized that a similar crosstalk can exist between dopamine and NPY, since both control important processes of energy balance, either through central or peripheral actions, and they were shown to regulate each other's function at the central level. It is possible that some of these beneficial effects observed with bromocriptine treatment in animal models can be explain by the modulation of NPYergic signaling in the peripheral tissues. However, it is necessary more investigation to confirm the existence of the crosstalk between dopamine and NPY in the adipose tissue.

Obesity and other metabolic diseases are characterized by a dysregulation of lipid and glucose metabolism in the periphery, processes that both NPY and dopamine appear to modulate, including in the adipose tissue. So, the understanding of the interactions between dopamine and NPY can be essential to the development of new therapeutics to the treatment of metabolic disorders. The main principle for these new therapeutics will be the modulation of this crosstalk, with agonists and antagonist for the hormone's different receptors, with the objective of potentiating the beneficial effects in energy balance processes.

CHAPTER 2 - SCIENTIFIC FRAMEWORK AND OBJECTIVES

Scientific Framework

The transition of MHO to MUO is a relatively new topic, that needs more study to be fully understood. AT dysfunction seems to be one of the key players that lead to the development of MetS and T2DM, which in consequence increases the risk of developing cardiometabolic diseases. So, understanding the phenomena leading to AT dysfunction and what is altered in the AT with the development of its dysfunction, is crucial to the development of therapies to treat obesity and to block the transition to an unhealthy phenotype.

In a study performed by our laboratory with a cohort of patients with obesity in different stages of metabolic (un)health, we observed that there were alterations in the expression of NPY and Dopaminergic machinery in VAT, along the evolution of the metabolic sequalae of obesity (102). Central control of energy balance processes by NPY and dopamine is extremely studied. In addition, several more recent studies demonstrate that these systems are present in peripheral tissues, such as liver, muscle, BAT and WAT, and that both NPY and dopamine can directly act in these tissues to control several important metabolic processes. For instance, in WAT, NPY and dopamine have been shown to regulate adipogenesis and lipid and glucose metabolism. So, the dysregulation of the machinery observed in the patients along the evolution of the metabolic sequalae of obesity can contribute to the dysregulation of lipid and glucose metabolism and to the development of AT dysfunction.

As it happens at the central level, where NPY system interacts with dopaminergic one to regulate energy balance, it can be suggested that this interaction and correlation between the two systems, also happens at the peripheral level. So, it would be interesting to understand how these systems can regulate each other in peripheral tissues and to know which are the metabolic outcomes of this regulation, to develop therapies where we can potentiate these beneficial metabolic outcomes. Indeed, in a study published by our laboratory, we show that dopaminergic modulation using bromocriptine (D2 receptor agonist) leads to the upregulation of catabolic pathways in WAT, improving metabolic profile of an obese T2D animal model (102). So, considering the importance of the interaction between NPY and dopamine systems at the central

level, it would be interesting to study how bromocriptine modulates NPY system in peripheral tissues and understand if this modulation contributes to the improvement in metabolic profile that we observe in the T2D obese animal model.

Next, we present some of our group already published results, that are fundamental to the understanding of the results obtain in this thesis. The data on the characterization of the human cohort of patients was published by Tiago Rodrigues et.al (108). All the raw data of the gene expression obtained with quantitative real-time PCR, was already done. The data of the variations of NPY system machinery expression among the groups was already published in a Master thesis, performed at University of Coimbra, named *"The gut-adipose tissue crosstalk: Unravelling the mechanisms of ghrelin-mediated regulation of adipose tissue angiogenesis"*, by Daniela Rosendo da Silva. Finally, the data of the variations of Dopamine system machinery among the groups is also already published (102).

Characterization of patients with obesity: Progressive development of metabolic dysregulation is parallel with AT

The obese patients, as previously show, were divided into four different groups according to their glycemic profile and insulin sensitivity: 1- IS NG (n=20) composed by patients with obesity in the MHO phase, that demonstrated normal levels of fasting glycaemia (≤ 100 mg/dL) and HbA1c (≤ 5.6%), and were insulin sensitive (Ox-HOMA2IR<1); 2- IR NG (n=66) composed by patients with obesity in That demonstrated normal levels of fasting glycaemia (≤ 100 mg/dL) and HbA1c (≤ 5.6%), but that were insulin resistant (Ox-HOMA2IR>1); 3- Pre-diabetic group (n=34) composed by patients with obesity that were insulin resistant (Ox-HOMA2IR>1) and had increased levels of fasting glucose (100 <FG≤ 125 mg/dL) and HbA1c (5.6%<HbA1c ≤6.4%); 4-Diabetic group (n=20) composed by patients with obesity that were insulin resistant (Ox-HOMA2IR>1) and diagnosed with T2DM, with the higher levels of fasting glucose (FG> 125 mg/dL) and HbA1c (HbA1c > 6.4%). This division reflets an evolution of metabolic dysregulation from the obese patients that can be considered metabolic healthy (group 1), followed by a constant development of metabolic unhealthy phenotype, from group 2 to group 4. Full

characterization of the different metabolic parameters from the patients was already published by our group (108).

BMI was similar between all the groups, which suggests the development of metabolic dysregulation isn´t correlated with the total quantity of fat in the body, although BMI is not also a good indicator of body fat. However, pre-diabetic (group 3) and diabetic (group 4) patients were significantly older than the insulin resistant normoglycemic group ($p<0.01$ vs IR NG (group 2)). Regarding leptin plasma levels, no severe alterations were observed among the groups, which is not surprising, considering BMI distribution among the groups. On the other hand, adiponectin was significantly decreased in patients from Diabetic group when compared with IS NG ($p<0.01$), IR NG and Pre-diabetic groups ($p<0.05$). Moreover, triglycerides levels are significantly higher in IR NG ($p<0.05$) and Diabetic ($p<0.01$) group compared with IS NG patients. Patients from the Diabetic group also demonstrated lower levels of high-density lipoprotein (HDL) cholesterol compared with group 1 ($p<0.001$) and 2 ($p<0.05$), similar to patients in the pre-diabetic group, that show lower levels of HDL cholesterol compared with group 1 ($p<0.05$). β-cell function is also altered along the groups. Patients from the group 2 and 3 demonstrate a higher β-cell function ($p<0.01$), compared with group 1, which suggests that in these groups there is a compensatory response in insulin secretion to compensate the development of insulin resistance. This compensatory response seems to vanish in the diabetic group, where is observed the lowest levels of β-cell function among the groups.

The progressive development of hypoadiponectinemia seen in pre-diabetic and diabetic groups, coupled with the progressive lower levels of HDL cholesterol and higher levels of triglycerides are markers of adipose tissue dysfunction. The progressive loss of insulin secretion compensatory mechanisms also appears to be correlated with AT dysfunction.

NPY and Dopaminergic system is altered in the visceral adipose tissue of patients with obesity in different stages of metabolic dysregulation

The evolution of metabolic dysregulation leads to alterations of visceral adipose tissue expression of NPY and dopamine receptors, which possibly reflects alterations in the NPYergic and Dopaminergic signalling. Regarding NPY system, we can observe in **Figure 4** that all the receptors that bind NPY and the peptide itself have alterations of gene expression profile along the different groups. *NPY1R* and *NPY5R* have a similar behavior, since both receptors demonstrate a peak increase of gene expression in group 3 (IR Pre-diabetic) compared with insulin sensitive patients, that is lost upon establishment of T2D in the group 4(IR Diabetic), where is observed a significant decrease of gene expression compared with group 3. Indeed, *NPY1R* gene expression significantly increased in group 3 ($p<0.05$ vs IS NG (group 1)) and decreased in group 4 ($p<0.05$ vs IR Pre-diabetic (group 3)) (**Figure 4B**). Moreover, NPY5R gene expression, despite not having a significant increase in group 3, decreases drastically in group 4 compared with IR NG and IR Pre-diabetic group ($p<0.05$ vs IR NG (group 2), $p<0.01$ vs IR Pre-diabetic (group 3)) (**Figure 4C**). On another hand, *NPY2R, PPY1R* and *NPY* gene expression shows a different alteration pattern among the groups, with a decreased of expression that occurs upon insulin resistance establishment. Regarding NPY2R it is possible to observe a significant decrease in group 3 ($p<0.0005$ vs IS NG (group 1)) and 4 ($p<0.05$ vs IS NG (group 1)) compared with IS NG group (**Figure 4E**). In patients that belong to IR NG group is possible to observe a tendency to a significant decrease compared with the IS NG group ($p=0.0518$ vs IS NG) (**Figure 4E**). *PPY1R*, also known as *NPY4R*, and *NPY* expression decreases significantly in group 3($p<0.05$ vs IS NG (group 1); $p<0.05$ vs IS NG (group 1)) and 4($p<0.05$ vs IS NG (group 1); $p<0.05$ vs IS NG (group 1)), compared with IS NG group (**Figure 4D and 4A**).

Dopaminergic machinery also is affected by the development visceral adipose tissue metabolic dysregulation. The expression of *D1R* and *D4R* in our patients similarly varies among the groups. In *D1R* case, we observe a significant reduction of gene expression in the IR NG group ($p<0.05$ vs IS NG (group 1)) that is maintain throughout group 3 ($p<0.05$ vs IS NG (group 1)) and 4 ($p<0.05$ vs IS NG (group 1)), compared to IS NG group (**Figure 5A**). Similarly, *D4R* expression is significantly reduced in group 3 ($p<0.05$ vs IS NG

(group 1)) and 4 ($p<0.05$ vs IS NG (group 1)) (**Figure 5D**). In fact, these two genes showed a similar variation as the *NPY2R*, *PPY1R* and *NPY*, with a reduction that happens with the establishment of insulin resistance and the development of T2DM. Regarding *D2R* (**Figure 5B**) and *D3R* (**Figure 5C**), no significant alteration were found among the groups.

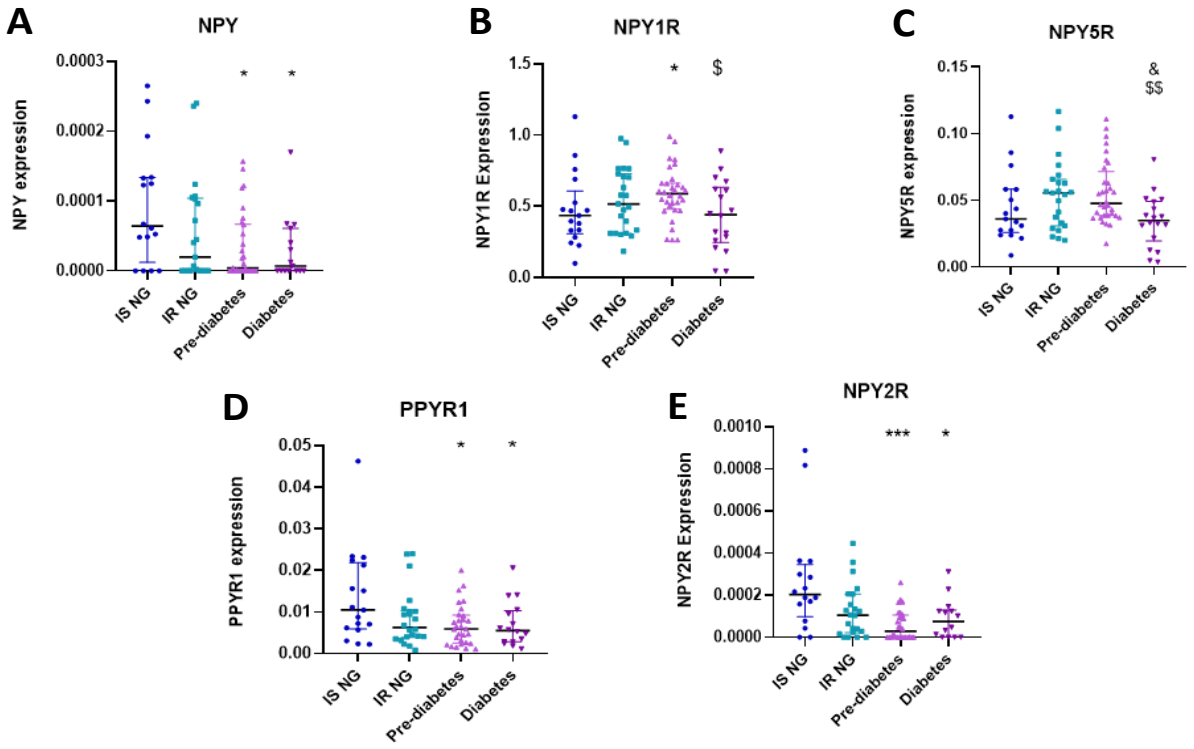

Figure 4. Gene expression of the NPYergic machinery in the VAT of patients with obesity at different stages of metabolic dysregulation. *NPY1R expression (**B**) was significantly increased in pre-diabetes patients, followed by a drastically reduction in the T2DM group. Similarly, NPY5R expression (**C**) also has a drop of expression with the aggravation of T2DM in group 4. The expression of NPY2R (**E**), PPY1R (**D**) and NPY (**A**) significantly decreases in pre-diabetes patients and this reduction is maintain in group 4 T2DM patients. Data is presented as median and interquartile range per group and Kruskal-Wallis comparations were conducted to compare among the groups, since groups had reduced sample size (<30). $p<0.05$ was considered significant. * vs IS NG; & vs IR NG; $ vs IR Pre-diabetes. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$. This data was obtained from a Master thesis, performed at University of Coimbra, named "The gut-adipose tissue crosstalk: Unravelling the mechanisms of ghrelin-mediated regulation of adipose tissue angiogenesis", by Daniela Rosendo da Silva.*

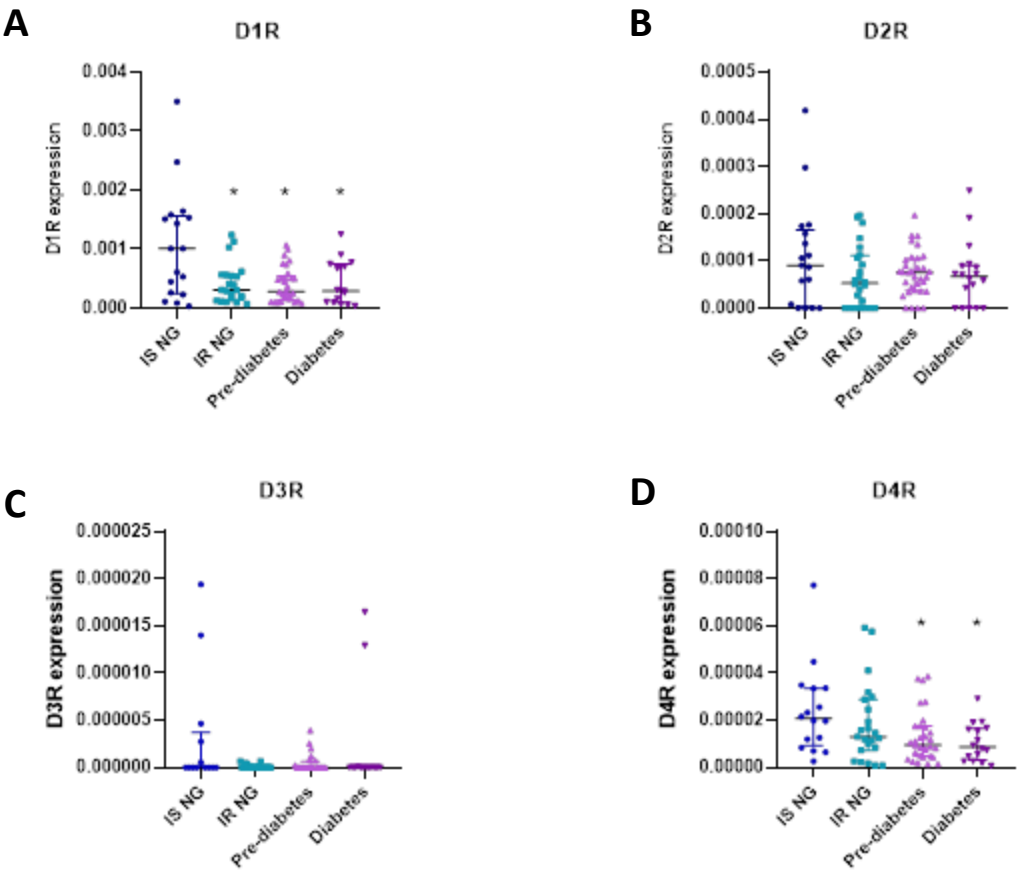

Figure 5. Gene expression of the Dopaminergic machinery in the VAT of patients with obesity at different stages of metabolic dysregulation *D1R expression (**A**) was significantly reduce in IR NG, pre-diabetes, and diabetic patients. Similarly, D4R expression (**D**) also reduces in pre-diabetes and diabetes group of patients. No alterations of expression were observed in D2R (**B**) and D3R (**C**) expression. Data is presented as median and interquartile range per group and Kruskal-Wallis comparations were conducted to compare among the groups, since groups had reduced sample size (<30). p<0.05 was considered significant. * vs IS NG; & vs IR NG; $ vs IR Pre-diabetes. 1 symbol p<0.05; 2 symbols p<0.01; 3 symbols p<0.001. This data was obtained from an article already published by our group* (102).

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Main Objectives

To study the crosstalk between NPY and Dopaminergic system in peripheral tissues, the metabolic outcomes that result from this interaction, mainly in the WAT, and to understand how obesity and the development of metabolic dysregulation affects these systems in the AT.

Specific Objectives

1- Study the correlations between NPY and dopamine system in WAT from obese individuals with different stages of metabolic dysregulation;
2- Understand how bromocriptine modulates NPY system in peripheral tissues from an obese T2DM rat model;
3- Determine the metabolic effects of the stimulation of *ex vivo* samples with dopamine, NPY and different antagonists for dopamine receptors;
4- Determine the effect of dopamine and NPY stimulation in the crosstalk, in adipocytes *in vitro*.

CHAPTER 3 – MATERIALS AND METHODS

Reagents

Unless stated elsewhere, all reagents and common products were bought from Sigma - Merck (USA) and Fisher Scientific - Thermo Fisher Scientific (USA).

In vitro study

Materials

Fetal Bovine Serum was purchased from Gibco (Barcelona, Spain). Cell plates were obtained from Corning (Tewksbury, Massachusetts, USA); Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, Oil Red O staining and isopropanol were obtained from Sigma (St. Louis, Missouri, USA).

3T3-L1 Cell Handling: Culture, differentiation and treatment

3T3-L1 Culture

3T3-LI pre-adipocytes were obtained from American type Culture Collection-LGC Promochem (Barcelona, Spain). The cells were cultured in Dulbecco's Modiefied Eagles Medium (DMEM) high glucose, purchased in Sigma (St. Louis, MO, USA), supplemented with sodium pyruvate (Gibco, Barcelona, Spain) at 400 mM, pH 7.4, and 1% antibiotic/antimycotic (Gibco, Barcelona, Spain). They were incubated in a 5% CO_2 95% air atmosphere incubator. Confluence was obtained after approximately 48 hours of culture.

3T3-L1 Differentiation and treatment

3T3-LI preadipocytes need to be differentiated to originate mature adipocytes. The cells, depending on the assay to be performed, were seeded in different multi-well plates, and incubate thill reaching 70-80% of confluence (normally after 2/3 days). After this, the medium was replaced for fresh one supplemented with the differentiation

cocktail (day 0). This cocktail was constituted by IBMX (0.5 mM), insulin (10 µg/mL) and dexamethasone (1 µg/mL). After incubation for 3 days, the medium was removed and, depending on the experiment, replaced for fresh one supplemented with the respective drugs, but without the differentiation cocktail (3rd day). In the fifth and in the seventh day, the medium was replaced as in the third day (5th or 7th day). After two days, the cells were either stained or collected to produce cellular extracts (9th day).

Cells in control conditions were incubated with IBMX and dexamethasone at day 0 and then, in 3rd, 5th and 7th were only incubate with fresh medium without any supplementation. Positive control cells were supplemented with insulin (10 µg/mL) in day 0, 3, 5 and 7. In order to observe the effects of NPY and Dopamine in adipocyte's metabolism, the cells were treated with different concentrations of NPY_{3-36} (1 and 3 µM) and Dopamine (1µM). The cells treated with dopamine were also supplemented with SCH 23390 (D1-like antagonist) and Domperidone (D2-like antagonist), to observe how the different modulation of Dopaminergic system will affect the NPY system in the adipocytes.

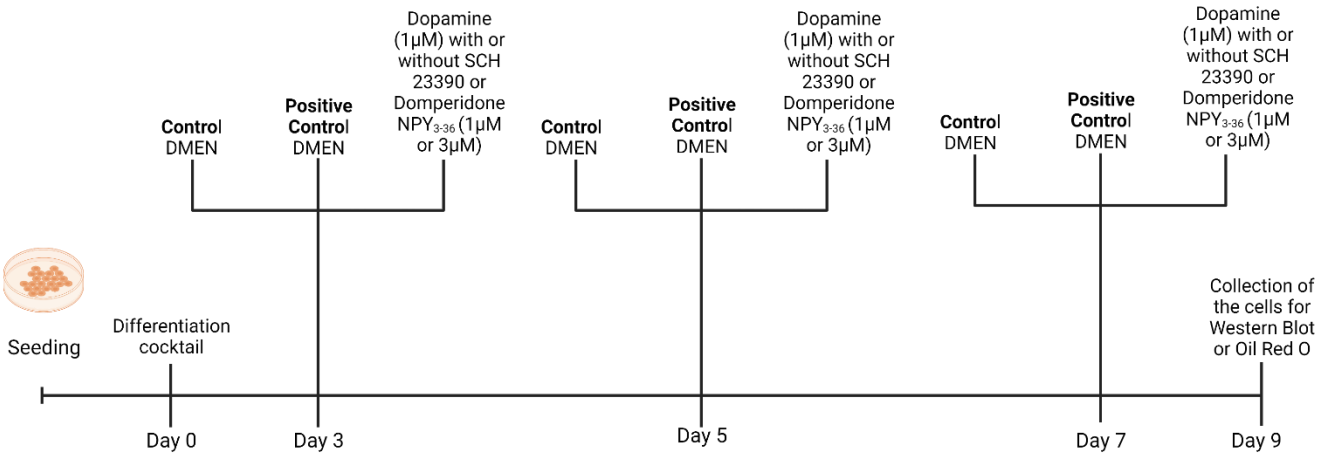


Figure 6. Experimental design for 3T3-L1 pre-adipocytes differentiation and treatment. *When the cells reached confluency were differentiated with differentiation cocktail (day 0). After this phase, the cells were treated in day 3, 5 and 7, and collected in day 9 for Oil Red O staining or Western Blot analysis. Created with BioRender.com.*

Western Blotting of cellular extracts

For Western Blot analysis of cellular protein, cells were seeded in 6-well plates and the differentiation protocol was applied when cells reached 70-80% of confluency (2-3 days). In the 9th day of experiment, the cells were washed with ice-cold phosphate-buffered saline (PBS) and disrupted in lysis buffer (0.25 M Tris-HCL, 125 mM NaCL, 1%Triton-X 100, 1mM EDTA, 1mM EGTA, 20mM NaF, 2mM Na_3VO_4, 10 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 10 mM phenylmethylsulphonyl, and 40 µL of protease inhibitor, pH 7.7). After this, cells went through 3 cycles of freezing/thawing and were both sonicated (10 seconds at 50% amplitude) and centrifuged at 14,000 rpm at 4ºC for 20 minutes. Then, the supernatant was collected and 10µL were separate to obtain protein through bicinchoninic acid (BCA) method (Alfa Aesar, USA). 2x Laemmli buffer (62.5 mM Tris-HCL, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) was added to the rest of the sample's supernatant, and the samples were re-sonicated and boiled at 95ºC for 3 minutes. Vertical electrophoresis was carried out in 10% polyacrylamide gel, divide in resolving (0.75 M Tris-HCl, 0.2% SDS, pH 8.8) and stacking (0.25 M Tris-HCl, 0.2% SDS, pH 6.8) plus acrylamide, Mili-Q water, ammonium persulfate and tetramethlyethylenediamine. Running buffer (125 mM Tris-base, 480Mm glycine, 1% SDS, pH 8.8) was prepared and added to the electrophoresis system (Bio-Rad, USA). After loading equal amounts of protein per sample into the gel, as well as protein standard marker (GRiSP, Research Solutions, Portugal), the protein migration started with a constant voltage of 120 V. SDS-polyacrylamide gels were transferred electrophoretically, at constant 750 mA for 2 hours, to polyvinylidene difluoride membranes (GRISP, Research Solutions, Portugal), that were previously activated in methanol, hydrated in ultra-pure water and washed 15 minutes in transfer buffer (50 mM CAPS, 2% NaOH, 10% methanol, pH 11). To block the membranes, these were incubated in 5% albumin in wash buffer (250 mM Tris, 1.5 mM NaCl pH=7.6 plus 0.5% Tween 20) for 2 hours. After this, membranes were incubated overnight with primary specific antibodies, followed by incubation with HRP-conjugated specific secondary antibodies for 2 hours at room temperature. All the antibodies were diluted in wash buffer plus 0.01% Tween20 and 1% bovine serum albumin, according to the specific optimal dilution of each antibody.

Chemiluminescent method using an ECL/substrate (1:1) was used to revealed membranes and the luminescence detection system used was ImageQuant LAS500 Software (GE Healthcare, United Kingdom). Image data was processed through Image Lab software (Bio Rad, USA).

Antibodies

Table 1. Primary antibodies used in Western Blot

Antibody	Molecular Weight(kDa)	Dilution	Manufacturer (Referência)	Secondary Antibody
Anti-Calnexin	≈ 83 kDa	1:1000	Sicgen, Portugal (AB0037)	Anti-Goat
Anti-GAPDH	≈ 37 KDa	1:1000	Sicgen, Portugal (AB0049-20)	Anti-Goat
Anti-NPY1R	≈ 52 KDa	1:1000	Bio-Rad, USA (6732-0150)	Anti-Sheep
Anti-NPY2R	≈ 52 KDa	1:1000	Abcam, UK (ab31894)	Anti-Goat
Anti-NPY5R	≈ 52 KDa	1:1000	Invitrogen, USA (PA5-106850)	Anti-Rabbit
Anti-D1R	≈ 48 KDa	1:1000	Abcam, UK (ab81296)	Anti-Rabbit
Anti-D2R	≈ 52 KDa	1:1000	Abcam, UK (ab85367)	Anti-Rabbit
Anti-phopho-ATGL	≈ 55 KDa	1:1000	Abcam, UK (ab135093)	Anti-Rabbit
Anti-phospho-AMPK	≈ 63 KDa	1:1000	Cell Signalling, USA (2535S)	Anti-Rabbit
Anti-phospho-ACL	≈ 125 KDa	1:1000	Cell Signalling, USA (4331P)	Anti-Rabbit

Oil Red O staining

Firstly, cells were seeded in 48-well plates and went through the differentiation and treatment protocol, already explained. On the 9th day, the cells were washed with ice cold PBS and fixed using formaldehyde 4% for 30 minutes at room temperature. Meanwhile, the Oil Red O working solution was prepared from the stock solution (0.5% Oil Red O in 100% isopropanol), in a proportion of 3 parts of stock with 2 parts of distilled water, followed by filtration in a 0.2 µm syringe filter. Before adding the Oil Red O

solution, cells were re-washed twice with PBS and one with distilled water and incubated with 60% isopropanol for 5 minutes at room temperature, that was removed before the addition of the Oil Red O solution. The staining phase was carried out in a plate shaker for 30 minutes at room temperature. Before the images were obtained, the excess of staining was removed from cells with distilled water and the wells dried.

Ex vivo study

All experimental and care procedures conducted on the animals were approved by the Ethics Committee of Faculdade de Ciencias Médicas|Nova Medical School and by Direccao-Geral de Alimentacao e Veterinária (DGAV), the Portuguese National Authority for Animal Health Animal Care. The animals used in this experience were 8- to 10- week-old male Wistar rats (200-300g) obtained from the vivarium of the Faculdade de Ciências Médicas|Nova Medical School, Portugal. The animals used were kept under controlled conditions (temperature: 21±1ºC; humidity: 55±10%), with a light/dark cycle of 12 hours and ad libitum access to food and water. After an overnight fasting episode, the animals were euthanized with sodium pentobarbital (60mg/kg, i.p.) and the epididymal and mesenteric white adipose tissue were collected and rapidly stored in an ice-cold 21% O_2/ 5% CO_2-equilibrated tyrode solution (in mM: 140 NaCl. 5 KCl, 2 CaCl2, 1.1 MgCl2, 10 HEPES and 5.5 glucoses, pH 7.40). After this, the tissue was divided in 3-4 pieces of tissue per Eppendorf tube (2mL) and were incubated at 37ºC during 15 minutes for stabilization. The following step was to move the pieces to other tube where they were incubated for 15 minutes per piece with different experimental conditions. The conditions that the samples were incubated were: I-Control; II-Dopamine (10 µM); III-NPY_{3-36} (500 nM); IV- Dopamine (10 µM) and NPY_{3-36} (500 nM); V-Dopamine (10 µM), NPY_{3-36} (500 nM) and SCH 23390 (10 nM); VI-NPY_{3-36} (500 nM) and SCH 23390 (10 nM); VII-Dopamine (10 µM), NPY_{3-36} (500 nM) and Domperidone (50 nM); VII-NPY_{3-36} (500 nM) and Domperidone (50 nM). All these drugs used were prepared in the day of the experiment.

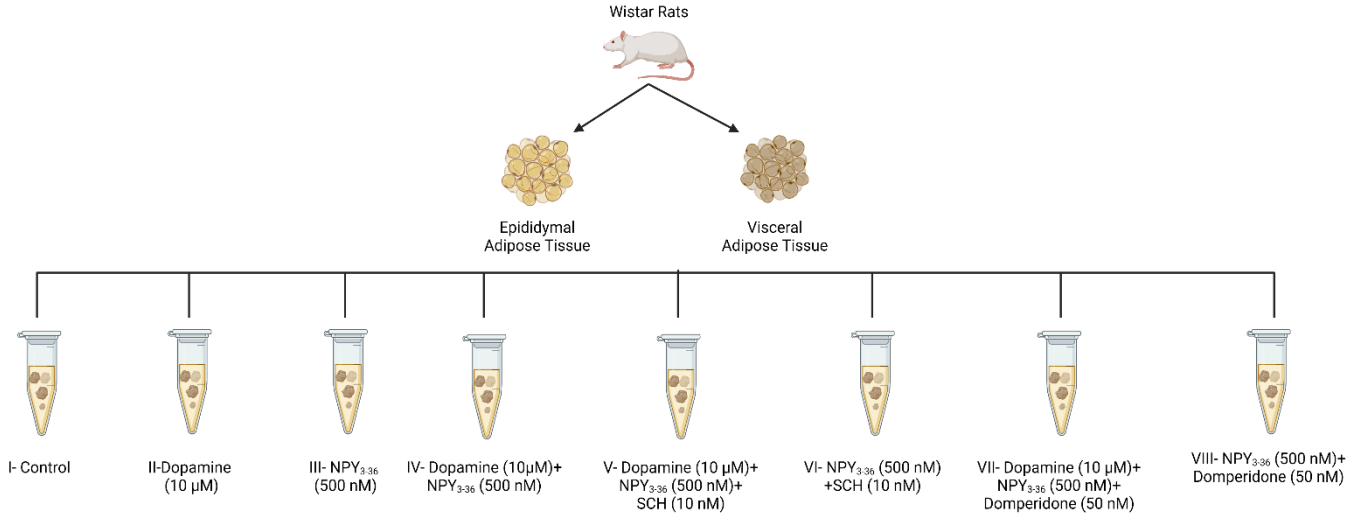


Figure 7. Ex vivo experimental design. *Wistars rats were kept at control conditions and euthanized when growth between 8 to 10 weeks old. WAT samples were collected and divided in 3-4 pieces off tissue for Eppendorf tube. After the stabilization of the samples, these were incubated during 15 minutes in different conditions: I-Control; II-Dopamine (10 µM); III-NPY_{3-36} (500 nM); IV- Dopamine (10 µM) and NPY_{3-36} (500 nM); V-Dopamine (10 µM), NPY_{3-36} (500 nM) and SCH (10 nM); VI-NPY_{3-36} (500 nM) and SCH (10 nM); VII-Dopamine (10 µM), NPY_{3-36} (500 nM) and Domperidone (50 nM); VIII-NPY_{3-36} (500 nM) and Domperidone (50 nM). Created with BioRender.com*

Western blotting analysis of *ex vivo* samples

After the collection of the tissues (mesenteric and epididymal WAT), these were washed with PBS. To produce the tissue homogenates, the tissues were disrupted in lysis buffer in a proportion of 250 mg of tissue/ 0.5 mL of lysis buffer (0.25 M Tris-HCl, 125 mM NaCl, 1% TritonX-100, 0.5% SDS, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 2 mM Na3VO4, 10 mM βglycerophosphate, 2.5 mM sodium pyrophosphate, 10 mM PMSF, 40 µL of protease inhibitor) using TissueLyser system (Quiagen, Germany). The lysates were cleared by centrifugation for 15 minutes at 14000 rpm, 4ºC, allowing a separation between the fat layer (top layer) from the protein fraction (supernatant) and from the membranes and nucleic acids present on the pellet. The supernatant was then collected and stored at a -80ºC. The following protocol is the same as the one described above for cellular extracts.

In vivo study

Diet-induced model of obesity and T2DM

This animal study involves three different groups of rats. The experimental protocol was approved by the local Institutional Animal Care and Use Committee (ORBEA 04-2015) of Faculty of Medicine if the University of Coimbra and DGAV, and all the procedures were performed by licensed users of Federation of Laboratory Animal Science Associations (FELASA) and in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Wistar rats were obtain from our breeding colonies (Faculty of Medicine, University of Coimbra) and were kept under standard conditions and in a controlled environment with day-night cycles of 12 h, a relative humidity of 50-60% and temperature between 22-24ºC. Male Wistar rats fed with a standard diet for 6 months were used as control (WSD). The other strain used was Goto Kakizaki (GK) rats, spontaneous type 2 diabetic rats, that were also acquired from local breeding colonies and kept in the same conditions. At 1 month old , GK rats were randomly divided in two groups, the first, maintained with the same standard diet (A03; 5% triglycerides and 45% carbohydrates, SAFE, France) until the end of the experiment at 6-months old (GKSD), while the other group was fed a high-caloric diet (HCD) (customized AO3 high-caloric diet with 20%fat plus 20% sucrose, SAFE, France), in the same period, to induce weight gain. In the last month of the protocol, GK HCD-fed group was randomly divided into three different groups: the first that maintain the diet without further treatment (GKHCD), the second group that were treated with bromocriptine in the last month (agonist for the dopamine 2 receptor) (GKHCDBr) and the last one that was treated only with the vehicle, without bromocriptine, during the same period of time (GKHCDVh). Bromocriptine was gently supplied by Generis® (Amadora, Portugal). The drug was diluted in DMSO in a 1:4 DMSO/H_2O proportion and was daily administered by intraperitoneal (i.p.) injection in a concentration of 10mg/Kg/day, during the period of treatment. The vehicle group was i.p. injected with the same volume (100 uL) of the vehicle 1:4 DMSO/H20, during the same period. The animals were sacrificed after 6 months by cervical displacement, and subcutaneous white adipose tissue (SAT), epididymal white adipose tissue (EWAT), brown adipose tissue (BAT), muscle and liver were collected, weighted, and stored at -80ºC.

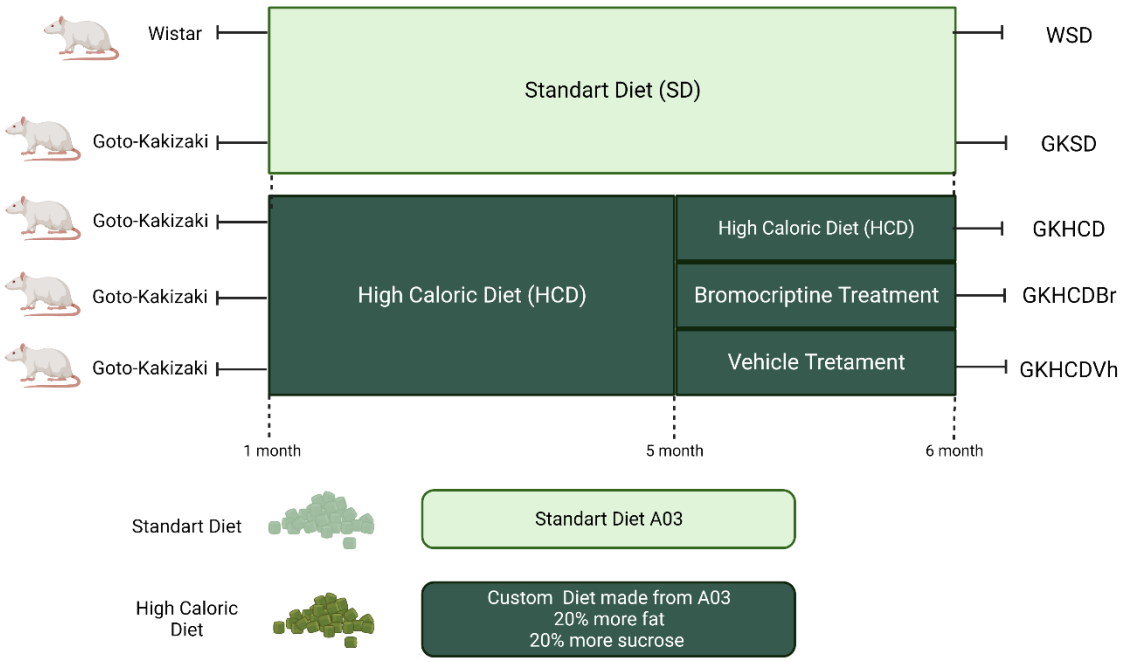


Figure 8. Animal model experimental design. *Wistars rats were fed a standard diet A03. Goto-Kakizaki rats were fed either standard or high caloric diet, that had 20% more fat and sucrose than the standard diet. In the 5th month, part of the diet-induce obese Goto-Kakizaki rats were treated either with bromocriptine (10mg/Kg/day) or with the vehicle (1:4 DMSO/H20). The experiment ended at the 6th month with the sacrifice of the rats and the collection of the tissues. Created with BioRender.com*

Western blotting analysis of tissue homogenates

To produce the tissue homogenates, visceral and subcutaneous WAT, BAT, liver and muscle were disrupted in lysis buffer (0.25 M Tris-HCl, 125 mM NaCl, 1% TritonX-100, 0.5% SDS, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 2 mM Na3VO4, 10 mM βglycerophosphate, 2.5 mM sodium pyrophosphate, 10 mM PMSF, 40 µL of protease inhibitor) using TissueLyser system (Quiagen, Germany). The lysates were cleared by centrifugation for 15 minutes at 14000 rpm, 4ºC, allowing a separation between the fat layer (top layer) from the protein fraction (supernatant) and from the membranes and nucleic acids present on the pellet. The supernatant was then collected and stored at a -80ºC. The following protocol is the same as the one described above for cellular extracts.

NPY ELISA Kit

NPY levels were assessed in epididymal white adipose tissue of the different animal models, using a Rat Neuropeptide Y ELISA Kit (MYBioSource, USA). For tissue homogenates preparation, 100 mg of tissue were homogenized in 1 mL of 1X PBS using TissueLyser system (Quiagen, Germany). After two freeze-thaw cycles, the samples were centrifuged for 5 minutes at 5000x g and the supernatant was collected and assayed immediately. The experiment was performed according to the protocol provide by the kit. Ultimately, the plate was read using a microplate reader set to 450 nm and using Gen5 software. A standard curve was construct and the concentrations of the samples were calculated.

Human study

Selection and characterization of patients

Patients from the obesity surgery consultation at the Hospital Geral de Coimbra (Covões) with obesity, with or without diabetes and with 25-65 years were recruited to participate on a prospective cross-sectional study entitled “Estudo Anatomo-Morfológico do Tecido Adiposo na Obesidade”. This study was approved by the Ethical Committee of Centro Hospitalar e Universitário de Coimbra and all patients that participated in the study signed an informed consent according to the principles defined in the Declaration of Helsinki. The exclusion criteria were active inflammatory and/or chronic diseases, previous restrictive/malabsorptive surgical procedures and other T2DM medication than metformim

Patient’s clinical data and tissue sample collection

Fasting blood samples were collected, one day before surgery, followed by isolation of serum and plasma. The samples were stored at -80ºC. An automatic analyzer was used to measure several blood parameters, such as insulin, fasting glucose, glycated haemoglobin (HbA1c), cholesterol and triglycerides levels. The percentage of β-cell

function was calculated using fasting glucose and insulin levels. The same was used to assess the homeostasis model assessment 2 insulin resistance index (Ox-HOMA2IR) (Oxford, United Kingdom), that, different from the normally used HOMA, this index accounts for variations in hepatic and peripheral insulin resistance, increases in insulin secretion curve for plasma glucose concentrations superior to 180 mg/dL and also the contribution of circulating proinsulin. The patients from which the VAT samples were collected were undergoing metabolic surgery, and after the biopsy the samples were kept in liquid nitrogen until arriving to our facilities, where they were stored at -80ºC. The generated clinical data was organized in a database, using SPSS Statistic Software, version 24.0 (IBM, USA) and kept in a cloud-based and confidential environment.

Characterization and criteria of assignment of each group

The samples were collected from a total of 93 patients, that were divided in four different groups, with patients with distinct glycaemic profile (fasting glucose levels, HbA1c and Ox-HOMA2IR). Group 1 (n=17) is composed by subjects that have insulin sensibility and are normoglycemic (NG) (Ox-HOMA2IR<1) - Insulin Sensitive group (IS). Group 2 (n=24) patients are characterized by having insulin resistance (IR) (Ox-HOMA2IR<1) and being normoglycemic (fasting glucose<100 mg/dL and HbA1c<5.7%) - Insulin Resistant and NG group (IR/NG). Group 3 (n=34) is constituted by individuals with IR and that have fasting glucose levels between 100-125 mg/dL or HbA1c between 5.7-6.4% - Pre-diabetes group. The patients from the last group (n=18) are characterized by being IR and having T2DM (fasting blood glucose>125mg/dL or HbA1c>6.4%)- T2DM group.

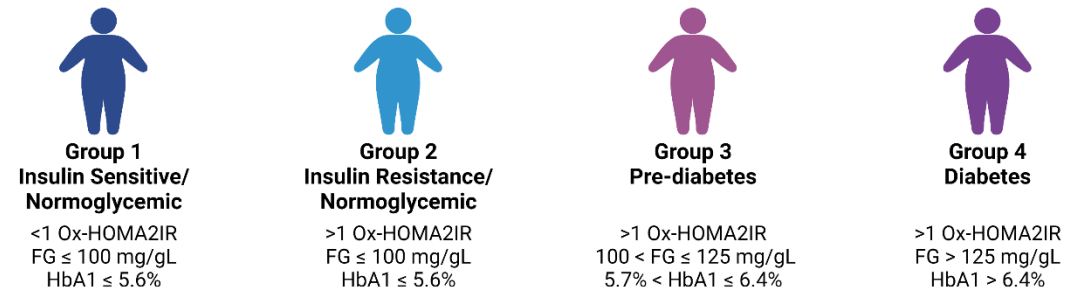


Figure 9. Groups created to divide the different patients. *Patients were divided according to the glycemic profile and the sensibility to insulin. The parameters taken in account to this division were fasting glycemia (FG), glycated haemoglobin (HbA1c) and homeostasis model assessment 2 insulin resistance index (Ox-HOMA2IR). Created with BioRender.com*

RNA extraction from human visceral adipose tissue samples

The total RNA was extracted from 100 mg of human VAT. It was used the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany) to perform the isolation of the RNA. After this step, the samples were analyzed by NanoDrop One/One spectrophotometer (ThermoFisher, Waltham, MA, USA) at a wavelength of 260 nm, in order to evaluate RNA concentration and to see if there was protein or phenol contamination. Capillary electrophoresis, with an Agilent RNA 6000 Nano Kit, was also used to analyze these samples and the results obtained with the Agilent 2100 Bioanalyser (Agilent Technologies, CA, USA), at the *Laboratório de Biomedicina Mitocondrial e Teranóstica, Centro de Neurociências e Biologia Celular*, provided information about the RNA integrity.

Quantitative Real-Time Polymerase Chain reaction using the Hign-Throughput Platform Biomark™ HD system

The RNA samples were all diluted in order to have the same concentration (25 ng/µL). To obtain cDNA, each 4 µL of RNA sample was mixed with Reverse transcriptase (1 µL) [qScript cDNA super mix (Quanta BioSciences)]. The cDNA was pre-amplified and, after that, 1.25 µL of each sample was mixed with 0.5 µL of a mix of pooled primers

(500nM final concentration each), 2.25 µL water and 1 µL of PreAmp Master Mix enzyme (Fluidigm). Thermal cycling was performed according to the enzyme manufacturer for 12 cycles. Exonuclease I (New England Biolabs) was used to remove unincorporated primers, and, after this treatment, the samples were diluted in 5x TE buffer, constituted by 10 mM Tris-HCL and 1mM EDTA. A Pre-Mix was prepared with 2.5 µL SsoFast Eva Green Supermix (Biorad), 0,25 µL of 20x DNA binding dye sample reagent (Fluidigm). The samples (5 µL) were pipetted to the respective inlet of a Fluidigm® 96.96 Gene expression IFC. To every gene measured, a mix of 12 µL was prepared with µL of 2x Assay loading reagent (Fluidigm), 5.4 µL of TE buffer, 1.2 µL from a stock of 50 µM each mixed forward and reverse primers. The assays (5 µL) were pipetted into their respective assay inlets on the chip. The assay and sample mixes were loaded with the Load mix (136x) script of the HX controller (HD Biomark). After loading the chip qRTPCR was carried out using the BioMark HDTM, accordingly to the cycling parameters recommended by Fluidigm® for 96.96 Gene expression Integrated Fluidic Circuit. Data was then collected using Dat Collection Software and all of it was analyzed in Fluidigm® Real Time PCR Analysis v2.1 software. To this analysis, genes that exhibit more than one peak in the melting curve, which reflect the amplification of non-specific products, were not included. The gene data was normalized for the reference gene that was *ATCB*. All the primers were obtained from Sigma and reconstituted to a final concentration of 100 µM in water.

Table 2. Gene of interest used in the human study

Gene	Gene I.D.	KiCqStart® SYBR® Green Primer Pair
NPY	4852	1
NPY1R	4886	1
NPY2R	4887	1
NPY5R	4889	1
PPY1R	5540	1
DRD1	1812	1
DRD2	1813	1
DRD3	1814	1
DRD4	1815	1
PLIN1	5346	1
UCP1	7350	1
PPARG	5468	1
PPARA	5465	1
PGK1	5230	1
INSR	3643	1
SOD1	6647	1
SOD2	6648	1
NFE2L2	4780	1
VEGFR-1/Flt-1	2321	1
TEK_Tie2	7010	1

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 (GraphPad Software, Inc, San Diego, USA). The normality of the data was assessed with Shapiro-Wilk normality test. Accordingly, data with two conditions were analysed with nonpaired t-test or Mann-Whitney test and data with more than two conditions analysed with Kruskal-Wallis test or with one-way ANOVA followed by Tukey´s post-hoc test. Differences were significant for $p < 0.05$.

CHAPTER 4 – RESULTS

Body mass index is not correlated with the progressive establishment of metabolic dysregulation, insulin resistance and AT dysfunction

To demonstrate that the BMI isn´t correlated with alteration of metabolic parameters and establishment of metabolic and AT dysfunction, a Spearman correlation analysis was performed to investigate if the alterations in BMI had a similar behavior as the alterations in the different metabolic parameters. Very low correlations were found between BMI and fasting glucose (r=0.1183; p=0.2751) (**Figure 10 C**), BMI and OX-HOMA (r=0.1578; p=0.1443) (**Figure 10 B**) and BMI and HbA1 (r=0.2988; p=0.0083) (**Figure 10 K**). BMI is also poorly correlated with β-cell function (r=0.02201; p=0.8397) (**Figure 10 D**) and insulin levels (r=0.1482; p=0.1707) (**Figure 10 E**). Regarding adipose tissue dysfunction markers, BMI demonstrate low correlations with adiponectin (r=0.09235; p=0.4093) (**Figure 10 J**), triglycerides levels (r=0.008987; p=0.9345) (**Figure 10 G**), free fatty acids levels (r=0.04981; p=0.6936) (**Figure 10 H**) and HDL cholesterol levels (r=0.1404; p=0.2027) (**Figure 10 F**), but, although also low, BMI correlation with leptin (r=0.2014; p=0.0946) (**Figure 10 I**) is higher compared with the other parameters, which should be expected since leptin is correlated with the total adipose tissue in the body. So, BMI isn't the best parameter to evaluate patient's metabolic condition and the division of the patients in the four groups suggested here, reflects better the evolution of obesity metabolic sequalae, from the MHO to the MUO.

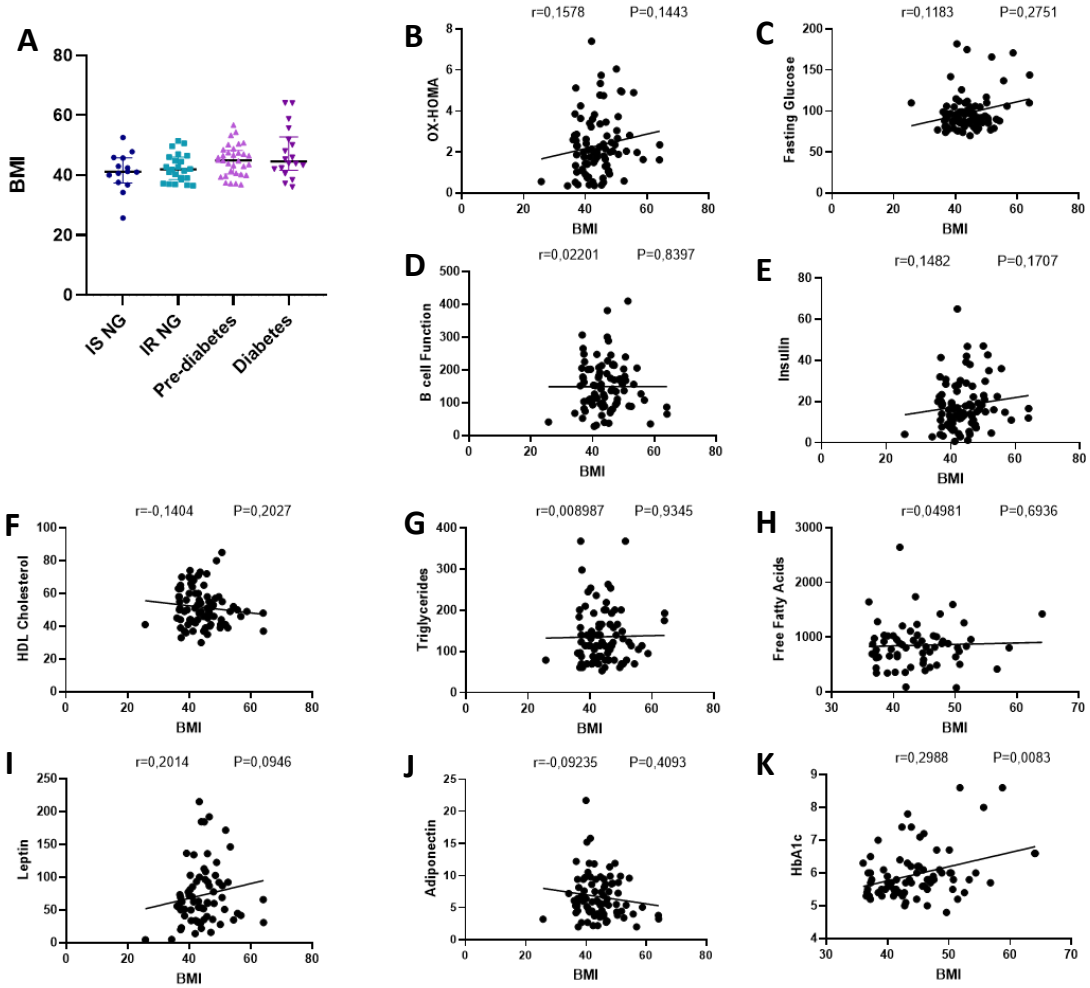


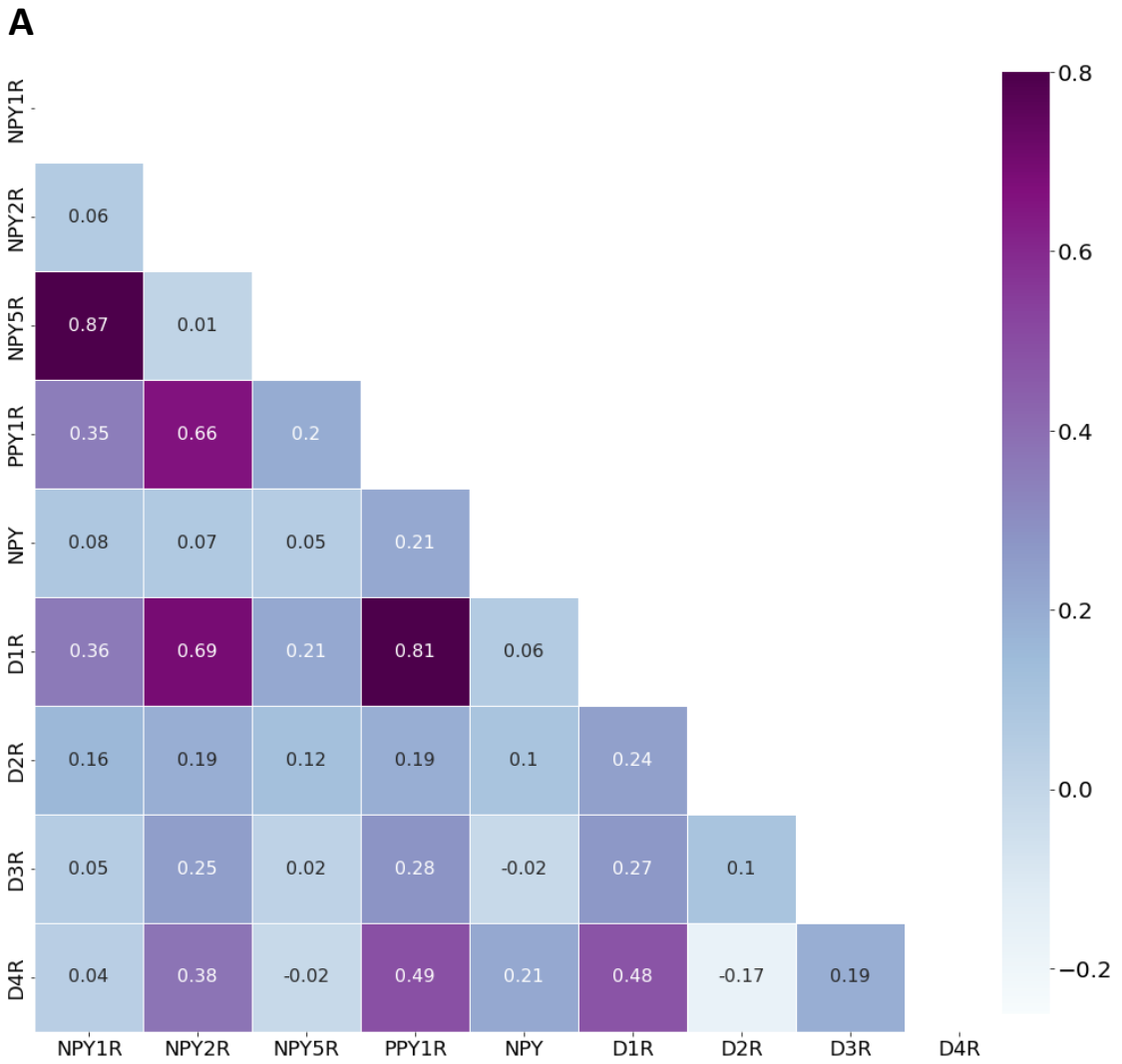
Figure 10. Spearman correlation analysis between BMI and different metabolic parameters in obese patients in different stages of metabolic dysregulation. *BMI is not significantly different among the groups of patients with obesity (**A**). BMI is poorly correlated with all metabolic parameters studied, since OX-HOMA (**B**), fasting glucose (**C**) and HbA1c (**K**), to β-cell function (**D**) and insulin (**E**), and even to the ones related with adipose tissue function as is HDL cholesterol (**F**), triglycerides (**G**), free fatty acids (**H**) leptin (**I**) and adiponectin (**L**).*

NPY and Dopaminergic machinery are correlated between each other in the visceral adipose tissue of patients with obesity in different stages of metabolic dysregulation

Since we previously observed that some receptors had a similar behavior regarding gene expressing along the different groups (described at the scientific framework), we then pursued to analyze the relation between the NPY and the Dopamine machinery in the visceral AT of the different obese patients. Using Spearman correlation analysis, we observed a strong correlation between the *NPY1R* and *NPY5R* gene (r=0.8704; p<0.0001) (**Figure 11A**), that agrees with the similar pattern of gene profile expression that we observed in the two genes along the groups, with a peak of expression in the pre-diabetes group that is lost upon the establishment of diabetes. Regarding the relations with the genes involved in the NPY or Dopamine system, neither *NPY1R* nor *NPY5R* show similar high correlations with other genes such as they have between each other, however, low positive correlations were found between *NPY1R* and *PPY1R* (r=0.349; p=0.001), *NPY1R* and *D1R* (r=0.357; p=0.00048) (**Figure 11A**). *NPY1R* and *NPY5R* correlation with other genes, such as *NPY2R*, *NPY*, *D2R*, *D3R* and *D4R* are all very low and non-significant (**Figure 11A**). Other significant high positive correlations are found between *NPY2R* and *PPY1R* (r=0.663; p<0.0001), *NPY2R* and *D1R* (r=0.692; p<0.0001) and *PPY1R* and *D1R* (r=0.812; p<0.0001) (**Figure 11A**). In agreement with what is observe in the *NPY1R* and *NPY5R* case, the expression of these three genes also demonstrate a similar pattern of gene expression along the groups, with a decrease of expression upon insulin resistance establishment. Low positive correlations, however significant ones are also found between *NPY2R* and *D4R* (r=0.383; p=0.00016), *D1R* and *D4R* (r=0.479; p<0.0001) and *PPY1R* and *D4R* (r=0.486; p<0.0001) (**Figure 11A**). The correlations between these 4 genes and other genes from the NPY and Dopaminergic system are all very low and not significant (**Figure 11A**). So, based on this data we can suggested that the two systems are correlated in VAT.

These results allowed us to identify two different clusters of gene expression. Cluster 2 constituted by *NPY1R* and *NPY5R* (**Figure 11C**), where the genes have a peak of expression in the pre-diabetes group and a significant decrease upon diabetes establishment, and cluster 1 constituted by NPY2R, D1R and PPY1R (**Figure 11B**), where

we observed a significant decrease early in the insulin resistance normoglycemic group, that is maintain in the pre-diabetes and diabetes group. *D4R* isn't present in the cluster 2, because, although it has significant positive correlations with the other genes of the cluster, the values of the correlations are low compared with the values of the correlations between *NPY2R*, *D1R* and *PPY1R*. So, NPY and Dopamine system appear to be correlated in the VAT and, since some of the genes have a similar pattern of expression, we can hypothesize that some of these genes could participate in similar functions or that they may have a common upstream regulator that controls the expression of these genes.



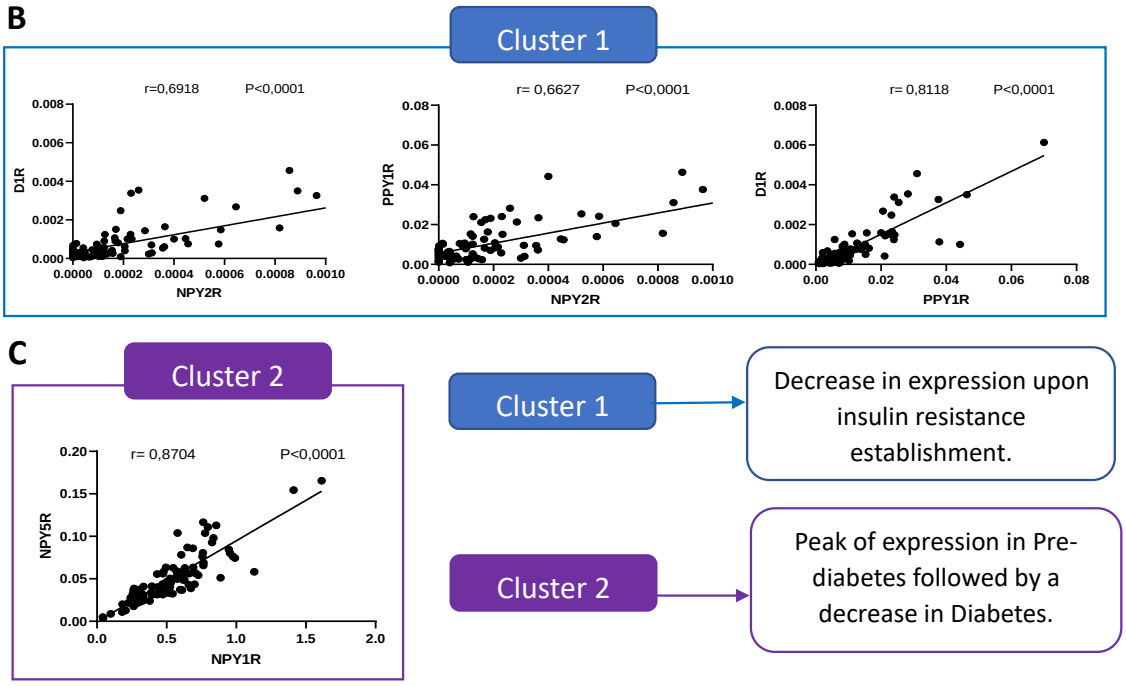


Figure 11. Spearman correlation analysis between NPY and Dopamine systems genes. *D1R, NPY2R and PPY1R expression are highly positive correlated between each other (NPY2R and PPY1R (r=0.663; p<0.0001), NPY2R and D1R (r=0.692; p<0.0001) and PPY1R and D1R (r=0.812; p<0.0001)) (**A**). NPY1R and NPY5R expression are also highly positive correlated in VAT of these patients r=0.8704; p<0.0001) (**A**). There was identified 2 cluster, one constituted by D1R, NPY2R and PPY1R (**B**) and other by NPY1R and NPY5R (**C**), that show similar profile of gene expression variation among the groups.*

NPY and Dopaminergic systems cooperate with each other in the regulation of important functions in visceral adipose tissue of patients with obesity in different stages of metabolic dysregulation

Since NPY and Dopaminergic system are known to participate in several metabolic processes, such as lipid and glucose metabolism, in the WAT and given that the two systems are, as we shown, correlated in the VAT, we then studied if the clusters that were identify where correlated with important proteins for VAT metabolism and function.

Using Spearman correlation analysis, we were able to observe that, in the cohort of patients with obesity, cluster 1 genes, constituted by *NPY1R* and *NPY5R*, showed strong positive correlations with important markers of AT function. Indeed, *NPY1R* and *NPY5R* are strongly correlated with two important genes responsible for lipid accumulation capacity in AT, *PPARG* (vs *NPY1R*: r=0.6788; p<0.0001; vs *NPY5R*: r=0.7794; p<0.0001) (**Figure 12A**), the master regulator of adipogenesis, and, although in a lower extend, with *PLIN1* (vs *NPY1R*: r=0.4305; p<0.0001; vs *NPY5R*: r=0.4841; p<0.0001) (**Figure 12A**), which encodes perilipin A, an important protein for lipid droplet formation. Cluster 1 is also strongly correlated with glucose metabolism markers, *INSR* (vs *NPY1R*: r= 0.5618; p<0.0001; vs *NPY5R*: r= 0.5481; p<0.0001) (**Figure 12B**), which originates insulin receptor protein, and *PGK1* (vs *NPY1R*: r= 0.6355; p<0.0001; vs *NPY5R*: r= 0.5775; p<0.0001) (**Figure 12B**), which originates phosphoglycerate kinase 1, an important protein in glycolysis. Moreover, strong correlations can also be found between cluster 1 genes and cellular antioxidant capacity markers, such as *SOD1* (vs *NPY1R*: r= 0.7471; p<0.0001; vs *NPY5R*: r= 0.7311; p<0.0001), *SOD2* (vs *NPY1R*: r= 0.4085; p<0.0001; vs *NPY5R*: r= 0.3798; p<0.0001) (**Figure 12C**), which encodes to superoxide dismutase 1 and superoxide dismutase 2, respectively, two important proteins in the antioxidant capacity of the cell, and *NFE2L2* (vs *NPY1R*: r= 0.6352; p<0.0001; vs *NPY5R*: r= 0.6008; p<0.0001) (**Figure 12C**), which induces the expression antioxidant response element-dependent genes. Genes from cluster 2 were not correlated with any of the analyzed genes.

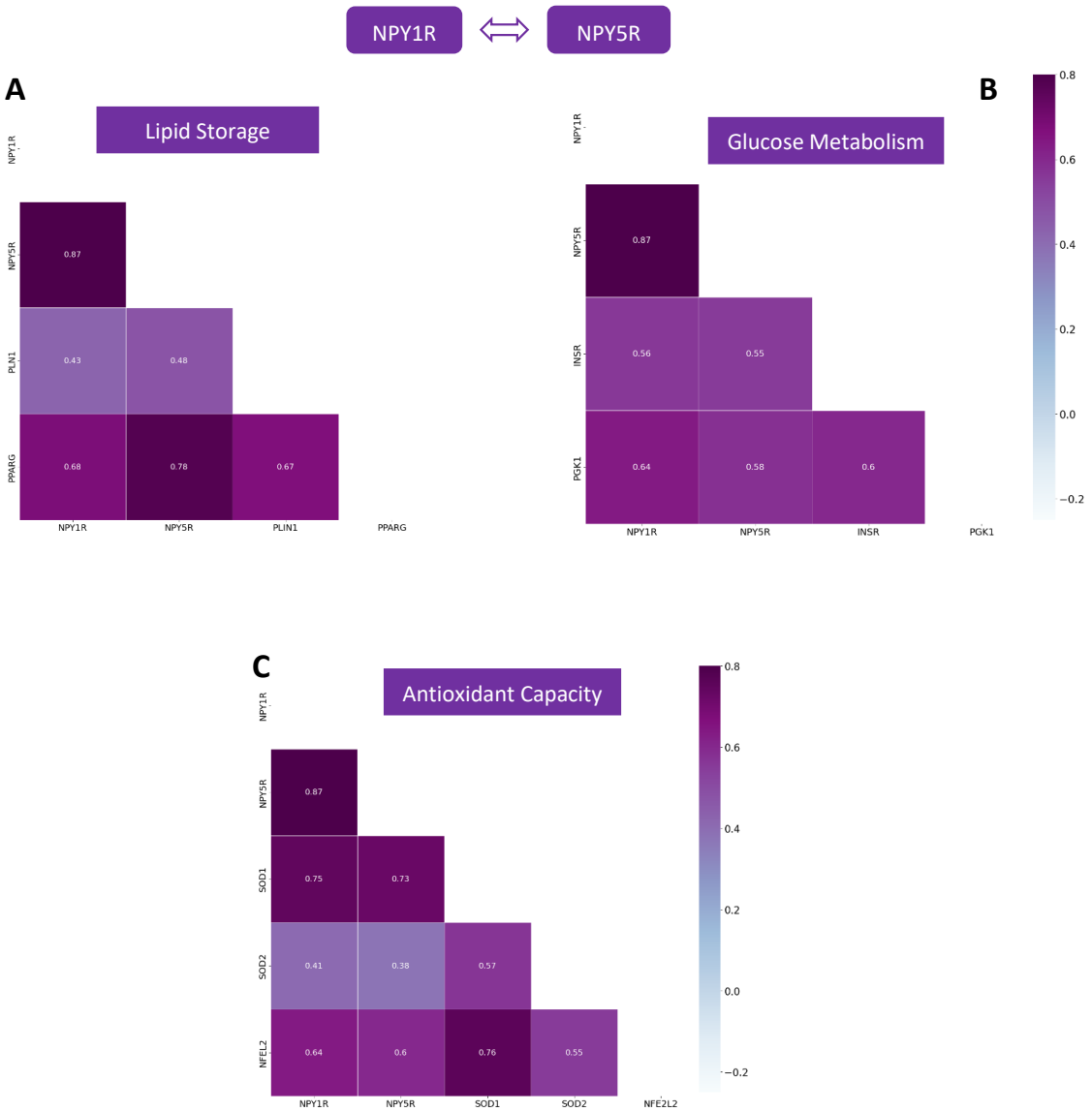


Figure 12. Spearman correlation analysis between Cluster 2 genes and lipid storage, antioxidant capacity and glucose metabolism markers. *NPY1R and NPY5R expression are greatly positively correlated with lipid storage markers, PPARG and PLN1 (**A**), glucose metabolism markers, INSR and PGK1 (**B**), and antioxidant capacity markers, SOD1, SOD2 and NFE2L2 (**C**).*

Regarding cluster 2, formed by *NPY2R*, *D1R* and *PPY1R* genes, these also appear to be correlated with genes that encode important proteins in AT metabolism. Indeed, these cluster genes showed high positive correlations with proteins involved in catabolism. For instance, *PPARA* expression, which regulates the transcription of genes involved in β-oxidation, positively correlated with all the genes from this cluster (vs *NPY2R*: r= 0.3949; p<0.0001; vs *D1R*: r= 0.5522; p<0.0001; vs PPY1R: r= 0.6338; p<0.0001) (**Figure 13A**). Similar to what is observed with the PPARA, UCP1 gene expression encoding for the key protein in thermogenesis mitochondrial uncoupling protein 1 is also highly correlated with the expression of cluster 2 genes (vs *NPY2R*: r= 0.7113; p<0.0001; vs *D1R*: r= 0.8501; p<0.0001; vs PPY1R: r= 0.7874; p<0.0001) (**Figure 13A**). AT metabolism requires a proper and plastic vascular network, to maintain a correct function. So, we wondered if there was a correlation between these systems and markers of microvascular function in AT. From this analysis, we observed that cluster 2 genes were positively correlated with some angiogenic markers. *VEGFR1* (vs *NPY2R*: r= 0.5302; p<0.0001; vs *D1R*: r= 0.6793; p<0.0001; vs PPY1R: r= 0.5218; p<0.0001) and TEK_Tie2 (vs *NPY2R*: r= 0.5429; p<0.0001; vs *D1R*: r= 0.7590; p<0.0001; vs PPY1R: r= 0.7073; p<0.0001) expression (**Figure 13B**), demonstrated high positive correlations with D1R, NPY2R and PPY1R, suggesting that this cluster of receptors can be involved in angiogenesis regulation.

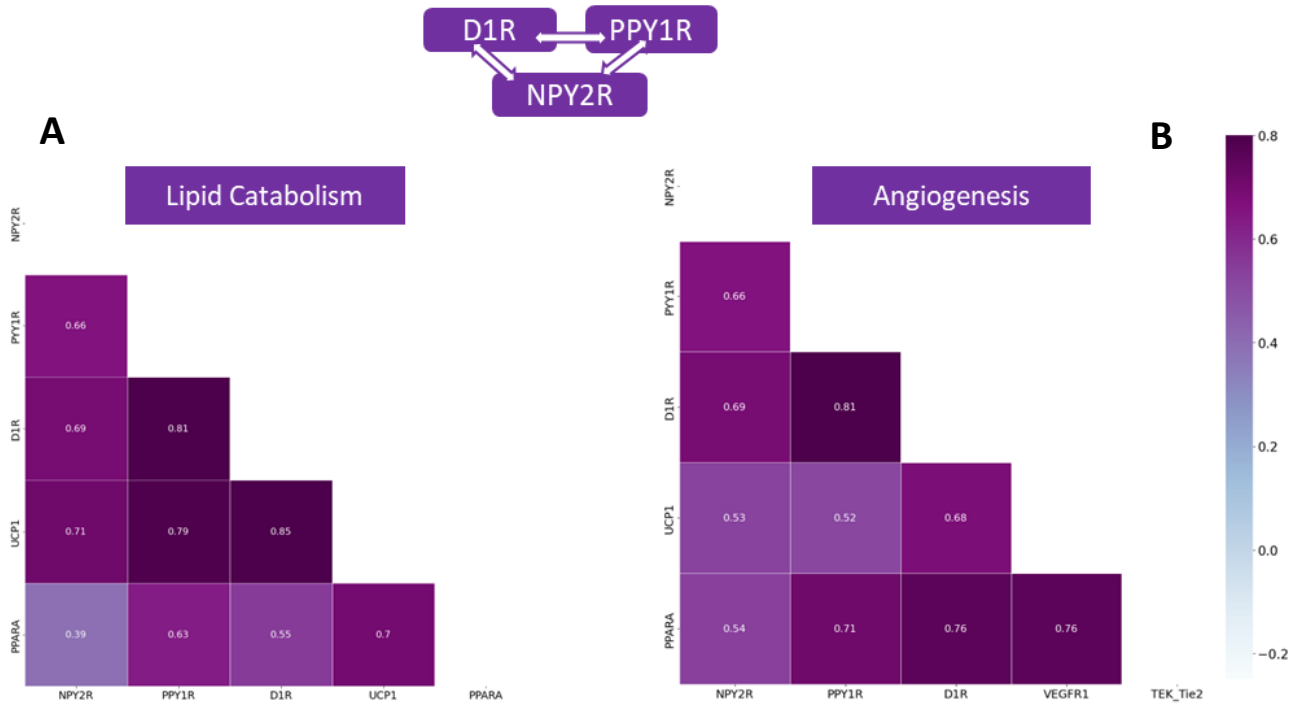


Figure 13. Spearman correlation analysis between Cluster 1 genes and lipid catabolism and angiogenic markers. *D1R, NPY2R and PPY1R expression are highly positively correlated with lipid catabolic markers, PPARA and UCP1 (**A**), and angiogenic markers, VEGFR1 and TEK_Tie2 (**B**).*

Bromocriptine remodels NPY system in pEAT of obese type 2 diabetic rats, but not in other peripheral tissues.

Previously, our group has found that bromocriptine improves metabolic profile of obese type 2 diabetic obese rats and remodels dopaminergic signaling in AT and in the liver, with the upregulation of D1R and TH protein levels in both tissues (102). Since we observe strong correlations between NPY and Dopamine system in VAT in the human study, we next aimed to evaluate if the bromocriptine's modulation of dopaminergic system in the periphery induces alterations in the NPY system in different peripheral tissues.

Indeed, in the peri-epididymal AT (pEAT), bromocriptine induces alteration in the NPY system. The NPY1R was significantly increased in the group treated with bromocriptine compared to GKSD ($p<0.005$), GKHCD ($p<0.05$) and GKHCDVh ($p<0.05$) group (**Figure 14A**). The increase in the group treated with bromocriptine was to levels similar to the control group. Regarding NPY2R levels, no alterations were found in the different groups, with all having similar NPY2R protein levels (**Figure 14B**). Despite not showing statistical difference, is possible to observe a trend to increased NPY5R protein levels in the GKHCDBr group ($p=0.084$ vs GKSD) (**Figure 14C**). Regarding the levels of NPY in the pEAT, we observee a significant decrease in NPY levels in the GKHCD group compared with control rats ($p<0.005$), followed by a significant increase in the GKHCDBr ($p<0.05$ vs GKHCD) (**Figure 14D**). Thus, the treatment with bromocriptine increases both NPY and NP1R protein levels in pEAT.

Regarding the other peripheral tissues studied, subcutaneous AT, liver, muscle and BAT, no significant alterations in the NPY1R, NPY2R and NPY5R protein levels were found between the groups (**Figure 15**). Bromocriptine´s modulation of NPY system only occurs in the pEAT.

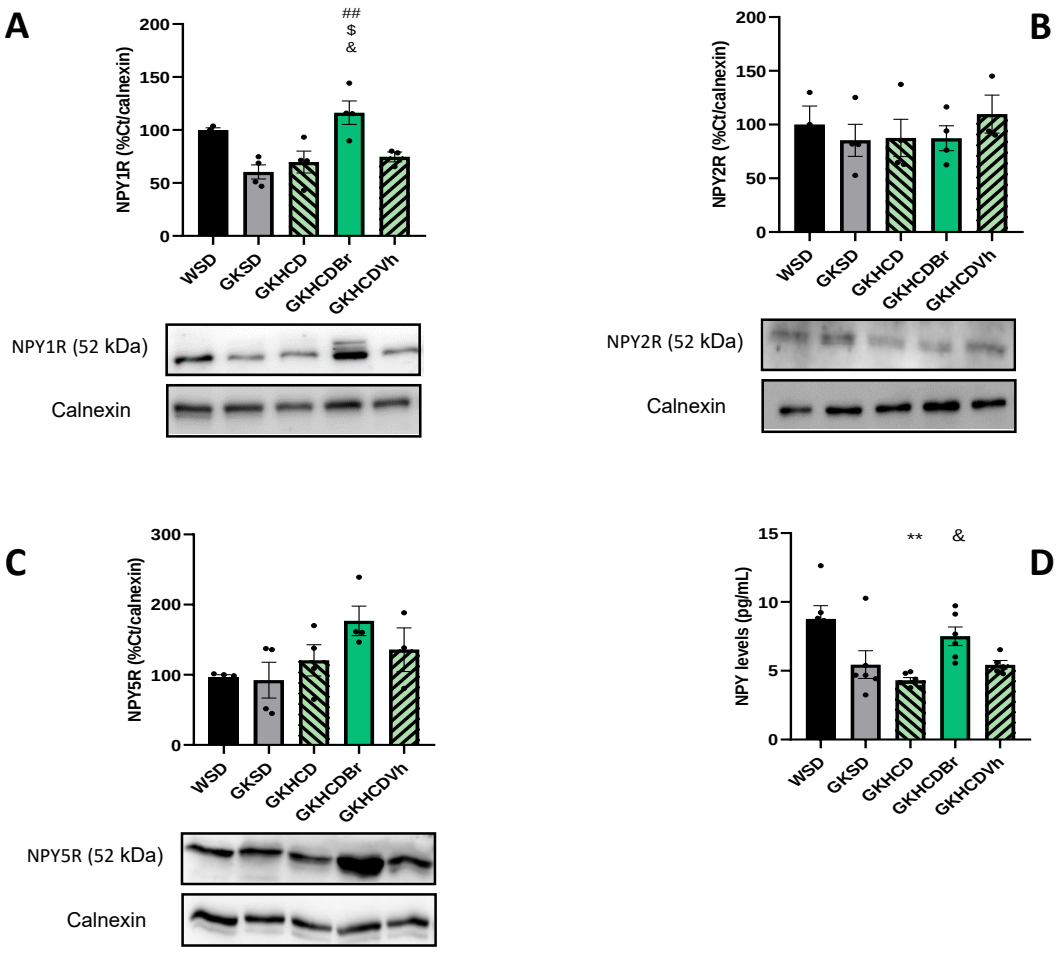


Figure 14. Bromocriptine modulates NPY system in the pEAT of type 2 diabetic obese rats. *Treatment with bromocriptine increased NPY1R (**A**) and NPY (**D**) protein levels, while not altering significantly NPY2R (**B**) and NPY5R (**C**) levels. Representative images of WB loading controls (calnexin) and proteins of interest are shown below of each graph. NPY levels were assessed with an ELISA Kit and are presented in pg/mL. WSD – Wistar rats standard diet-fed, GKSD – GK rats standard diet-fed, GKHCD – GK rats high caloric diet-fed, GKHCDBr - GK rats high caloric diet-fed treated with bromocriptine and GKHCDVh - GK rats high caloric diet-fed treated with vehicle. Bars represent mean ± SEM and Kruskal-Wallis comparisons were conducted to compare among the groups. * vs WSD, # vs GKSD, & vs GKHCD and $ vs GKHCD_Sh. 1 symbol p<0.05; 2 symbols p<0.01*

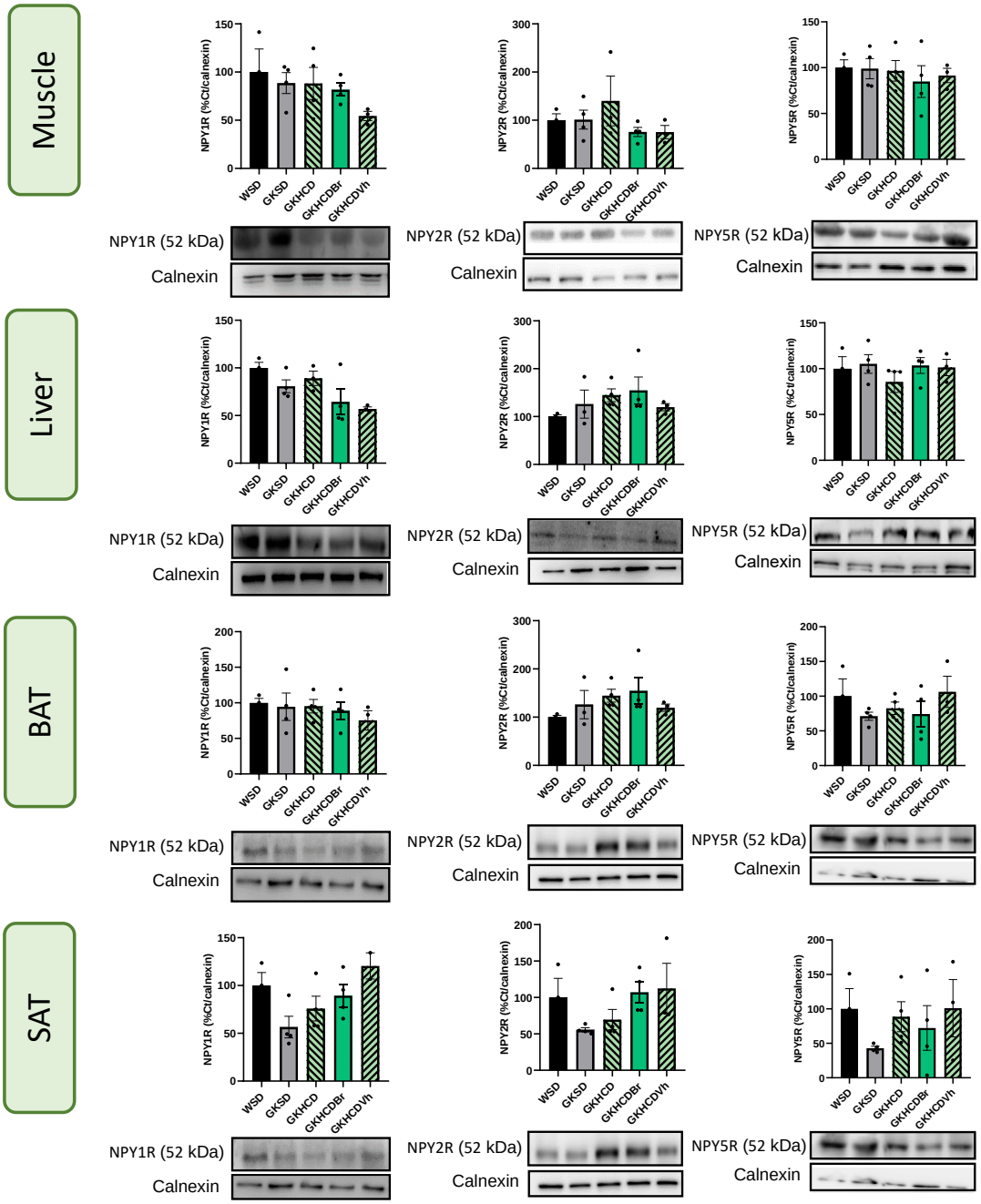


Figure 15. Bromocriptine doesn't affect NPY system in the any other peripheral tissues of obese type 2 diabetic rats. *Treatment with bromocriptine doesn't alter NPY machinery in the muscle, liver, brown adipose tissue, and subcutaneous adipose tissue. Representative images of WB loading controls (calnexin) and proteins of interest are shown below of ich graph. WSD – Wistar rats standard diet-fed, GKSD – GK rats standard diet-fed, GKHCD – GK rats high caloric diet-fed, GKHCDBr - GK rats high caloric diet-fed treated with bromocriptine and GKHCDVh - GK rats high caloric diet-fed treated with vehicle. Bars represent mean ± SEM and Kruskal-Wallis comparisons were conducted to compare among the groups.*

Ex vivo samples of epididymal and mesenteric AT acutely increase catabolic activity when incubated with Dopamine, NPY3-36 and Domperidone

Given that the most significant results in the animal and human study were found in the AT, we next studied what were the acute metabolic effects that result from the stimulation with dopamine and NPY. To assess this, *ex vivo* samples of epididymal and mesenteric AT from Wistar rats were incubated in different conditions of dopamine, NPY_{3-36} and antagonists for the dopamine receptors.

In the epidydimal AT, modulation of NPY and Dopaminergic signaling induce a great improvement of catabolic activity in this tissue. The condition where both dopamine and NPY_{3-36} are present did not show any metabolic effects in the adipose tissue. However, in the presence of domperidone, antagonist for the D2-like receptors, the same co-stimulation demonstrated a significant increase of ATGL and AMPK phosphorylation compared with several other conditions. No changes were observed when inhibiting D1 receptors with SCH 23390. In the case of phospho-ATGL, protein levels were significantly higher in the condition of dopamine + NPY_{3-36} + domperidone compared with the control condition ($p<0.05$), only dopamine condition ($p<0.01$), only NPY_{3-36} condition ($p<0.01$), Dopamine+ NPY_{3-36} condition ($p<0.01$), Dopamine+ NPY_{3-36}+SCH 23390 ($p<0.05$) and NPY_{3-36}+ SCH 23390 ($p<0.05$) (**Figure 16A**).

The alteration of phospho-AMPK levels in the dopamine + NPY_{3-36} + domperidone condition is not so extensive as the one found in the phospho-ATGL levels, however, is still significant when compared with only dopamine condition ($p<0.05$) and only NPY condition ($p<0.05$) (**Figure 16B**). Regarding phospho-ACL protein levels, no significant alterations were found among the different conditions (**Figure 16C**).

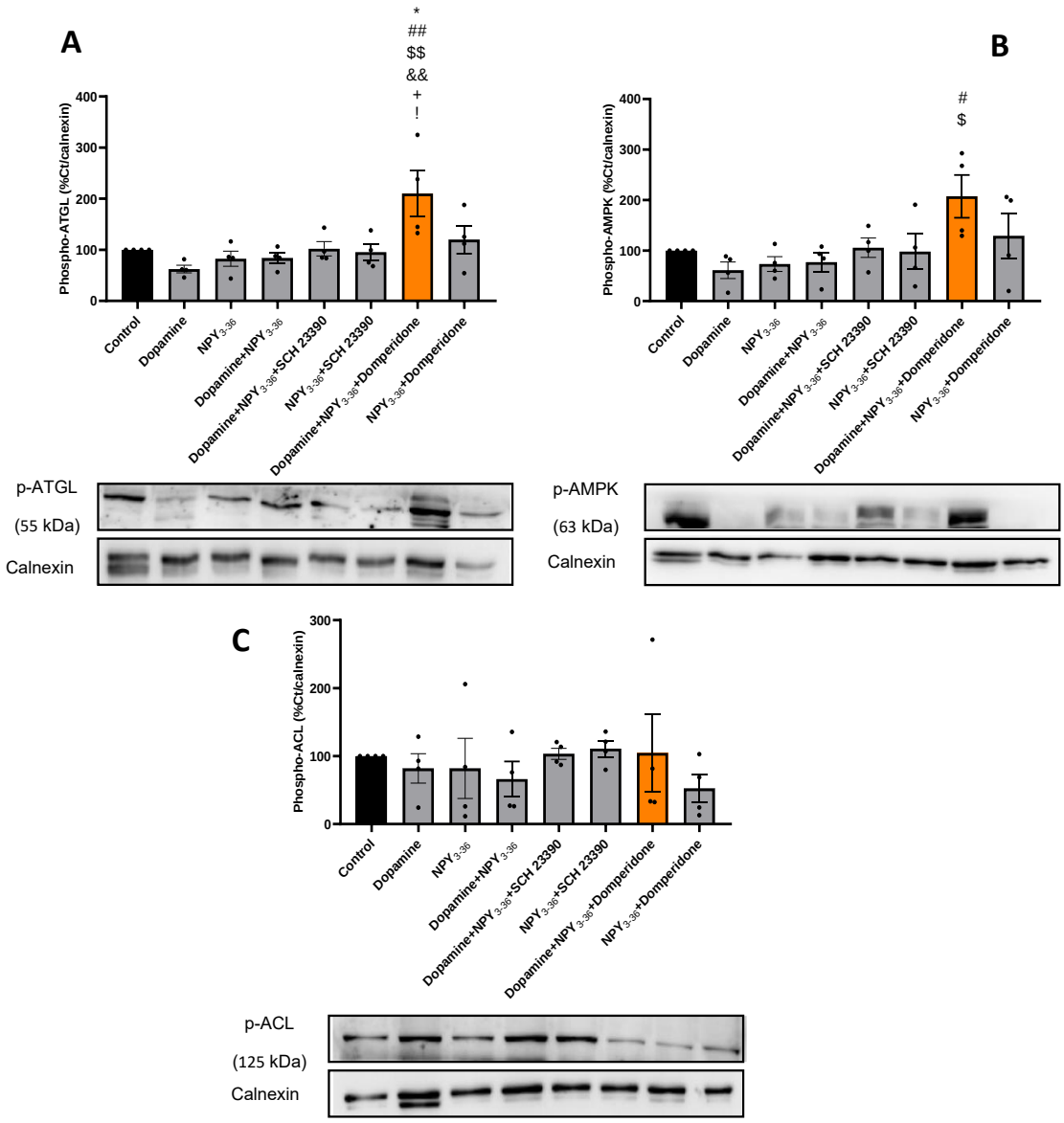
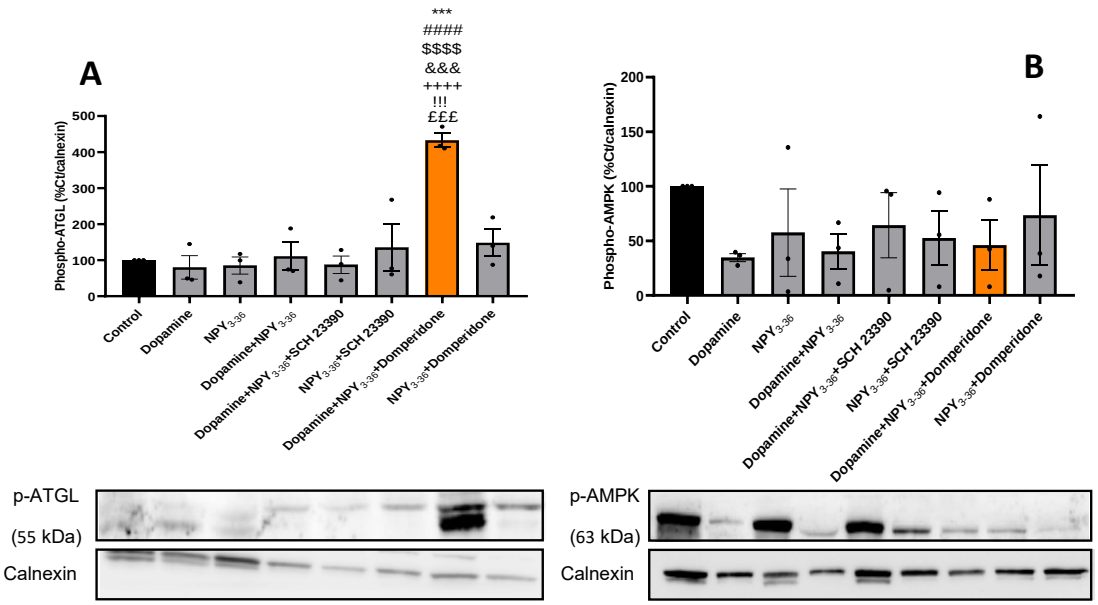


Figure 16. Incubation of epididymal AT ex vivo samples with dopamine, NPY_{3-36} and domperidone potentiates catabolic activity. *The incubation of epididymal AT with dopamine, NPY_{3-36} and domperidone resulted in a significant increase of the phosphorylation form of ATGL (**A**) and AMPK (**B**), while not altering phospho-ACL (**C**) protein levels. Representative images of WB loading controls (calnexin) and proteins of interest are shown below of ich graph. Bars represent mean ± SEM and Kruskal-Wallis comparisons were conducted to compare among the groups. * vs Control, # vs Dopamine, $ vs NPY_{3-36}, & vs Dopamine+ NPY_{3-36}, + vs Dopamine+ NPY_{3-36}+ SCH 23390, ! vs NPY_{3-36} + SCH 23390. 1 symbol p<0.05; 2 symbols p<0.01.*

In the mesenteric AT, the condition where the samples are incubated with dopamine + NPY_{3-36} + domperidone is also the one that leads to the more remarkable alterations in catabolic activity. The samples treated with this condition demonstrated phospho-ATGL protein levels that where significantly different of all the other conditions (vs control: $p<0.001$; vs dopamine: $p<0.0001$; vs NPY_{3-36}: $p<0.0001$; vs dopamine + NPY_{3-36}: $p<0.001$; vs dopamine + NPY_{3-36}+ SCH 23390: $p<0.0001$; vs NPY_{3-36} + SCH 23390: $p<0.001$; NPY_{3-36} + domperidone: $p<0.001$)) (**Figure 17A**).

Regarding the other proteins measured, the levels of phosphorylated AMPK (**Figure 17B**) and ACL (**Figure 17C**) in the mesenteric AT don´t change with the incubation in these conditions. However, the superb increase observed in ATGL phosphorylation in the mesenteric AT is more extensive compared to the one observed in the epididymal adipose tissue, pointing to a greater induction of lipolysis in this tissue.



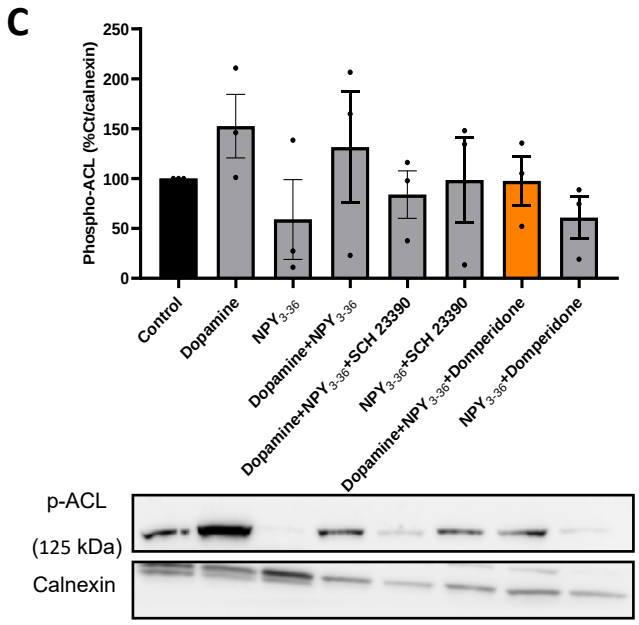


Figure 17. Incubation of mesenteric AT ex vivo samples with dopamine, NPY_{3-36} and domperidone potentiates catabolic activity. *The incubation of visceral AT with dopamine, NPY_{3-36} and domperidone resulted in a significant increase of phospho-ATGL (**A**), but not of AMPK (**B**) and ACL (**C**) phosphorylated form. Representative images of WB loading controls (calnexin) and proteins of interest are shown below of ich graph. Bars represent mean ± SEM and Kruskal-Wallis comparisons were conducted to compare among the groups. * vs Control, # vs Dopamine, $ vs NPY_{3-36}, & vs Dopamine+ NPY_{3-36}, + vs Dopamine+ NPY_{3-36}+ SCH 23390, ! vs NPY_{3-36} + SCH 23390, £ vs NPY_{3-36} + Domperidone. 1 symbol p<0.05; 2 symbols p<0.01; 3 symbols p<0.001; 4 symbols p<0.0001.*

Dopamine stimulates lipid accumulation via D2R in a concentration-dependent manner in 3T3-L1 cell line and incubation with NPY3-36 does not alter dopamine system in this cell line

Since we observed a crosstalk between NPY and dopamine system in the AT, we then aimed to study the effects of the stimulation of NPY and dopamine in cultured adipocytes. To understand this, we used 3T3-L1 fibroblasts cell line, which can differentiate into mature adipocytes, so that we can study the effect of NPY and dopamine in lipid accumulation and how the stimulation of one system can modulate the other.

We started by performing a staining with Oil Red O with cells treated with different concentrations of dopamine to identify the best concentration for further studies and the effects of dopamine in lipid accumulation and differentiation of the adipocytes. Furthermore, we observed the effect of inhibiting dopamine receptors on lipid accumulation in adipocytes, by using SCH 23390 and domperidone as antagonists for D1-like and D2-like receptors, respectively.

Negative control cells did not show any lipid droplets stained with OIL Red O in the last day of the treatment (**Figure 18A**). In contrast, in the positive control it's possible to observe a more rounded shape of the adipocytes and the formation of several lipid droplets, which points to lipid accumulation (**Figure 18B**). Dopamine treatment appear to increase lipid accumulation in a concentration dependent manner, from the lowest concentration of dopamine (0.05 µM), which is like the negative control, to the highest concentration of dopamine (1 µM), where we can observe similar or, maybe, higher levels of lipid accumulation compared with the positive control cells (**Figure 18C-F**).

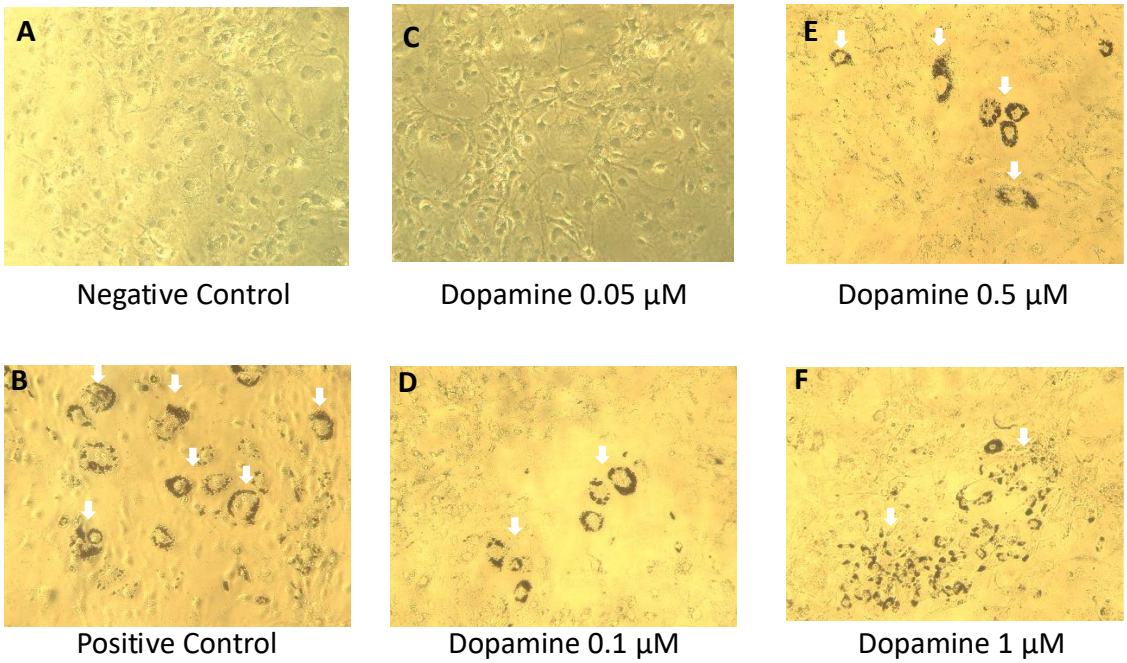


Figure 18. The effect of dopamine in lipid accumulation in 3T3-L1 adipocytes. *Representative images showing adipocytes stained with OIL Red O after treatment with dopamine at 0.05 µM (**C**), 0.1 µM (**D**), 0.5 µM (**E**) and 1 µM (**F**), and in the absence of dopamine (**A** and **B**).*

Afterwards, in order to understand the mechanisms that lead to the effect observed in lipid accumulation, cells were treated with the same concentrations of dopamine plus an antagonist for D1-like receptors, SCH 23390 (5 μM), or an antagonist for D2-like receptors, domperidone (5 or 1 μM), or both. In the cells treated with dopamine and SCH 23390) (**Figure 19C-F**) we can observe a similar growth as found in the cells treated only with dopamine, with the condition with highest concentration of dopamine + SCH 23390 having similar lipid droplet formation levels as the dopamine 1 μM and the positive control. This behavior is not observed in the cells treated with dopamine and the highest concentration of domperidone (**Figure 19G-J**). Here we observed that, although it appears that the level of lipid accumulation increases with the dose of dopamine, the growth of these structures is not similar to what is observed in the cells treated with dopamine and dopamine + SCH 23390. Regarding the cells treated with the lowest concentration of domperidone (**Figure 19K-N**), the cells appear to accumulate lipids in a dopamine dependent manner with a more substantial and great effect than the one observed in the cells treated with the highest concentration of domperidone, however, still not so profound as the one observed in dopamine and dopamine + SCH 23390 treatment. Finally, in the treatment with both antagonists (**Figure 19O-R**) we can observed the lowest levels of lipid accumulation. Coupled with this, treatment with both antagonists appears to decrease the rate of differentiation of the fibroblasts in mature adipocytes, since it's possible to observed that many cells still present spindle shape, typical of fibroblasts. So, generally, it appears that blocking the D2-like receptors with domperidone result in a negative effect in preadipocyte differentiation and lipid accumulation.

Finally, in the treatment with both antagonist (**Figure 19O-R**) we can observed the lowest levels of lipid accumulation. Coupled with this, treatment with both antagonists appears to decrease the rate of differentiation of the fibroblasts in mature adipocytes, since it's possible to observed that lots of cells still present spindle shaped, typical of fibroblasts. So, generally, it appears that blocking the D2-like receptors with domperidone result in a negative effect in lipid accumulation.

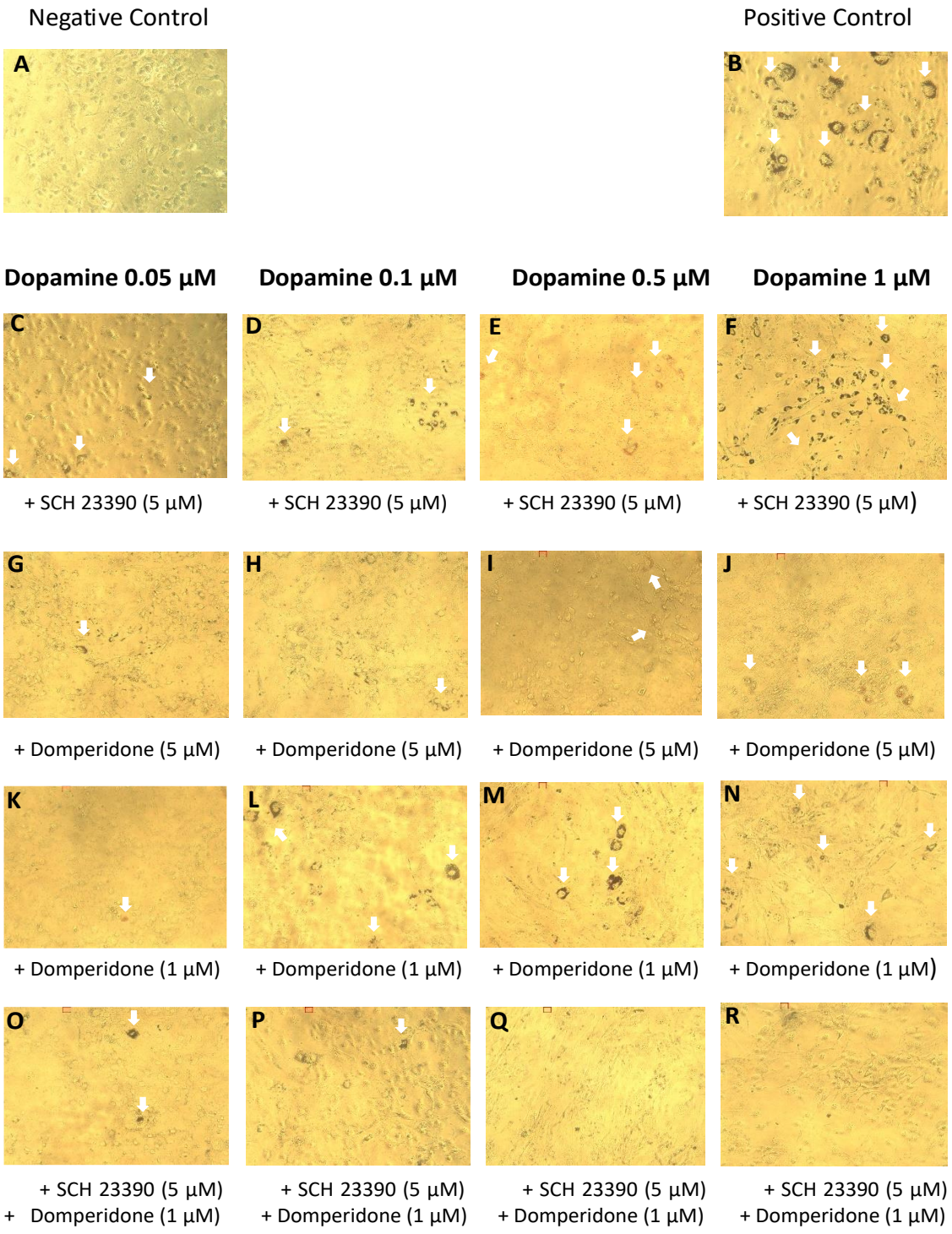


Figure 19. The effect of dopaminergic system modulation in lipid accumulation in 3T3-L1 adipocytes. *Representative images showing adipocytes stained with OIL Red O after treatment with dopamine at 0.05 µM, 0.1 µM, 0.5 µM and 1 µM, and different antagonists for the dopamine receptors: SCH 23390 at 5 µM (**C**, **D**, **E**, **F**), domperidone at 5 µM (**G**, **H**, **I**, **J**), domperidone at 1 µM (**K**, **L**, **M**, **N**) and both SCH 23390 (5 µM) and domperidone (1 µM). Negative and positive control were not treated with dopamine or any of the antagonist (**A** and **B**).*

The study with the animal model revealed that dopaminergic system stimulation potentiates NPY system in the pEAT of obese type 2 diabetic GK rats. To understand if this is a one-way effect or if the opposite also happens, adipocytes were treated with two different concentrations of NPY_{3-36} (1 and 3 µM) and D1R protein levels were measured by WB. The chronic treatment of 3T3-L1 adipocytes with NPY_{3-36}, after a pre-incubation with a differentiation cocktail, didn't lead to significant alteration of D1R protein levels in any of the two concentrations of NPY_{3-36} used (1 and 3 µM) (**Figure 20**).

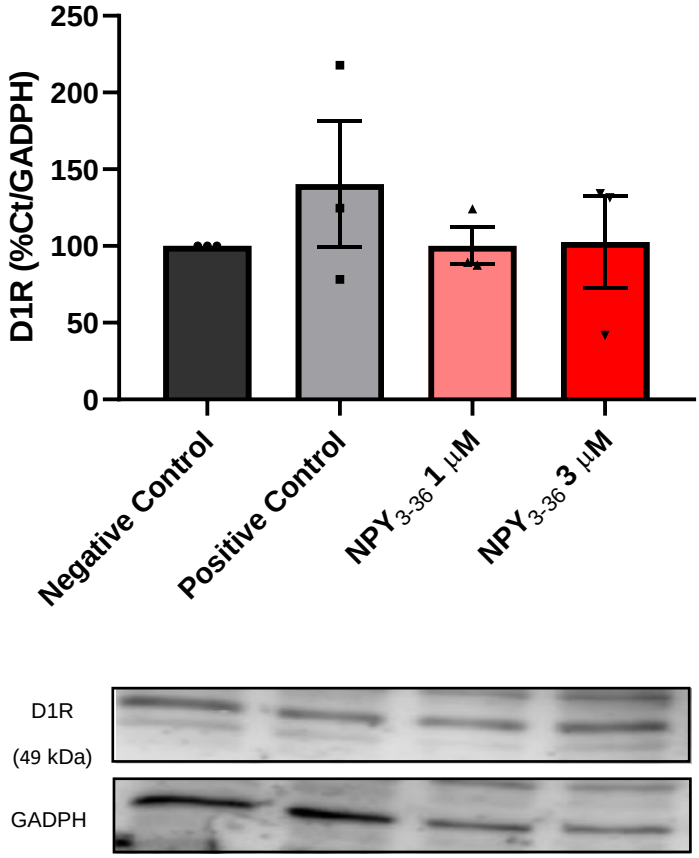


Figure 20. Incubation off 3T3-L1 differentiated adipocytes with NPY_{3-36} does not alter D1R levels. *Treatment of the differentiated 3T3-L1 adipocytes with two different concentrations of NPY_{3-36}(1 and 3 µM) doesn't lead to alterations in D1R protein levels. Representative images of WB loading controls (calnexin) and proteins of interest are shown below of ich graph. Bars represent mean ± SEM and Kruskal-Wallis comparisons were conducted to compare among the groups*

CHAPTER 5 – DISCUSSION

Obesity is characterized by an excessive and chronic accumulation of body fat, that can ultimately lead to the development of several comorbidities such as T2DM. Nowadays, is possible to distinguish two different profiles of obesity: MHO and MUO (14). Despite it's not known if MHO is a permanent state (14), the onset of the unhealthy phenotype is strongly correlated with a progressive loss of AT function and the dysregulation of energy homeostasis. Indeed, obesity was shown to interfere with the important interactions and communication between the different central and peripheral tissues to maintain energy homeostasis.

NPY and dopamine systems are important players in energy balance control at the central level. It was also observed that these systems can interact and regulate each other in a crosstalk that is very important to the control of energy balance. In the recent years, NPY and dopamine have also been appointed for their direct actions in several peripheral tissues, regulating important metabolic processes. However, it has not been studied yet if these systems can interact and modulate each other on a peripheral level like it happens in brain regions such as the hypothalamus. The study of this crosstalk could be important to the development of new therapies to prevent the metabolic dysregulation or even restore the correct function of these systems.

In this work, we explored the crosstalk of NPY and dopaminergic in the periphery and how the interactions between the systems affect metabolism. We have previously described that NPY and dopaminergic machinery expression are altered in human VAT in obese individuals, with the progressive development of insulin resistance and metabolic dysregulation. As major discoveries of this work, we can highlight that: 1- NPY and dopaminergic system are correlated in the VAT and they appear to be involved in the control of important metabolic processes; 2- Dopaminergic system modulation with bromocriptine treatment in diabetic obese rats remodels NPY system only in the pEAT ; 3-Dopamine and NPY_{3-36}, when domperidone is present, acutely potentiates catabolic activity in epididymal and visceral AT *ex vivo* samples; 4- Dopamine stimulates lipid accumulation in adipocytes in a concentration-depend manner, with a mechanism that seems to involve D2-like receptors signaling; 5- NPY_{3-36} does not appear to modulate dopaminergic machinery in adipocytes.

Obesity is usually diagnosed according to the BMI, which is a measure but does not completely reflect the total quantity of body fat in the organism (5). However, several studies demonstrated that the difference between MHO and MUO individuals, is not associated with the total amount of body fat, but rather to the distribution of this fat in the different body regions and the function of such tissue (11). Indeed, in our study we demonstrated that in our cohort of patients with obesity, BMI does not correlate with any of the metabolic parameters measured, showing that the total quantity of fat does not correlate to metabolic dysregulation. Moreover, the BMI does not significantly change among the groups formed by our patients. This confirms that dividing the patients according to the BMI will not permit us to study the progressive loss of metabolic health. However, the division that we used in this study reflects as we observed, a progressive loss of metabolic health with the establishment of insulin resistance, seen by the compensatory increase of β -cell function in group 2, that is progressively lost with the onset of prediabetes and T2DM. Progressive AT dysfunction is also observed through the continuous development of hypoadiponectinemia, coupled with lower levels of HDL-cholesterol and higher levels of triglycerides. So, this division will undoubtedly permit a more accurate vision of what happens in the transition from MHO to MUO.

We previously described the changes occurring in the NPY and dopaminergic systems in VAT of this cohort of obese patients (102). The expression of almost all the genes involved in these systems machinery was significantly altered among the groups, which shows that the development of metabolic dysregulation affects these important systems in VAT. However, the alterations between groups were not equal for all the genes. The expression of *NPY1R* and *NPY5R* were similar, with an increase in the IR Pred-diabetic group (3), only observed in *NPY1R* expression, but with both decreasing in the IR Diabetic group (4), suggesting that the progressive evolution of metabolic dysregulation, triggers a compensatory mechanism that potentiates *NPY1R* and *NPY5R* expression, but it's then lost when the patients develop diabetes. *NPY2R*, *PPY1R*, *NPY*, *D1R* and *D4R*, also had a similar expression behavior among the groups in the VAT, with a significant decline that happens with establishment of insulin resistance(102). So,

these genes appear to be connected and to be dysregulated earlier in the onset of metabolic dysregulation.

Given these similarities in the pattern of expression among the groups, we then wanted to see if these genes were correlated in the human VAT. We found that NPY1R and NPY5R are strongly correlated between each other, which suggests they may have similar functions. Indeed, we found that both the genes are strongly positively correlated with genes involved in lipid storage, glucose metabolism and antioxidant capacity, which suggests that NPY1R and NPY5R are involved in the regulation of these processes. In fact, NPY1R activation in AT has been shown to be involved in lipid storage by increasing the levels of important lipogenesis markers, such as PPARγ (68), which expression we showed to be correlated with NPY1R in human VAT. Such result suggests that in a state of stress and chronic higher nutrient availability like the one observed in patients in the IR-Prediabetes group, VAT potentiates lipid storage as a last resource, to try to store nutrients, while maintaining tissue function. However, this compensatory mechanism apparently ends up failing which lead to the breakdown of lipid storage capacity. Accordingly, we observed higher circulating levels of triglycerides in the circulation in the Diabetes group, which are known to increase ectopic fat accumulation, the development of IR and the progression of diabetes.

Regarding the other set of genes with similar behavior, *NPY2R*, *D1R* and *PPY1R* are also strongly correlated between each other. D4R was excluded from this cluster because, despite showing modest correlations with the others, the magnitude of these is not the same as observed between the other genes. These results demonstrated that indeed dopaminergic and NPYergic systems are correlated in the human VAT, possibly collaborating in the regulation of important AT functions. For instance, these cluster of genes is highly correlated with the expression of catabolic and angiogenesis markers. This is not surprisingly since the effect of D1R on potentiating catabolism in the AT has already been described (98), such as the effect of NPY2R in angiogenesis (68). However, the involvement of each gene in the other function, and of PPY1R in both as not been studied yet. Thus, it seems that the first thing that declines with the progressive development of metabolic dysregulation is the tissue catabolic capacity and angiogenic capacity, the last being fundamental to maintain healthy hyperplasic tissue growth.

After demonstrating that NPY and dopaminergic system are correlated in the human VAT and correlated with the expression of several gene involved in the regulation of important processes for tissue function, we then aimed to study if this peripheral crosstalk was confirmed in an animal model. To assess this, a diabetic obese rat model was treated with bromocriptine, an agonist for D2R, aiming to disclose if the peripheral modulation of dopaminergic system with bromocriptine will induce alterations in the NPY system in the different peripheral tissues. Bromocriptine succeeded in altering NPY system, with an increase in NPY1R, NPY5R and NPY levels in a tissue-dependent manner, only affecting pEAT. This is not surprising, since in previous studies with the same animal model, the most significant alterations where also found in the pEAT. In fact, bromocriptine was shown to upregulate D1R protein levels in this tissue being this associated with an induction of catabolic activity in the tissue (102). Indeed, in our cohort of patients with obesity, D1R gene expression in VAT is also correlated with catabolic markers. However, in the same study it was also found that the treatment with bromocriptine significantly increases glucose metabolism markers, insulin receptor (InsR) and GLUT4, and PPARγ levels. Since the genes encoding for InsR and PPARγ were highly correlated in human VAT with NPY1R and NPY5R we may hypothesize that NPY system may also be involved in such effects of Bromocriptine. Regarding, the increase in NPY levels observed after Bromocriptine treatment, they may reflect an increase in neuronal NPY production in the AT, however, if this upregulation is caused by the increase of neuronal projections to the AT or direct action of bromocriptine in the tissue is still not known.

Overall, the results obtained in the animal study demonstrated that bromocriptine modulation of dopaminergic system remodels NPY system in pEAT, confirming the existence of the crosstalk between the two systems in this tissue. Moreover, these interactions appear to be important in the regulation of glucose and lipid metabolism. A similar interaction was found between dopamine and GLP-1 system. In the same animal model, our group found that bromocriptine treatment led to increase GLP-1 signaling in pEAT. However, diabetic rats treated with liraglutide, a GLP-1R agonist, didn’t show alterations in dopaminergic signaling, suggesting that dopamine

is an upstream regulator of this system (107). Something similar can be happening in the case of NPY system.

After demonstrating the crosstalk between NPY and dopamine system in the AT, we wondered what where the acute effects of the modulation of these systems in the AT metabolism. We observed that in both epidydimal and mesenteric AT, the condition where both NPY_{3-36} and dopamine are present together with domperidone, leads to a huge increase in catabolic activity of both tissues, with upregulation of the phosphorylated form of ATGL in both tissues and of the phosphorylated form of AMPK, only in epididymal one. Such results suggest that blocking the signaling via D2-like receptors and potentiating D1R signaling in the presence of dopamine and NPY_{3-36}, acutely augments the catabolic activity of both mesenteric AT and EAT. This was expected since several studies point to the catabolic activity induced by the D1R in AT. However, in a previous study performed in our laboratory used a similar experimental design and demonstrated that similar concentrations of dopamine and domperidone didn't result in a similar activation of AMPK, phospho-ATGL was not tested at the time (100). Although our results suggest that the presence of NPY_{3-36} Potentiates dopamine effects under D2 receptor inhibition, such result must yet to be confirmed in future experiments. Nevertheless, in human VAT, *NPY2R* gene is strongly correlated with several catabolic markers and this form of NPY peptide used (NPY_{3-36}) has a stronger affinity for NPY2R. In the future, it should be interesting to add a new condition to this study, with the incubation of the *ex vivo* samples with only dopamine and domperidone. With this condition we will be able to understand if the NPY system is involved in this augmented effect or if the blockage of D2-like receptor signaling alone promotes these catabolic effects.

On the opposite, when signaling via D2R, dopamine stimulation seems to lead to adipogeneses and lipogenesis. Indeed, our results from the treatment of the 3T3-L1 adipocytes with dopamine and different antagonists, suggest that when blocking the signaling via D2R, with domperidone, the level of lipid accumulation is much more reduced than the one we observe with the treatment with only dopamine or dopamine plus SCH 233390. So, the outcome of dopaminergic stimulation in adipocytes seems to depend on which receptor is activated. Contrarily to what we observe, Mukherjee et.al

demonstrated that bromocriptine, that is an agonist for D2R, had anti-lipogenic effect on 3T3-L1 adipocytes (109). However, they attributed this effect to α2-Adrenergic Receptor, since when they incubated the cells with an antagonist for this receptor, the bromocriptine's anti-lipogenic effect was abolished.

Lastly, although dopaminergic stimulation could alter NPY machinery in the pEAT of obese diabetic rats, the opposite is not yet known. D1R levels are not altered in the 3T3-L1 adipocytes after treatment with NPY_{3-36}. Although *NPY2R* and *D1R* genes are highly correlated in the human VAT of our cohort of patients with obesity, this was not observed *in vitro*, since an agonist with more affinity to activate NPY2R, fails to alter the D1R levels. One possible explanation to this is that dopamine system is an upstream regulator of NPY system, so, as it happens in the case of GLP-1, NPY system does not regulate dopaminergic one, and the relation between the two systems is unilateral. However, to have more accurate conclusions about this topic, the other dopamine receptors should be determined in the same, and NPY receptors should be determined in adipocytes treated with dopamine.

CHAPTER 6 – CONCLUSIONS AND FUTURE PERSPECTIVES

The numbers of people with obesity are increasing with the years. The development of new therapeutic strategies to stop this growing numbers is fundamental, mainly to the treatment of MUO and associated MetS, since they constitute risk factors to the development of T2DM and CVD's. This unhealthy phenotype of obesity is associated with AT dysfunction and the dysregulation of energy homeostasis. NPY and dopamine system participate in this control of energy balance having important central and peripheral function, and are known to regulate each other at a central level. Although, the interactions between these systems in the periphery are not yet studied. Thus, in this work we aimed to unravel the existence of a peripheral crosstalk between NPY and dopamine system and the importance of this interaction to the peripheral whole-metabolism.

We found that the NPY and dopamine system are correlated between each other in the human VAT of patients with obesity. These results allow us to distinguish between two different cluster, one formed by *NPY1R* and *NPY5R,* that are highly correlated with genes involved in lipid storage, antioxidant capacity and glucose metabolism, and the other formed by *NPY2R*, *D1R* and *PPY1R*, strongly related with genes involved in catabolic function and angiogenesis. Thus, NPY and dopamine systems seem to cooperate in the regulation of important functions in the VAT. So, the dysregulation of these system's machinery observed in VAT of patients with obesity along the development of metabolic dysregulation, is associated to the AT dysfunction observed in MUO individuals. Another discover that suggests the existence of this peripheral crosstalk is that bromocriptine, a D2R agonist, restores NPY signaling in the pEAT of diabetic obese rat model, contributing to the improvement of AT function and metabolic profile of these rats. Thus, the pharmacologic modulation of these systems and the restoration of these in the periphery could be an important therapeutic target for MUO. Regarding the acute effects of this modulation, we observed that dopamine and NPY_{3-36} in the presence of domperidone significantly increased catabolic activity in both mesenteric and epididymal AT. However, conclusions about if this effect reflects a cooperative effect of dopamine and NPY_{3-36} or if this is the result of blocking D2R and potentiating dopamine signaling via D1R, were not possible to fully. Contrarily, D2R stimulation appears to induce lipid accumulation and adipogenesis. This is not

surprising, since signaling via D1R or D2R also have opposite effects at a central level. Finally, we observed that treatment of adipocytes with NPY_{3-36} didn´t result in any changes in D1R levels, suggesting that the regulation of these systems may be unilateral. However, in the future, to have a full picture, we should measure the levels of the other receptors of dopamine and of the dopamine in the medium. An animal model treated with agonists and antagonists for the NPY system, also should be a possible option to study this interaction. Moreover, we can also treat adipocytes with dopamine, to study if the effects that we observe in the animal model, are maintained in the cells.

Notwithstanding, this work made it possible to obtain knowledge and awareness on the potential of the crosstalk between dopamine and NPY system as therapeutic target for AT dysfunction, one of the markers of metabolic diseases, such as MetS and T2DM, as well as clarifying the function of these systems in regulating AT plasticity, catabolic activity and, in general, metabolic function. Pharmacological modulation of these systems and their peripheral and centrally interactions could be, indeed, a powerful therapy to treat obesity and associated metabolic dysregulation in the future.

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