Gabriela Pires Tavares

Peripheral Dopamine action in Insulin Sensitive Tissues: A Regulator of Metabolic Activity and Therapeutic Target to Enhance Insulin Sensitivity

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Tese no âmbito do Programa Doutoral Interuniversitário em Envelhecimento e Doenças Crónicas – PhDOC, orientada pelo Professor Doutor Paulo Nuno Centeio Matafome e pela Professora Doutora Silvia Margarida Vilares Conde e apresentada à Faculdade de Medicina da Universidade de Coimbra

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Faculdade de Medicina da Universidade de Coimbra

Unravelling the Role of Peripheral Dopaminergic Signalling in Metabolism

Gabriela Pires Tavares

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The experimental research was performed in the Institute of Physiology and Coimbra Institute for Clinical and Biomedical Research (iCBR) - Faculty of Medicine - University of Coimbra, and at Chronic Diseases Research Center (CEDOC) from NOVA Medical School, Faculdade de Ciências Médicas, Universidade Nova de Lisboa. The clinical study was conducted at Centro Hospitalar e Universitário de Coimbra – Hospital Geral - Covões, Coimbra, Portugal.

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ABSTRACT

Introduction: Dopamine is a key cellular modulator for the control of metabolism in the central nervous system. Accordingly, it has been proposed a role for dopamine, its precursor (L-Dopa) and dopaminergic drugs, in peripheral glucose homeostasis. However, the direct actions of dopamine on peripheral insulin-sensitive tissues have never been addressed before, which inevitably requires research and clarification in this area of knowledge.

Aims: Thus, in the present work we aimed to study whether dopamine presents a direct role on glucose uptake and metabolic activity in peripheral insulin sensitive organs. We also aimed to investigate if dopamine receptors' gene expression is altered or dysregulated in human visceral adipose tissue (VAT) samples from a cohort of patients with metabolic disorders. Finally, we aimed to study the metabolic function of peri-epididymal white adipose tissue (pEWAT) and insulin sensitivity in an animal model of type 2 diabetes and obesity, after modulation of the dopaminergic signalling with bromocriptine (selective D2R agonist).

Materials & Methods: To accomplish our first goal, young and healthy Wistar rats were used, after an overnight fasting to perform *in vivo* (100nmol intravenous injected dopamine) and *ex vivo* tissue-specific glucose uptake experiments (liver, soleus muscle, white (WAT) and brown adipose (BAT) tissues). Tissue explants were incubated at 37º in normoxic conditions with dopamine (10μM) or bromocriptine (10μM), both in the presence or absence of insulin ($10mU.ml^{-1}$) and the selective D2R antagonist, domperidone (50nM) and non-selective (D1R and D2R) antagonist, haloperidol (500nM). This pharmacologic approach was used to discriminate the contribution of each dopamine receptors family for tissue glucose uptake and for the modulation of metabolic pathways (insulin receptor (InsR) and AMPK activation and , and HSL,

ACC ACL activation specifically in white adipose tissue). Incubation with insulin constituted the positive control of the experiment.

To study dopamine receptor gene expression, as well as markers of adipose tissue metabolic function, samples from VAT were collected from patients with obesity and used for RNA extraction, followed by gene expression quantification. This cohort of patients with obesity was divided into insulin sensitive (IS) or insulin resistant (IR) groups according to Ox-HOMA2IR, and then IR ones into normoglycemic, pre-diabetic and diabetic patients according to fasting glycemia and HbA1c levels.

The animal model with dysmetabolism studied were the Goto-Kakizaki rats, a genetic model of spontaneous type 2 diabetes, which were fed for 6 months with high caloric diet to induce obesity. They were used to study the contribution of dopaminergic modulation with bromocriptine treatment (10mg/kg/day, 28 days i.p.), in peripheral organs (pEWAT and BAT, liver, and muscle).

Results: Dopamine increases *in vivo* glucose uptake in insulin sensitive tissues. The e*x vivo* experiments showed that dopamine and bromocriptine increased liver glucose uptake while enhanced insulin-mediated glucose uptake in the white adipose tissue (via D2R activation, since this effect was abolished in the presence of domperidone). In the soleus muscle dopamine but not bromocriptine increased glucose uptake, thought a D1R modulated mechanism. Additionally, D2R antagonism, increased AMPK, HSL and ACL activation in white adipose tissue, being more evident in the presence of bromocriptine. Regarding the DR's gene expression study in human VAT samples, *DRD4* gene expression was decreased in patients with pre-diabetes and diabetes compared to IS patients. *DRD1* expression ($p = 0.06$) decrease in IR patients compared

to IS patients. Moreover, decreased *DRD1* expression was correlated with decreased expression of *UCP1*, *PPARA* and insulin receptor gene *(INSR*).

The use of bromocriptine as a therapeutical strategy to modulate peripheral dopaminergic signalling in an animal model of dysmetabolism resulted in increased D1R and TH levels in liver and pEWAT. Furthermore, decreased pEWAT weight, adipocytes' volume, hepatic steatosis, liver weight and hepatic triglycerides were observed after bromocriptine treatment. Interestingly, in pEWAT, the total levels of GLUT4, InsR and PPARγ increased after bromocriptine treatment. Additionally, in pEWAT, AMPK activation and β-hydroxybutirate also increased in a post-absorptive state while InsR activation increased postprandially. The improvement of pEWAT metabolic function towards increased lipid catabolism and glucose uptake may contribute to the improvement of systemic lipid and glucose profile (decreased plasma triglycerides and cholesterol and fasting glycemia) and peripheral insulin sensitivity (decreased AUC of insulin tolerance test).

Conclusion: We showed for the first time that dopamine directly regulates glucose uptake and metabolic pathways in insulin sensitive tissues, through specific activation of distinct dopamine receptors families in a tissue specific manner. Moreover, D2R antagonism promotes lipid catabolism in white adipose tissue. Furthermore, samples from VAT from patients with insulin resistance presented a downregulation of dopamine receptors genes, and decreased *D1DR* expression was correlated with decreased expression of genes responsible for lipid catabolism. At last, we showed for the first time that modulation of peripheral dopaminergic system through bromocriptine treatment increased both D1R and TH levels in liver and pEWAT. Altogether, these data led us to conclude that modulation of peripheral dopaminergic signalling and its modulation could constitute a novel strategy toward the treatment of metabolic disorders.

Keywords: Dopamine, Bromocriptine, Insulin sensitive tissues, Insulin resistance, Type 2 diabetes, and Obesity

RESUMO

Introdução: A dopamina é um importante modulador do metabolismo no sistema nervoso central para além disso, esta, o seu percursor (L-Dopa) e agentes farmacológicos dopaminérgicos, desempenham funções modulatórias no controlo da homeostasia sistémica da glucose. Contudo, as suas ações diretas nos tecidos periféricos sensíveis à insulina nunca foram investigadas, sendo por isso, relevante desenvolver estudos neste contexto.

Objetivos: Neste trabalho, pretendemos primariamente, estudar se a dopamina tem um papel direto na modulação da captação de glucose e na modulação de vias metabólicas nos tecidos periféricos sensíveis à insulina. Foi ainda, objetivo, investigar se a expressão dos genes que codificam os recetores dopaminérgicos se encontra alterada em amostras de tecidos adiposo omental (VAT) humano, de um *cohort* de doentes com disfunção metabólica. Por fim, estudou-se a função metabólica e sensibilidade à insulina do tecido adiposo branco peri-epididimal (pEWAT) de animais com diabetes tipo 2 (T2D) e obesidade, após a modulação da sinalização dopaminérgica com bromocriptina (agonista seletivo dos D2R).

Materiais & Métodos: Ratos Wistar jovens e saudáveis e em jejum prolongado foram utilizados para estudar a captação de glucose em cada tecido sensível à insulina, tanto *in vivo* (injeção intravenosa de 100nmol de dopamina), como *ex vivo* (explantes de fígado, musculo soleus, tecido adiposo branco (WAT) e castanho (BAT)). Os explantes foram incubados a 37º em condições de normóxia com dopamina (10μM) ou bromocriptina (10μM), na presença ou na ausência de insulina (10mU.ml^{-1}), assim como dos antagonistas dos recetores dopaminérgicos: domperidona (50nM), antagonista seletivo dos D2R e haloperidol (500nM) antagonista não seletivo (D1R e D2R). A contribuição de cada subtipo dos recetores dopaminérgicos na captação da glucose e na modulação das vias metabólicas (ativação do recetor de insulina (InsR) e da

AMPK e, em particular no WAT, a ativação da HSL, ACC e ACL) foi analisada. A incubação com insulina serviu de controlo positivo da experiência. No estudo da expressão génica dos recetores dopaminérgicos e de marcadores da função metabólica, amostras de VAT foram recolhidas de doentes com obesidade durante cirurgia abdominal, para extração de RNA e posterior quantificação da expressão génica. O *cohort* estudado de doentes com obesidade foi dividido de acordo com o Ox-HOMA2IR em insulino-sensíveis (IS) e insulino-resistentes (IR), posteriormente divididos em doentes com normoglicémia, pré-diabetes e diabetes, de acordo com os níveis plasmáticos da glucose em jejum e a HbA1c.

O modelo animal de disfunção metabólica utilizado, foram ratos Goto-Kakizaki, um modelo genético de T2D espontânea, alimentados durante 6 meses com dieta hipercalórica, induzindo obesidade. Foi estudada a contribuição da modulação dopaminérgica pelo tratamento com bromocriptina (10mg/kg/dia, durante 28 dias i.p.) nos órgãos periféricos (pEWAT, BAT, fígado e músculo).

Resultados: A dopamina aumentou *in vivo* a captação da glucose nos tecidos sensíveis à insulina. E*x vivo*, verificou-se que a dopamina e a bromocriptia aumentaram a captação da glucose no fígado e potenciaram a ação da insulina na captação da glucose no WAT (pela ativação dos recetores D2R - com a domperidona perde-se este efeito). No músculo sóleo, apenas a dopamina promoveu a captação de glucose, mecanismo modulado pelos D1R. Adicionalmente, o antagonismo D2R promoveu a ativação da AMPK, HSL e ACL no WAT, sendo mais evidente na presença de bromocriptina. No estudo da expressão génica dos DR's no VAT de amostras humanas, observou-se uma diminuição da expressão de *DRD4* em doentes com pré-diabetes e diabetes em relação aos doentes IS. A expressão do *DRD1* (p=0.06) diminuiu nos doentes IR

relativamente aos doentes IS. Ainda, a diminuição da expressão do *DRD1* correlacionou-se com a diminuição da expressão dos genes *UCP1, PPARA e INSR.*

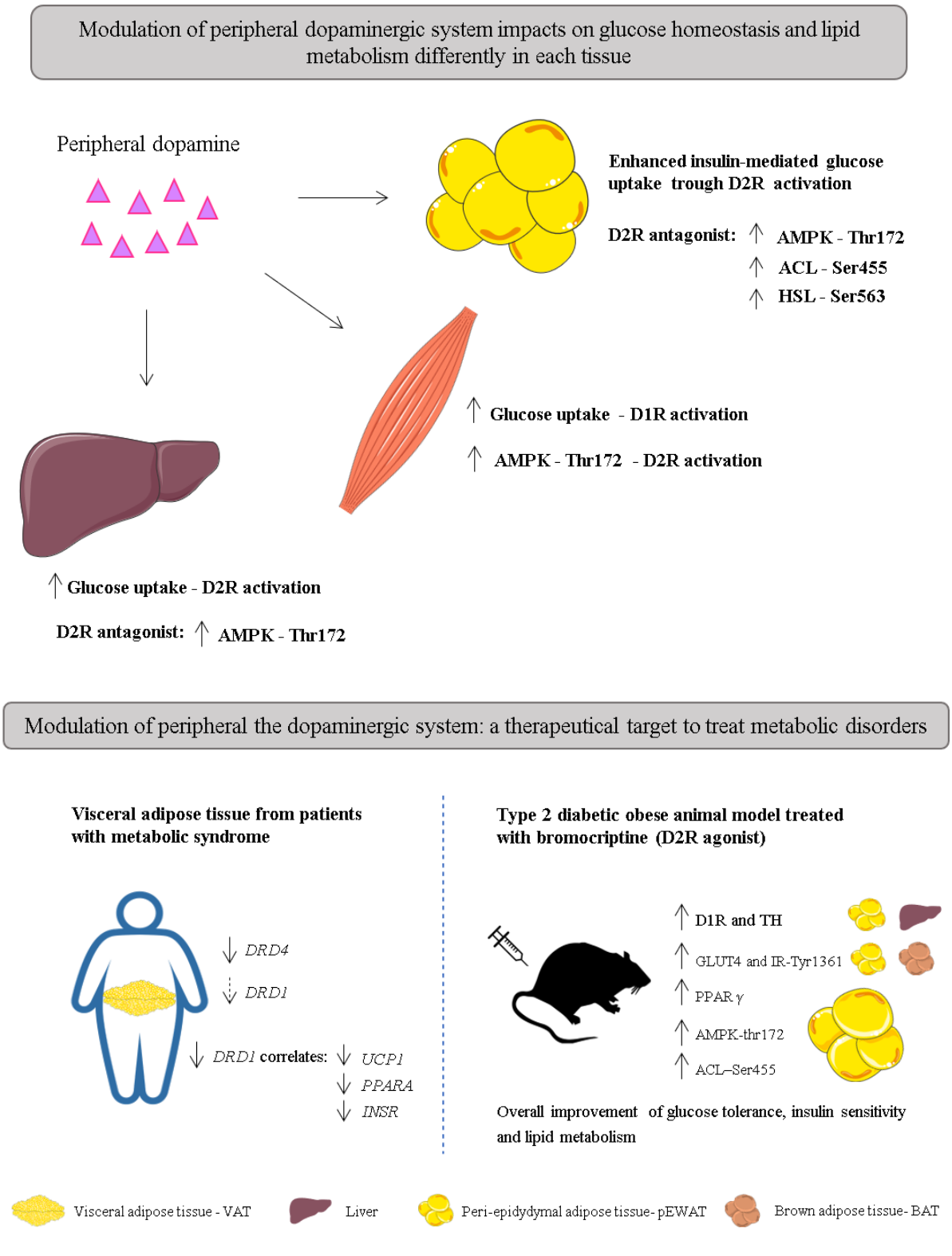
O uso da bromocriptina como estratégia terapêutica na modulação da sinalização dopaminérgica periférica no modelo animal de disfunção metabólica, demonstrou o aumento dos níveis do D1R e TH no fígado e pEWAT. Além disso, o peso do pEWAT e o volume dos adipócitos foram reduzidos com o tratamento, assim como a esteatose hepática, o peso do fígado e o conteúdo hepático de triglicerídeos. No pEWAT observou-se o aumento dos níveis totais de GLUT4, InsR e PPARγ, bem como o aumento da ativação da AMPK e dos níveis de β-hidroxibutirato em jejum e o aumento da ativação do InsR em pós prândio. A melhoria da função metabólica do pEWAT, pelo aumento do catabolismo lipídico e da captação da glucose, associou-se à do perfil lipídico e glicémico sistémicos (diminuição dos níveis plasmáticos de triglicerídeos e colesterol e dos níveis de glucose em jejum) e melhoria da sensibilidade periférica à insulina (diminuição da AUC durante o teste de tolerância à insulina).

Conclusão: Neste trabalho, mostrámos pela primeira vez que a dopamina regula de forma direta a captação da glucose e a modulação de vias metabólicas nos tecidos periféricos sensíveis à insulina, através da ativação, especifica em cada tecido, dos distintos recetores de dopamina (família D1R ou D2R). Ainda, o antagonismo D2R promoveu o catabolismo lipídico no WAT. Demonstrámos também, que, em amostras de VAT de doentes com resistência à insulina existe uma diminuição da expressão dos genes que codificam os DR's. A diminuição da expressão do *D1DR* correlacionou-se com a diminuição da expressão de genes responsáveis pelo catabolismo lipídico. Por fim, observámos que a modulação da sinalização dopaminérgica periférica com a bromocritpina promoveu o aumento do D1R e da TH no fígado e pEWAT.

Estes resultados, levaram-nos a concluir que, a modulação da sinalização dopaminérgica periférica pode constituir uma nova estratégia terapêutica no tratamento de doenças metabólicas.

Palavras-chave: Dopamina, Bromocriptina, Tecidos sensíveis à insulina, Insulinorresistência, Diabetes tipo 2 e Obesidade

GRAPHICAL ABSTRACT



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LIST OF ABREVIATIONS

#:

[^{3}H]2-deoxyglucose – Radiolabelled glucose

β- hydroxybutyrate – ketonic body also known as 3-hydroxybutyric acid

A:

AADC – Aromatic amino-acid decarboxylase, converts L-DOPA into dopamine

ACC – Acetyl-CoA carboxylase

ACC-Ser79- phosphorylated Acetyl CoA Carboxylase on serine79 residue

ACL-Ser455- phosphorylated ATP Citrate Lyase on 455 serine residue

AKT-Ser473 – phosphorylated protein kinase B on 473 serine residue

AMPK – AMP-activated protein kinase

AMPK-Thr172 – Phosphorylated AMP-activated protein kinase on 172 threonine residue

Ap2 - Adaptor protein complex, also known as FABP4, fatty acid binding protein 4.

APDs – Antipsychotic drugs

ARSA – Arylsulfatase, converts sulphated dopamine (DA-S) into dopamine

ATGL - Adipose triglyceride lipase

B:

BAT – Brown adipose tissue

C:

CA - Catecholamines

cAMP – cyclic adenosine monophosphate

CB – Carotid body

CD36 receptor – Cluster of differentiation 36 receptor

CSN – Carotid sinus nerve

D:

DAG – Diacylglycerol

D1R – Dopamine receptor 1

D2R – Dopamine receptor 2

DA – Dopamine

DA-S – Dopamine sulphate

DARPP 32 – Dopamine- and cAMP-regulated neuronal phosphoprotein

DRD1– Gene encoding for human dopamine receptor 1

DRD2 – Gene encoding for human dopamine receptor 2

DRD4 – Gene encoding for human dopamine receptor 4

E:

ECM – extracellular matrix

eWAT – epididymal white adipose tissue

F:

FAs – Fatty acids

FATP – Fatty acid transporter protein

FFA's – Free fatty acids

G:

GI – Gastro-intestinal tract

GIP - glucose-dependent insulinotropic polypeptide

GLP1 - glucagon-like pep- tide-1

GLUT1 – Glucose transporter 1

GLUT2 – Glucose transporter 2

GLUT4 – Glucose transporter 4

GP3 – Glycerol-3-phosphate

H:

HCD – High caloric diet

HIFs – hypoxia-inducible factors

HOMA-IR – homeostatic model assessment for insulin resistance

HOMA-IR2 – modified homeostatic model assessment for insulin resistance

HSL – Hormone sensitive lipase

HSL-Ser563 – phosphorylated hormone sensitive lipase on serine 563 residue

I:

IBMX – phosphodiesterase inhibitor (prevents cAMP decline)

IFG – impaired fasting glucose

IFNγ – Interferon gamma

IGT – impaired glucose tolerance

IL1 – interleukin 1 (pro-inflammatory IL)

IL4 – interleukin 4 (pro-inflammatory IL)

IL6 – interleukin 6 (pro-inflammatory IL)

IL8 – interleukin 8 (pro-inflammatory IL)

IL10 – interleukin 10 (anti-inflammatory IL)

IPGTT – Intraperitoneal glucose tolerance testing

IKKβ – Inhibitor of nuclear factor kappa-B kinase subunit beta

iNKT – Invariant natural killer T

INSR – Gene encoding for human insulin receptor

InsR – Insulin receptor

InsR-Tyr1361 – Phosphorylated insulin receptor on 1361 tyrosine residue

InsR-Tyr972 – Phosphorylated insulin receptor on 972 tyrosine residue

IR – Insulin resistance

IRS1 – Insulin receptor substrate 1

IS – Insulin sensitive

J:

JKN – c-Jun N-terminal kinases

L:

L-DOPA– L-3,4-dihydroxyphenylalanine, an intermediator of dopamine biosynthesis

LPL – lipoprotein lipase

M:

MAO A – Monoaminoxidase A

MAO B – Monoaminoxidase B

mWAT – mesenteric white adipose tissue

N:

NEFA – non-esterified free fatty acids

NG – normoglycemic

NGT– normal glucose tolerant

NF-κB – nuclear factor kappa B

P:

PDE3β – Phosphodiesterase-3β

PDK4 – Pyruvate dehydrogenase kinase 4

PEPCK– Phosphoenolpyruvate carboxykinase

pEWAT – peri-Epididymal adipose tissue

PI3K – Phosphatidylinositol-4,5-bisphosphate 3-kinase

PGC1α – Peroxisome proliferator-activated receptor-gamma coactivator

PKC – Protein kinase C

PPARA – Gene encoding for human peroxisome proliferator-activated receptor alfa

PPARα – Peroxisome proliferator-activated receptor alfa

PPARγ – Peroxisome proliferator-activated receptor gamma

S:

SCN – Suprachiasmatic nuclei of the hypothalamus

SHR – rat model of genetic spontaneous hypertensive condition

SNS – sympathetic nervous system

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

STZ – Streptozotocin

SULT1A3 – Sulfotransferase 1A3, converts dopamine into dopamine sulphate

T:

T2D – Type 2 Diabetes

TBZ – Tetrabenazine

TH – Tyrosine hydroxylase

TG – triacylglycerol or triglycerides

U:

UCP1 – uncouple protein 1

UCP1 – Gene encoding for human uncoupling protein 1

V:

VAT – Visceral adipose tissue

VEGF-A – Vascular endothelial growth factor A

VEGFR2 – Receptor 2 for VEGF

VLDL – Very low-density lipoprotein

VMAT1 – Vesicular monoamine transporter type 1 (found at intestinal enterochromaffin cells)

VMAT2 – Vesicular monoamine transporter 2 (found at β-pancreatic cells)

VMH – Ventromedial hypothalamus

VTA – Ventral tegmental area

CHAPTER 1 - INTRODUCTION

1. Adipose tissue dysfunction, insulin resistance and type 2 Diabetes

1.1 Type 2 Diabetes: physiopathology, risk factors and associated comorbidities.

The prevalence of type 2 diabetes (T2D) has continued to increase over the past 50 years, distributed from western countries to the western Pacific, as well as to Asia and African countries (DeFronzo & Abdul-Ghani, 2011; Roden & Shulman, 2019). In 2017, the International Diabetes Federation has estimated that by the year of 2015 8.8% of world-wide population between the age of 20–79 years meaning that 1 in 11 adults suffers from T2D and it was predicted that these percentage will increase over the years (Ogurtsova et al., 2017). Particularly, in Portugal it was estimated by the year 2015 that approximately 13.3% of the population suffered from T2D (Matafome et al., 2015; Raposo, 2016, Relatório Anual do Observatório Nacional da Diabetes, Edição de 2019 search on Sociedade de Diabetologia site on 13.04.2021). These data demonstrated how T2D constitutes a highly socio-economical and heath burden worldwide (Glovaci et al., 2019; Pradeepa & Mohan, 2017). The increasing prevalence of T2D is driven by a complex interplay of socioeconomic and demographic (physical inactivity and overnutrition), genetic and environmental factors (pollution, smoking and sleep deprivation) (Roden & Shulman, 2019, International Diabetes Federation search at 13.04.2021). T2D risk factors include a combination of both genetic and metabolic factors and, despite the genetic background being a contributor to insulin resistance and β-cell failure, weight gain, physical inactivity and aging, can seriously aggravate T2D prognosis (Glovaci et al., 2019; Kwon et al., 2017; Roden & Shulman, 2019).

Although T2D is a chronic and heterogeneous disease, its prognosis could be improved or even its remission achieved if substantial lifestyle modifications are implemented regarding dietary and physical activity habits (avoiding the intake of highly palatable or energy dense refined food

and sedentary behavior) (Bódis & Roden, 2018; Guilherme et al., 2008; Roden & Shulman, 2019).

Insulin resistance is considered the core feature for the development of T2D onset, followed by glucose intolerance, which culminates in T2D (Roden & Shulman, 2019). Current evidences favour the two-step development of T2D premise, where during step one, normal glucose tolerant (NGT) individuals progress to impaired glucose tolerance (IGT) with insulin resistance as the primary condition. Then, plasma insulin levels remain elevated to maintain glucose homeostasis, although β-cell function begins to be compromised. In the step 2, IGT progresses to T2D due to the decline in β-cell function and failure, leading to chronic hyperglycaemia (DeFronzo & Abdul-Ghani, 2011; Forbes & Cooper, 2013; Javeed & Matveyenko, 2018; Skyler, 2004). Insulin resistance affects peripheral tissues such as the liver, skeletal muscle and adipose tissue, including an impairment insulin receptor availability, function and signal transduction, (DeFronzo & Abdul-Ghani, 2011; Guilherme et al., 2008; Javeed & Matveyenko, 2018; Patel & Goyal, 2019; Skyler, 2004).

The major complications of T2D are denominated as ‘diabetic triopathy’ including: diabetic kidney disease (nephropathy), diabetic retinopathy and diabetic neuropathy, which are manifested by microvascular injuries (Eid et al., 2019; Forbes & Cooper, 2013; Glovaci et al., 2019; Pradeepa & Mohan, 2017) . Diabetic kidney disease affects 30–40% of all individuals with diabetes and is the leading cause of renal function failure, characterised by increased urinary albumin excretion and declined glomerular filtration rate (Eid et al., 2019; Forbes & Cooper, 2013).

The most severe and primary cause of blindness worldwide at the working-age population is diabetic retinopathy, affecting more than 60% of T2D patients. Proliferative diabetic retinopathy

is characterized by an increased pathological retinal neovascularisation, which can lead to visual loss within 5 years upon the onset of the disease in patients with T2D (Eid et al., 2019; Ljubimov, 2017).

Diabetic neuropathy is a highly prevalent complication affecting 50% of individuals with diabetes. Various types of peripheral nerve disorders can be developed, being distal symmetric polyneuropathy the most common affecting nerves extremities. From this point forward, this highly debilitating complication could be associated with an increased susceptibility to ulcerations and infections (most commonly known: the diabetic foot) that could eventually lead in the worst-case scenario to lower-limb amputations (Eid et al., 2019; Megallaa et al., 2019).

Several complications associated to T2D also affect the cardiovascular system (the leading cause of premature death in adults worldwide including the Portuguese population (Eid et al., 2019; Glovaci et al., 2019; Henning, 2018). Cardiovascular disorders comply the macrovascular complication of T2D, where it increases two to three times the risk of developing heart failure, peripheral arterial disease, coronary heart disease, atherosclerosis and cerebrovascular events (transient ischemic strokes) (Eid et al., 2019; Forbes & Cooper, 2013; Glovaci et al., 2019; Pradeepa & Mohan, 2017).

Weight gain and obesity are major contributors to the development of peripheral insulin resistance and T2D. In fact, body fat distribution and weight gain throughout adulthood are important predictors of T2D (Berry et al., 2013; Goossens, 2008; Kwon et al., 2017; Roden & Shulman, 2019). Obesity and obesity-related disorders have been increasing and reached an alarming epidemic proportion. By 2008, it was estimated that more than 1 billion adults were overweight and, from those, 300 million were obese (Berry et al., 2013; Goossens, 2008; Kwon et al., 2017). There is an association between increased body mass index (BMI) (overweight and

obesity) with the development insulin resistance and ultimately f TD2 (Kwon et al., 2017; Marcelin et al., 2019; Qatanani & Lazar, 2007; Scherer, 2006), additionally in the Portuguese population, according to data obtained from PREVADIAB, 90% of diabetic patients presented overweight or obesity. Thus, as presented at figure 1, the prevalence of diabetes in obese subjects (BMI > 30) is 4 times higher than in non-obese diabetic patients (BMI < 25) (Raposo, 2016, Relatório Anual do Observatório Nacional da Diabetes, Edição de 2019 search on Sociedade de Diabetologia site at 13.04.2021).

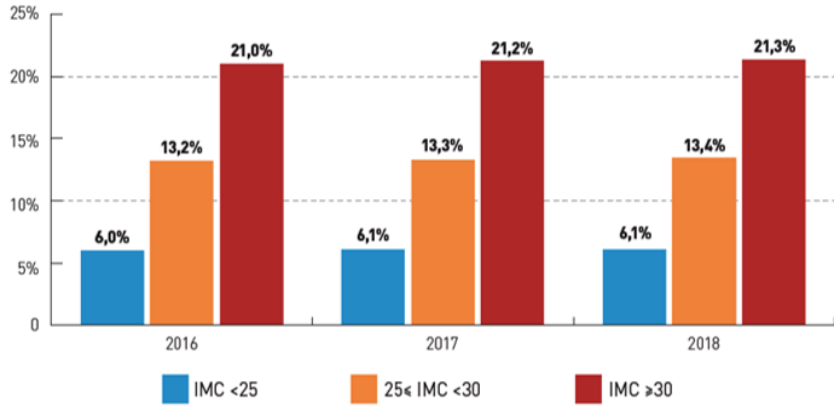


Figure 1 - Prevalence of Diabetes in Portugal according to BMI in 2018. Data extracted from PREVADIAB – SPD.

Understanding the association and cause-effect between obesity, adipose tissue dysfunction, insulin resistance and T2D may clarify our knowledge about its pathophysiology, pointing out to more effective or new therapeutical approaches for metabolic disorders.

1.2. Adipose tissue as a central organ modulating peripheral metabolism

1.2.1 Adipose tissue function

Adipose tissue (AT) is a highly organized and heterogenous tissue comprising adipocytes, the cells specified to store the fat content from the circulation, surrounded by a stromovascular fraction responsible for providing nutrients, O_2, hormones and growth factors, and proper angiogenesis to adipocytes (Crewe et al., 2017; Lemoine et al., 2013; Rajala & Scherer, 2003). Thus, the stromovascular fraction includes mesenchymal stem cells, preadipocytes and fibroblasts, which communicate with adipocytes, ensuring the proper AT angiogenesis and adipogenesis, or adipo-vascular coupling. Furthermore, it also safeguards a proper immune-vigilance by the resident macrophages responsible the waste removal, such as apoptotic cells or organelles and also guarantee the degradation of extracellular matrix components (ECM) providing healthy adipose tissue growth and plasticity (Belligoli et al., 2019; Christiaens & Lijnen, 2010; Crewe et al., 2017; Guilherme et al., 2008; Lemoine et al., 2013; Rajala & Scherer, 2003).

Adipose tissue comprises various discrete depots throughout the body, such as inguinal, interscapular, perigonadal, retroperitoneal and mesenteric depots, presenting distinct morphologies (Berry et al., 2013; Bódis & Roden, 2018; Kwon et al., 2017).

According to histological, molecular features and function, in mammals, adipose tissue has been divided into 2 major categories: the white adipose tissue (WAT) and brown adipose tissue (BAT) (Berry et al., 2013; Cai et al., 2018). Furthermore, from an anatomic point of view WAT is separated into subcutaneous (SAT) and visceral depots (VAT) (Belligoli et al., 2019; Bódis & Roden, 2018; Kwon et al., 2017). While white adipocytes are mainly responsible and specialized for nutrients storage (fatty acids, triglycerides and glucose) brown adipocytes are enriched in mitochondria and uncoupling protein 1 (UCP1) responsible for oxidizing lipids and glucose to

generate body heat (thermogenesis) (Berry et al., 2013; Cai et al., 2018; Guilherme et al., 2008; Kohlie et al., 2017; Tran et al., 2012).

Besides being a highly active metabolic tissue, AT is also an endocrine organ that exerts autocrine, paracrine and/or endocrine effects by secreting a variety of adipokines, important for modulating its metabolic actions and also mediating thermoregulation, shield against trauma and cold, reproduction and satiety (Berry et al., 2013; Cai et al., 2018; Czech, 2020; Goossens, 2008; Guilherme et al., 2008; Juge-Aubry et al., 2005; Scherer, 2006; Townsend et al., 2017).

Adipose tissue metabolic function:

One of the main functions of adipose tissue is the storage of lipid content from the circulation, namely non-esterified fatty acids (NEFA) to be oxidized or esterified into triglycerides (TG). By exerting this buffering action, it provides a whole-body regulation of lipid flux (Cai et al., 2018; DeFronzo & Abdul-Ghani, 2011; Guilherme et al., 2008). Thus, after a meal, lipids coming from intestinal absorption (chylomicrons) bind to the plasma membrane CD36 receptor, where then are hydrolysed by lipid protein lipase (LPL) and transported to adipocyte cytoplasm as NEFA's trough the fatty acid transporter protein (FATP/CD36). Then, the adaptor protein complex Ap2 captures NEFA, avoiding its freely circulation inside the adipocyte, which could mediate an inflammatory response, to be esterified into TG and stored into the lipid droplet. Importantly lipid droplet surface is coated by perilipin protein, namely perilipin A, critical to regulate proper lipid storage and release (Furuhashi et al., 2014; Lafontan & Langin, 2009; Letra & Seiça, 2017; Tansey et al., 2004; Townsend et al., 2017).

Lipid handling in the adipose tissue is regulated by the peroxissome proliferation activated receptor-gamma (PPARγ), a nuclear receptor which regulates the transcription of genes involved in fatty acid uptake: FATP, CD36 and LPL; metabolic pathways, storage (perilipin A) and lipid

oxidation such as UCP-1and adiponectin. (Lee et al., 2011; Letra & Seiça, 2017; Liang & Ward, 2006; Tansey et al., 2004; Townsend et al., 2017; S. Wang et al., 2016; Zhu et al., 2021).

Moreover, PPARγ activation by endogenous (fatty acids) or exogenous (thiazolidinediones) stimuli also prevents the activation of intracellular inflammatory pathways, as nuclear factor kappa B-mediated signalling (NF-κB), and play an important role in improving insulin signalling and consequently sensitivity by reducing the cytoplasmatic amount of free fatty acids (Goossens, 2008; Guilherme et al., 2008; Scherer, 2006; S. Wang et al., 2016; Wellen & Hotamisligil, 2005).

The control of fat storage and mobilization according to energy flux changes such as the transition from postabsorptive to postprandial state is a tightly regulated process (Dimitriadis et al., 2021). Thus, when required (fasting periods with lower energy sources) TG can be mobilized from the lipid droplet and hydrolyzed into fee fatty acids (FFA) through a process named lipolysis (Lafontan & Langin, 2009; Tansey et al., 2004; Townsend et al., 2017; Zimmermann, 2004).

AMP-activated protein kinase (AMPK) is responsive to cellular energy state, hormonal and nutritional signals being an important regulator of lipid metabolism in adipocytes (Kahn et al., 2005; Mihaylova & Shaw, 2011; Saha et al., 2014; Townsend et al., 2017). Thus, AMPK phosphorylates perilipin A allowing the fragmentation of a larger droplets into smaller ones ensuring the action of lipolytic enzymes to convert TG into FFAs and glycerol in a sequential activation of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL) (Townsend et al., 2017; Zimmermann, 2004).

Re-esterification of FFA's depends on glycerol-3-phosphate (G3P) availability in the cell which main source comes from glyceronegensis a process dependent on pyruvate dehydrogenase kinase

4 (PDK4) and phosphoenolpyruvate carboxykinase (PEPCK). While PDK4 inhibits the pyruvate dehydrogenase complex, preventing pyruvate conversion into acetyl-CoA and shuttling pyruvate toward G3P (Cadoudal et al., 2008), PEPCK catalyses oxaloacetateto decarboxylation to form phosphoenolpyruvate, ultimately converted into G3P (Townsend et al., 2017).

Interestingly, while lipolysis does not consume energy FFA re-esterification consume ATP through the acetylation of FFA by acyl-CoA synthetase which generates AMPK (Gauthier et al., 2008; Townsend et al., 2017).

Besides being an energy-sensor responding primary to the cellular energy state due to the increased AMP:ATP ratio (low nutritional apport and or prolonged exercise), AMPK is also involved in the modulation of food intake, energy expenditure and control whole-body energy balance by integrating hormonal (leptin, adiponectin) and nutritional signals as well as sympathetic nerve stimulation through adrenergic signalling activation (figure 2). (Jocken & Blaak, 2008; Kahn et al., 2005; Mihaylova & Shaw, 2011; Townsend et al., 2017). AMPK is kwon as a promoter of catabolic pathways to generate more ATP, and inhibitor of anabolic pathways (Kahn et al., 2005; Mihaylova & Shaw, 2011; Shackelford & Shaw, 2009). In the adipose tissue, AMPK is responsible for regulating: i) lipolysis, by activating HSL and ATGL; ii) lipid oxidation; iii) lipogenesis, inhibiting Acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS); iv) glucose uptake by assisting glucose transporter 4 (GLUT4) trafficking and v) mitochondrial biogenesis through of PGC1α activation (figure 2) (Mihaylova & Shaw, 2011; Saha et al., 2014; Townsend et al., 2017; Zimmermann, 2004).

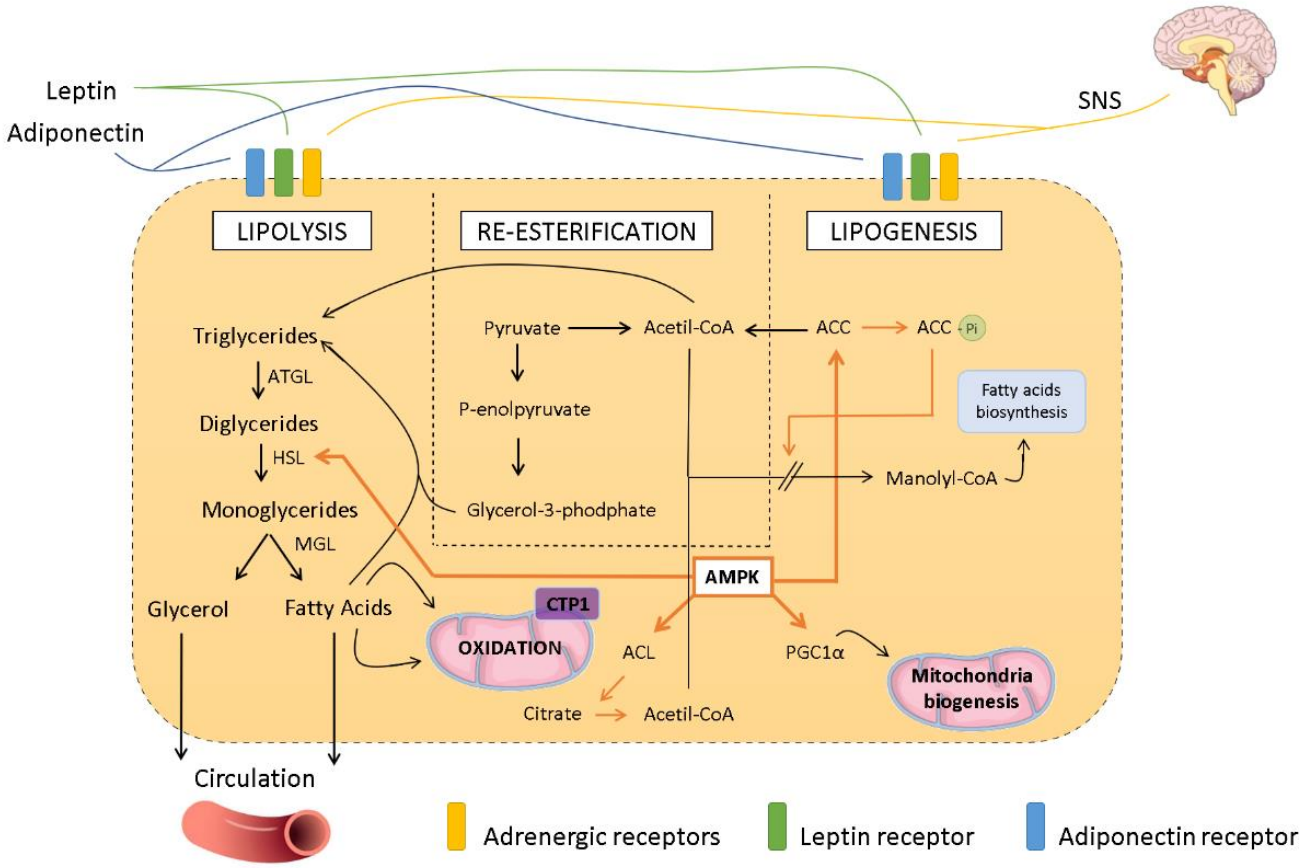


Figure 2 - The complex and highly coordinated mechanism lipid handling in the adipose tissue (storage, lipolysis, lipid oxidation and lipogenesis).

The role of AMPK in biochemical pathways regulating lipolysis, lipid oxidation and lipogenesis. Besides cellular energy status to activate AMPK, lipolysis and lipogenesis can also be mediated by hormonal factors (leptin and adiponectin) as well as the activation of sympathetic nervous system modulating adrenergic signalling. Adapted from Townsend et al., 2017.

The action of insulin in adipose tissue is crucial to modulate lipid metabolism since during post-prandial phase it activates anabolic pathways (lipogenesis) and lipid storage while at the same time inhibit lipolytic pathways. Once insulin reaches its receptor (tyrosine hydroxylase receptor) at adipocyte cellular membrane, it phosphorylates specific tyrosine residues, namely at Tyr 1160, Tyr1361 and Tyr972 promoting its dimerization and consequent activation of insulin receptor

substrate 1 (IRS1). Then, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) is phosphorylated and subsequently the protein kinase b (Akt/PKB) which, among other actions, is responsible for the activation of the signaling cascade resulting in GLUT4 translocation from endocytic vesicles to the plasma membrane allowing post-prandial glucose uptake (Bódis & Roden, 2018; Haeusler et al., 2018; Patel & Goyal, 2019; Roden & Shulman, 2019; Wellen & Hotamisligil, 2005).

Furthermore, insulin signaling pathway also activates phosphodiesterase enzymes, namely PDE3β, limiting the intracellular amount of cyclic adenosine monophosphate (cAMP) and consequently, the activation of PKA that results in decrease activation of HSL and Perilipin A (Roden & Shulman, 2019).

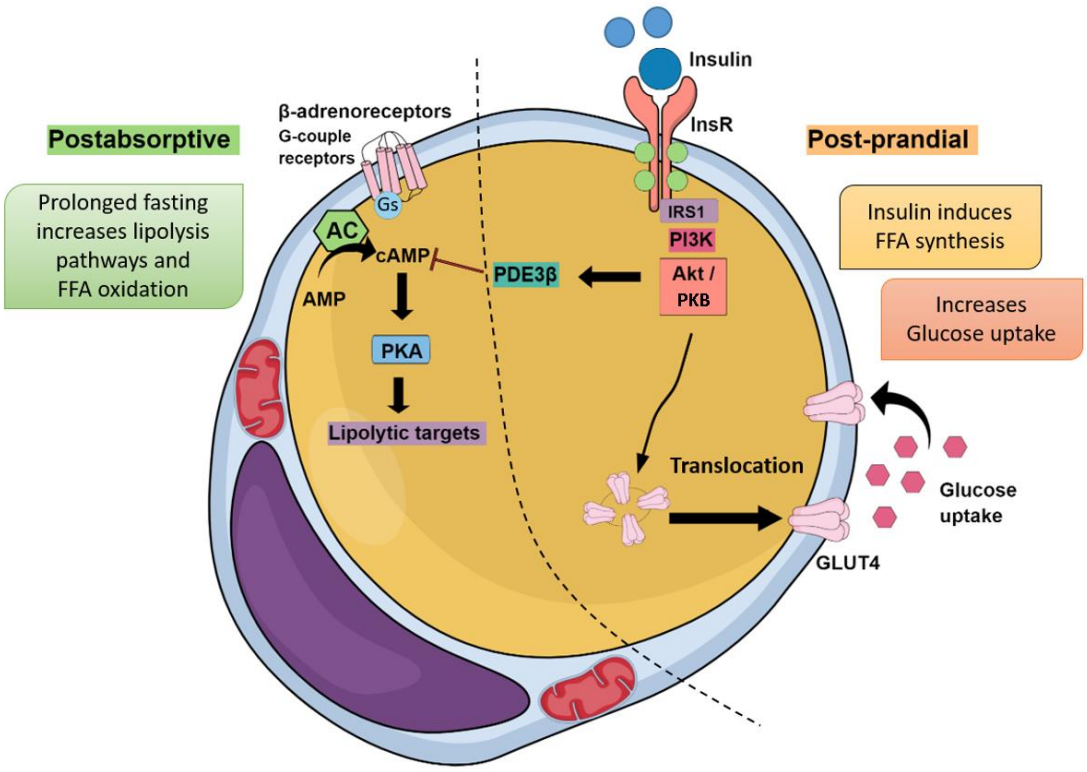


Figure 3 – Insulin receptor pathway is activated in a post-prandial stage, promoting glucose uptake and fatty acids synthesis, while in postabsorptive state, lipolytic and oxidation pathways are activated instead, due to the metabolic shift from glucose (post-prandial) to fatty acids (post-absorptive) as fuel source.

Endocrine function of adipose tissue:

Despite its main function being lipid buffering, the AT is more than a passive reservoir of lipids and glucose and adipocytes secrete many hormones and cytokines that modulate energy homeostasis and peripheral insulin sensitivity (Dimitriadis et al., 2021; Galic et al., 2010; Matafome et al., 2012a; Townsend et al., 2017) Among these hormones and adipokines are leptin, adiponectin, resistin, omentin-1, visfatin, vasoactive effects, and inflammatory factors such as IL6, IL10 and TNFα (pro and anti-inflammatory cytokines) (Boydens et al., 2012; Galic et al., 2010; Letra & Seiça, 2017; Scherer, 2006; Wellen & Hotamisligil, 2005; Yiannikouris et al., 2010).

Leptin and adiponectin will be highlighted here. Leptin is abundantly and almost exclusively produced by adipocytes and its circulating levels closely correlate with the total amount of body fat (Galic et al., 2010; Kahn et al., 2005; Rajala & Scherer, 2003; Scherer, 2006). Leptin is responsible for controlling food intake, body weight and energy expenditure by activating afferent nerve fibres that enervate directly the hypothalamus (Galic et al., 2010; Kahn et al., 2005; Kiess et al., 2008; Rajala & Scherer, 2003). Moreover, it also stimulates fatty acid uptake and oxidation in skeletal muscle and liver (Meier & Gressner, 2004; Rajala & Scherer, 2003) glucose uptake (Haque et al., 1999; Kahn et al., 2005; Kamohara et al., 1997), inhibit fatty acid synthesis by supressing the transcription factor SREBP-1c and the activity of key proteins of fatty acids biosynthetic pathway – ACC and FAS (Kahn et al., 2005; Ueki et al., 2004).

Leptin also activates AMPK as previously mentioned, stimulating FFA oxidation in the adipose tissue, preventing its returning to the circulation and ectopic accumulation (Juge-Aubry et al., 2005; Kahn et al., 2005; Meier & Gressner, 2004; Rajala & Scherer, 2003). Furthermore, leptin acutely supresses insulin secretion in the pancreas ensuring long-term β cell survival and function, although leptin receptor is linked to the activation of proteins and transcription factors responsible for stimulating insulin synthesis (Kahn et al., 2005; Rajala & Scherer, 2003).

Adiponectin, also produced by the adipose tissue in response to the activation of PPARγ, is a key mediator of lipid metabolism by stimulating FAA oxidation (Xu et al., 2003). PPARγ modulates the expression of adiponectin levels both via transcriptional and post-transcriptional mechanisms (Hwang et al., 2019; Rajala & Scherer, 2003; Scherer, 2006; S. Wang et al., 2016; Zhu et al., 2021). Adiponectin circulates in three main molecular forms in the plasma (trimeric, hexametric, and high-molecular weight isoform) and binds to two membrane receptors, AdipoR1 (mostly express in skeletal muscle cells) and AdipoR2 (most predominate in hepatocytes). It is well described that adiponectin activates both AMPK and peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PPARα) through the binding to AdipoR1 and AdipoR2, promoting the uptake and oxidation of glucose and lipids, and inhibiting gluconeogenesis, ensuring whole-body energy balance (Juge-Aubry et al., 2005; Kahn et al., 2005; Nawrocki et al., 2006; Scherer, 2006; Yamauchi et al., 2001, p. 4). AMPK activation trigger GLUT4 trafficking to the cell membrane, facilitating glucose uptake, while PPARα initiates the expression of enzymes involved in lipid oxidation and regulators of mitochondrial biogenesis (Kahn et al., 2005; Mihaylova & Shaw, 2011; Saha et al., 2014; Yamauchi et al., 2001). Moreover, adiponectin directly inhibits the enzymes involved in the FFA synthesis, through the inhibition of ACC and other lipogenic key enzyme (Nawrocki et al., 2006; Ouchi et al., 2001; Xu et al., 2003).

Moreover, adiponectin also has shown effects on β cells, improving insulin secretion, and on the vasculature exerting an anti-inflammatory affect that prevents atherosclerosis and confer long term cardio-protection (Juge-Aubry et al., 2005; Kahn et al., 2005; Xu et al., 2003; Yiannikouris et al., 2010).

Adipose tissue plasticity:

WAT is highly dynamic organ with remodelling capacity to enlarge its volume according to nutritional signals, food availability and metabolic shift from postabsorptive to postprandial states. Adipose tissue plasticity is tightly regulated by 3 main factors: hypoxia-responsive pathways, angiogenesis and mild acute inflammatory response (Christiaens & Lijnen, 2010; Crewe et al., 2017; Dimitriadis et al., 2021; Matafome et al., 2012b, 2015; Nawrocki et al., 2006; Scherer, 2019; Townsend et al., 2017). Adipose tissue plasticity is essential to ensure peripheral metabolic homeostasis: guaranteeing lipid buffering capacity preventing lipotoxicity (ectopic lipid accumulation) according to whole-body energy status and food supply (Bódis & Roden, 2018; DeFronzo & Tripathy, 2009a; Guilherme et al., 2008). Hypoxia stimuli is a key driver to ensure heathy AT plasticity. During postprandial stage AT expansion was shown to lead to the formation of small hypoxic regions. Therefore, a mild degree of hypoxia is sufficient to trigger the activation of hypoxia-inducible factors (HIFs), which enhances angiogenesis pathways activation and ECM remodelling to reduce hypoxia and facilitate expansion of AT (Christiaens & Lijnen, 2010; Crewe et al., 2017; Matafome et al., 2015; Scherer, 2019; Trayhurn, 2013). Also, lipid oxidation mediated by UCP1 in the mitochondria increase O_2 necessity, consequently reducing its availability, what may also transiently reduce oxygen availability (Crewe et al., 2017; Roden & Shulman, 2019). The adipose tissue of obese subjects was however suggested to

be transiently hyperoxic, due to a reduced metabolic activity (Boydens et al., 2012; Goossens et al., 2011; Goossens & Blaak, 2012).

Moreover, an adequate vascularization and angiogenesis, is critically to ensure a healthy AT and VEGF-A/VEGFR2 signalling pathway is considered to be particularly important in this process (Christiaens & Lijnen, 2010). Besides being regulated by HIFs, this pathway is also upregulated by insulin, promoting a healthy adipose tissue expansion. Moreover, adipose tissue ECM remodelling promoted by the resident macrophages is essential to adipocyte proliferation and enlargement, also playing a key role as source in degrading ECM constituents in response to acute/mild inflammatory stimuli (Christiaens & Lijnen, 2010; Coats et al., 2017; Crewe et al., 2017; Juge-Aubry et al., 2005; Scherer, 2019).

Adipose tissue plasticity is essential to ensure peripheral metabolic homeostasis: guaranteeing lipid buffering capacity preventing lipotoxicity (ectopic lipid accumulation) according to whole-body energy status and food supply (Bódis & Roden, 2018; DeFronzo & Tripathy, 2009a; Goossens, 2008; Guilherme et al., 2008, 2019; Roden & Shulman, 2019).

1.2.2 Adipose tissue dysfunction

From an evolutionary point of view, periods of fasting or starvation were recurrent. Nevertheless, this reality has change in our days leading our society to be confronted with excess of food availability which can persuade some individuals to face a passive and persistent state of chronic overnutrition and physical inactivity (Bódis & Roden, 2018; Crewe et al., 2017; DeFronzo, 2004; DeFronzo & Tripathy, 2009). Thus, recurrent seeking for high-fat / high caloric diets lead to an imbalance between energy supply and its expenditure, resulting in increased incidence of overweight and obesity (Boden, 2011; Bódis & Roden, 2018; Guilherme et al., 2008). As consequence, lipid uptake and storage by the AT become an unceasing process leading to

continuous AT expansion until the diffusional limit of O_2 is reached. Then AT losses its buffering capacity allowing TG and NEFA's to flow to the circulation and ectopically accumulate in non-adipose tissues. The excessive influx of lipids to non-adipose tissues, namely the liver and skeletal muscle contributes to peripheral insulin resistance, mostly because liver and skeletal muscle capacity to oxidize fatty acids is not sufficient (Bódis & Roden, 2018; Crewe et al., 2017; Czech, 2020; DeFronzo & Tripathy, 2009a; Goossens, 2008; Matafome et al., 2012b; Scherer, 2019).

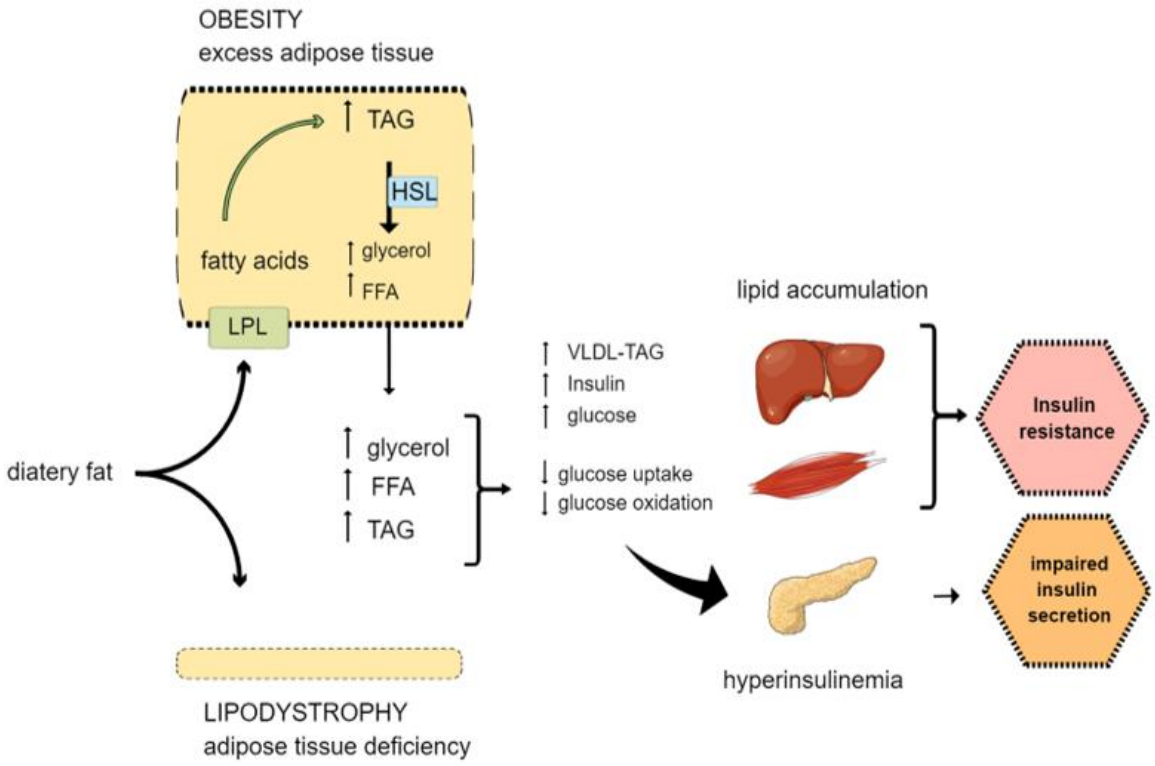


Figure 4 - Decreased buffering capacity for lipid storage in the adipose tissue in obesity exposes non-fat tissue to excessive fatty acids and triacylglycerols (TAG) interfering with insulin sensitivity (skeletal muscle and liver) and insulin secretion (pancreas). Adapted from Gooses et al., 2008.

Lipid oxidation is a limited process therefore FFA and its metabolites such as fatty acyl CoA, ceramides and diacylglycerols accumulate inside adipocytes, hepatocytes and myocytes contributing to insulin resistance development. Moreover, these FFA intermediates can activate several proteins with Ser/Thr kinase function (PKC, IKKβ, JKN) leading to the inhibition of insulin pathway through the phosphorylation serine residues of insulin receptor and insulin receptor substrate 1 (IRS1) (Boden, 2011, 2011; Bódis & Roden, 2018; Galic et al., 2010; Goossens, 2008; Guilherme et al., 2019; Haeusler et al., 2018). By compromising insulin signalling pathway, glucose uptake became impaired due to the reduction of GLUT 4 trafficking to adipocytes and myocytes cell membrane. Such mechanisms when chronically activated, result ultimately in insulin resistance as well as unsolved hyperglycaemia leading to the onset of T2D (Bódis & Roden, 2018; Czech, 2020; Guilherme et al., 2008, 2019; Roden & Shulman, 2019).

Enlarged adipocytes as consequence of increased adiposity leads also to disturbances in adipokine secretion. Such changes consequently alter AT inflammatory response towards a pro-inflammatory profile, with increased secretion of MCP1, TNFα, IL1, among others pro-inflammatory cytokines. This cytokines function as chemoattract agents, recruiting macrophages to AT, which generates infiltrates (Crewe et al., 2017; Goossens, 2008; Guilherme et al., 2008; Scherer, 2019; Townsend et al., 2017). Chronic and unsolved local inflammation in AT due to macrophage infiltration dysregulates ECM remodelling and angiogenesis, perpetuating the cycle of inflammation which impairs AT expandability and plasticity. The increase of ECM constituents ultimately leads to fibrotic AT and recruitment of more macrophages. In turn, an exacerbated inflammatory response also alters adipokines release, as it has been observed in obese patients, namely by decreasing adiponectin and increasing leptin, IL-6 and TNFα creating a vicious cycle that results in vasoconstriction and endothelial dysfunction resulting in impaired

AT angiogenesis. Furthermore, these exacerbated and unsolved mechanisms also resulted in cellular senesce and AT necrosis (Bódis & Roden, 2018; Crewe et al., 2017; Scherer, 2019; Townsend et al., 2017; Wellen & Hotamisligil, 2005).

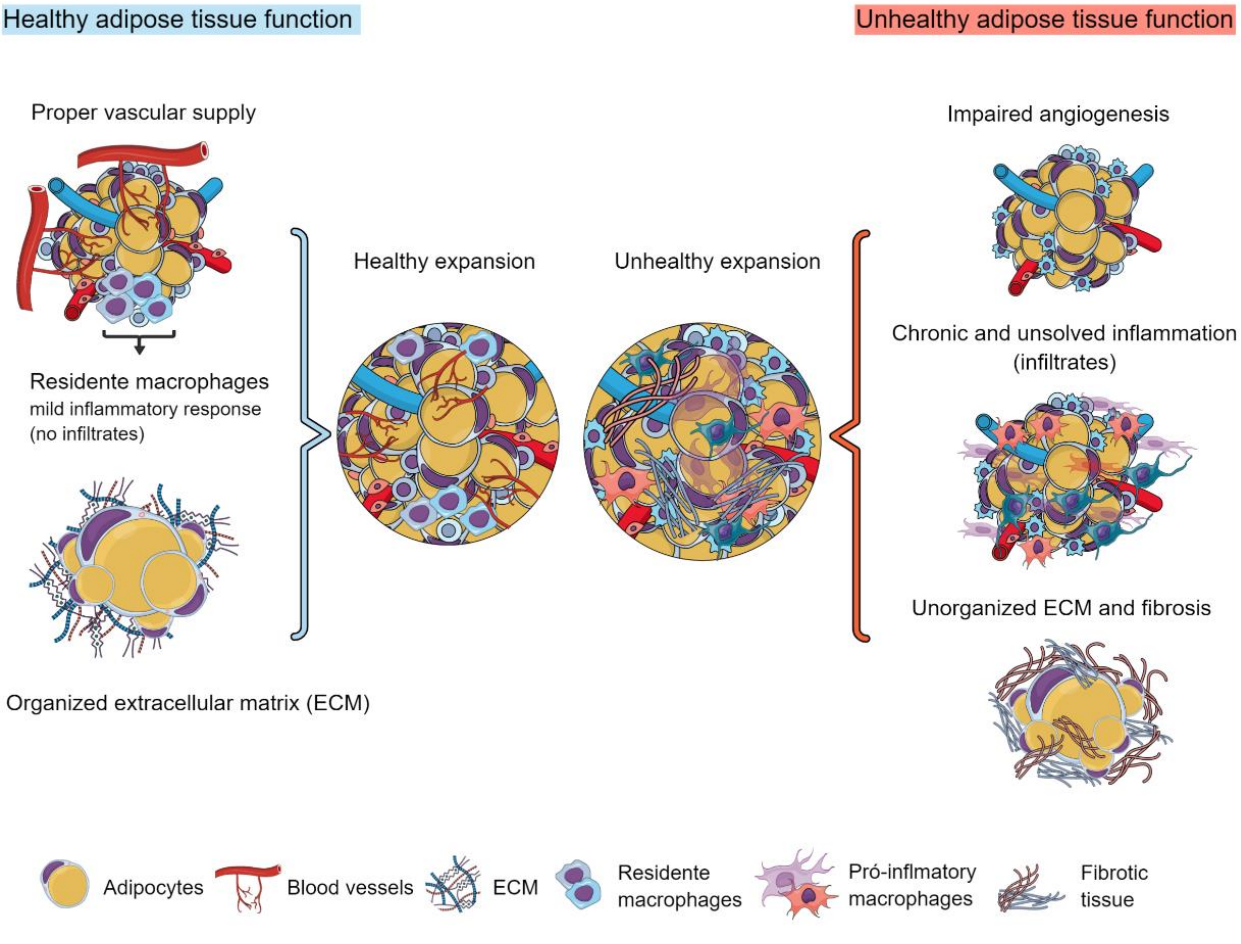


Figure 5 – Schematic figure representing the complex regulation between the three main mechanisms involved in heathy adipose tissue expansion: mild hypoxia stimuli, angiogenesis and controlled inflammatory response, that when lost, compromises adipose tissue plasticity and impairs its metabolic and secretory function. Adapted from Crew et al., 2019.

Thus, loss of AT healthy plasticity into unhealthy AT expansion (chronic hypoxia, unsolved inflammation response, macrophages infiltration and impaired angiogenesis) contributes to systemic metabolic disturbances, characteristic of obesity and T2D (Christiaens & Lijnen, 2010; Coats et al., 2017; Crewe et al., 2017; Lemoine et al., 2013; Scherer, 2019; Townsend et al., 2017; Trayhurn, 2013). From a molecular point of view, this switch compromises lipolysis, lipogenesis, and lipid oxidation by downregulating PPARy (Guilherme et al., 2008; S. Wang et al., 2016), AMPK, UCP1, lipolytic proteins and other metabolic mediators such the responsible ones for mitochondrial function (PGC1α) (Bódis & Roden, 2018; Guilherme et al., 2008; Liang & Ward, 2006). Moreover, as a result of decreased PPARy expression, adiponectin secretion becomes compromised as well (Hwang et al., 2019; Rajala & Scherer, 2003; S. Wang et al., 2016; Zhu et al., 2021).

Besides, peripheral insulin resistance, namely in AT, liver and skeletal muscle, the pancreas itself with the excess of NEFA accumulation upon AT loses it lipid buffer capacity, suffers from insulin resistance which compromises β cell function and insulin secretion (DeFronzo, 2004; Goossens, 2008; Rajala & Scherer, 2003; Scherer, 2019).

2. Control of lipid metabolism by catecholamines and adrenergic signalling

Catecholamines (CA) are known to stimulate lipolysis via the β1- β2- and β3-adrenoceptors. Adrenoceptors are G couple proteins receptors inducing the increase of intracellular cAMP concentration via cAMP-dependent protein kinase activation, which leads to the phosphorylation and activation of HSL (Zouhal et al., 2013).

The physiological effects of catecholamines can result from a combination of increased activity of the sympathetic nervous system and secretion from the adrenal medulla (Macdonald et al., 1985), thus their actions could be referred as part of the sympatho-adrenal system (Zouhal et al., 2013).

Adipose tissue is connected to the central nervous system (CNS) via afferent and efferent nerve fibbers directly innervating each fat depot, which are entirely catecholaminergic (Garretson et al., 2016; Youngstrom & Bartness, 1995). All three beta-adrenoceptors are functional in human AT but β1 and β2 are the most active, whereas β3 is the mostly active in rodents (Jocken & Blaak, 2008). Despite of the role of β3-adrenoceptors in human adipocytes being less clear, some authors have demonstrated a lipolytic role, while others report a negligible effect or no effect (Lafontan & Langin, 2009; Macdonald et al., 1985; Zouhal et al., 2013). Garretson et al. 2016 has described that during an energetic challenge such as cold exposure in humans, norepinephrine binds β3-adrenorecptors initiating a lipolytic cascade dependent on HSL activation, which leads to release of long-chain FFA's and glycerol as source of energy supply (Garretson et al., 2016), while other authors have suggested a β2-adrenorecptors driven mechanism (Blondin et al., 2020).

Disturbances in lipolytic pathways may play a role in the development and maintenance of lipid storage. Thus, a reduced catecholamine-induced lipolysis may contribute to increased AT stores

that at some point could progress to metabolic disorders such as obesity (Jocken & Blaak, 2008). In fact, it has been described a blunted whole-body effect of catecholamines-induced lipolysis in obese patients, while some *in vitro* studies suggest a site-specific catecholamine resistance in the abdominal SAT (Jocken & Blaak, 2008).

This blunted catecholamine-induced lipolysis in AT of obese subjects may be a primary defect leading to increased adiposity in obesity or may, on the other hand, be an adaptational response to the obese insulin resistant state, which remains to be better elucidated in the future (Jocken & Blaak, 2008).

Dopamine (DA) is also a catecholamine and it is an important regulator of energy expenditure, food intake and goal-oriented behaviours (de Leeuw van Weenen et al., 2010; J. P. Dunn et al., 2012; Garcia-Tornadú et al., 2010; Lopez Vicchi et al., 2016). It has been described a defective DA neurotransmission in both obese humans and animals, with some imaging studies showing that obese individuals presented decreased striatal D2R availability. Davis et al., 2009 have shown by quantitative autoradiography, that genetically obese Zucker rats presented decreased D2R-binding capacity, in all striatal regions (Striatum, NA shell and core, VTA and lateral hypothalamus) comparatively with lean controls. It has also been hypothesized that reduced dopamine signal transduction may promote overeating stimuli and decreased energy expenditure, which can contribute to the observed energy imbalance observed in obesity (Bódis & Roden, 2018; Davis et al., 2009; DeFronzo & Abdul-Ghani, 2011; G.-J. Wang et al., 2001). These observations suggested a hyposensitivity of the central dopaminergic system involving the D2R in obesity and obesity-related metabolic disturbances (Davis et al., 2009). Whether such individuals are born with inherently lower numbers of D2R, or whether obesity results from

pathological eating behaviours altering DA signalling (affectingD2R expression) remains to be elucidated (Davis et al., 2009; Haltia et al., 2007).

2.1 Peripheral dopaminergic system: a metabolic modulator and therapeutic target

Besides acting as a neurotransmitter, DA is known to act peripherally in non-neuronal tissues, e.g., pituitary, kidney, and blood vessels, controlling electrolyte transport, vasodilation, hormone production and cell proliferation (Borcherding et al., 2011; Bucolo et al., 2019; Lopez Vicchi et al., 2016).

Moreover, all components of dopaminergic machinery essential for its synthesis, catabolism and signalling are present in several peripheral organs such as the carotid body (CB) (Gonzalez et al., 1994; Wakai et al., 2015), the adrenal gland medulla and the gut (Chaudhry et al., 2016), the main ones responsible for peripheral dopamine production. Dopamine receptors are also found in pancreatic β-cells, kidney, retina, the vascular system (Bucolo et al., 2019; Nash, 2017) and in insulin sensitive tissues, such as the adipose tissue (Borcherding et al., 2011; Vargovic et al., 2011).

Dopamine receptors are G-coupled receptors categorized as either D1-like type (D1 and D5) or D2-like type (D2, D3 and D4) based on structural, pharmacological and biochemical properties. The D2 receptors are usually coupled to a Gi/Go inhibitory protein and are typically located presynaptically in the brain (Bucolo et al., 2019; Lopez Vicchi et al., 2016; Nash, 2017). On the other hand, D1 receptors are coupled to a Gs stimulatory protein, responsible for the activation of adenylyl cyclase and increasing cAMP levels. D1-like receptors are exclusively postsynaptic.

Similarly, to dopaminergic neurons in the central nervous system, peripheral organs also express tyrosine hydroxylase (TH), the rate limiting enzyme in DA biosynthesis, which converts tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), as well as, the aromatic amino-acid decarboxylase

(AADC) that converts L-DOPA into dopamine (Farino, 2018; Vargovic et al., 2011). Furthermore, the vesicular monoamine transporter 2 VMAT2, responsible for storing dopamine into vesicles, is also expressed in pancreatic β-cells (Chaudhry et al., 2016; Farino et al., 2019; Maffei et al., 2015).

Peripheral DA does not interfere with the amount of DA in the CNS since its major form in human circulation is its biologic inactive form, dopamine sulphate (DA-S) which does not cross the blood-brain barrier (Borcherding et al., 2011; Chaudhry et al., 2016; Ustione et al., 2013). Sulfoconjugation is carried out in the gastrointestinal tract by the sulfotransferase SULT1A3 in order to increase serum DA half-life. Basal serum dopamine (DA-S), has half-life of 3–4 hr compared to the several minutes of the unmodified form of DA (Borcherding et al., 2011). Further, DA-S levels at <10 nM exceeds by 5-fold the combined levels of free DA (1.5 nM) found in the circulation (Chaudhry et al., 2016; Maffei et al., 2015). Sulfoconjugations are a reversible process carried out by arylsulfatase (ARSA), which forms dopamine through de-conjugation of DA-S and it has been found in adipocytes (Borcherding et al., 2011).

2.1.1 Major peripheral dopamine-producing tissues

The carotid body:

Carotid bodies (CB) are chemo-sensorial organs located at the bifurcation of the common carotid artery, being the major peripheral sensors of arterial blood chemical composition of the blood, like O_2, CO_2, pH and several hormones (Conde et al., 2017; Leonard et al., 2018; López-Barneo et al., 2016; Wakai et al., 2015). The CB is composed by glomeruli highly vascularized by an organized network of capillaries. Each glomerulus contains several neuron-like glomus cells (type I) and glia-like sustentacular cells (type 2). Type 1 cells hold numerous secretory vesicles filled with neurotransmitters, in particular DA, ATP, acetylcholine, histamine, serotonin, GABA,

and several neuropeptides: glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor and neurotrophin-3 (Leonard et al., 2018; López-Barneo et al., 2016; Shukla et al., 2004; Wakai et al., 2015). Upon stimulation of type 1 cells, neurotransmitters are release in order to activate/inhibit the carotid sinus nerve (CSN). Hypoxia is the standard stimulus for the carotid body activation, which increases CSN activity, that is integrated in the brain stem to induce sympathetic nervous system (SNS) activation and consequently stimulate hyperventilation and cardiovascular reflex (Conde et al., 2017). On the other hand, CB stimulation is also modulated by these same neurotransmitters (DA, ATP, acetylcholine, histamine, serotonin, GABA) and hormones (insulin and leptin).

Interestingly, it appears that both DA and GABA (the main inhibitory neurotransmitter) decrease the magnitude of CNS excitatory response (Alcayaga et al., 1999; Iturriaga et al., 2003; Limberg, 2018). Regarding DA it is possible that its release from afferent C fibres may induce an autocrine-paracrine stimulation of D2R receptors (inhibitory response) on type I cells or afferent nerve terminals (Benot & Lopez-Barneo, 1990; Iturriaga et al., 2003; Leonard et al., 2018; Limberg, 2018).

These DA autocrine-paracrine may involve either negative or positive feedback mechanisms and the most well studied is the negative feedback of DA action on inhibitory D2R from Type I cells. For instance, it is known from the presynaptic side that acute hypoxia causes inhibition of background K^+ channels in type I cells, leading to Ca^{2+} entry trough voltage-gated channels and consequently neurotransmitter release (Benot & Lopez-Barneo, 1990; Leonard et al., 2018).

Although, from intact rat CB, when D2R are blocked by domperidone or haloperidol (D2R antagonists) an increased dose dependent of basal levels of K^+ occurs and hypoxia-evoked catecholamine release is enhanced (Conde et al., 2008; Leonard et al., 2018). These could be a

result of a negative feedback inhibition of intracellular Ca^{2+} signalling, since it is known that DA inhibit L-type Ca^{2+} currents in isolated type I cells (Benot & Lopez-Barneo, 1990; Leonard et al., 2018).

Moreover, D2R stimulation on chemosensory petrosal neurons and their terminals causes intracellular cAMP decrease causing cAMP-gated ion channels inhibition which results in decreased membrane excitability in chemosensory petrosal neurons (Leonard et al., 2018).

The adrenal gland:

In adrenal gland, it had been described the presence of both D1- and D2-like receptors either in human and several animals' species (Mannelli et al., 1995, 1997), where D2-like receptors are mainly expressed in the zona glomerulosa of the adrenal cortex (Amenta et al., 1994; Pivonello et al., 2004). Interestingly, the rat adrenal cortex contains quantities of DA that are compatible with its function as a neurotransmitter, suggesting that locally released DA may act as a neuro-regulator within the gland (Porter et al., 1992). In humans, it had been shown that endogenous DA, co-secreted with the other CA, modulates sympathetic-adrenal discharge during periods of high sympathetic stimulation in an autocrine manner to limit the excessive sympathetic adrenal discharge as occurs during physical exercise where oxygen consumption is elevated (Pivonello et al., 2004; Manneli et al., 1995, 1997).

According to *in vitro* studies using bovine cultured chromaffin cells from adrenal gland, the activation of D2R (both by dopamine and quinpirole - D2R and D3R agonists) resulted in an inhibitory effect on CA release (Huettl et al., 1991; Mannelli et al., 1995). On the other hand, *in vivo* studies performed in heathy subjects have shown the opposite effect or no alteration on CA release (Mannelli et al., 1995;Carey et al., 1983; Whitfield et al., 1980).

Kidney:

It is well described that the dopaminergic system regulates the renin-angiotensin-aldosterone system. DA had been postulated as an intrarenal natriuretic hormone opposing the sympathetic nervous system and the renin-angiotensin system, decreasing renal tubular ion transport in almost all nephron segments (Armando et al., 2015; Jose et al., 1991). Dopamine receptors are expressed along the nephron and are responsible for over 50% sodium excretion when NaCl intake is increased. Disturbance in the renal dopaminergic system may play a role in the pathogenesis of some forms of hypertension, since it had been observed salt-sensitive hypertension in genetically altered mice for renal dopamine production (pt*Aadc*$^{-/-}$ mice) (Armando et al., 2015; Jose et al., 1991; Zhang et al., 2011).

Regarding specific receptor function, adenylyl cyclase-independent dopamine receptor with some pharmacological features of the D2 receptor was described in the renal cortex and inner medulla, but only D3 receptor has been reported at the mRNA level (Jose et al., 1991). Despite of the role of D2R-like receptors remain poorly understood, it was suggested to regulate renal proximal convoluted tubule ion transport together with D1-like receptors (Armando et al., 2015). On the other hand, D1-like receptors functions have been more well described and is associated with cAMP-dependent renal vasodilation and increase of electrolyte excretion (Jose et al., 1991; Li et al., 2015). The impairment in D1R recruitment to the plasma membrane in renal proximal tubule cells is associated with impaired renal sodium homeostasis and hypertension (Armando et al., 2015). It is also recognized that the majority of DA is produced locally in renal tubules, independent of innervation, and not metabolized to norepinephrine, where dopaminergic system is regulated by tubular paracrine and autocrine mechanisms, rather than hemodynamic mechanisms (Armando et al., 2015; Jose et al., 1991).

2.1.2 Other peripheral sources of dopamine

Gastrointestinal tract:

Dopamine was shown to be produced along the gastrointestinal tract, including the parietal cells of the stomach and several regions of the small intestine epithelium. Intestinal TH is expressed in enterochromaffin cells, Lieberkühn crypts, ileal epithelial cells, and throughout the lamina propria of the small intestine. Furthermore, the vesicular monoamine transporter type 1 (VMAT1), responsible for dopamine transport into storage vesicles, is expressed by enterochromaffin cells (Chaudhry et al., 2016).

It has been shown in healthy volunteers that plasma levels of both DA and its biosynthetic precursor L-Dopa increase after ingestion of a standard mixed meal. Moreover, the same effect was observed in rats after performed the mixed meal testing model (Chaudhry et al., 2016; Farino et al., 2019; Maffei et al., 2015). Indeed, it is believed that the nutrient sensing by the gastrointestinal tract is the trigger for plasma DA and L-Dopa increase in the bloodstream, since it is known that: 1) the kinetics of DA and L-DOPA appearance in the serum match with the postprandial rise and fall of plasma incretins; 2) the foregut (including the stomach) is the major source of peripheral circulating DA and L-DOPA (Chaudhry et al., 2016). Therefore, it could be postulated that the foregut is the origin of postprandial elevations of circulating DA and L-DOPA where both TH (DA biosynthesis) and VMAT1 (DA transport and storage) are also expressed (Chaudhry et al., 2016; Maffei et al., 2015).

Pancreatic β cell

As reviewed by Chaundy and other authors, pancreatic β cells express all the machinery responsible for DA synthesis, catabolism and signaling, including TH, AADC, VAMT2, MAO-A and MAO-B, DAT and dopamine receptors (Chaudhry et al., 2016; Farino et al., 2019; Maffei

et al., 2015). Chaundy and others, have suggested that circulating dopamine released from the gastrointestinal tract is transported into and stored in the β-cells trough VMAT2 (Chaudhry et al., 2016; Maffei et al., 2015). Farino and collaborators have shown that the uptake of L-DOPA is also essential for establishing intracellular DA stores in β-pancreatic cells (Farino et al., 2019). Thus, in humans, DA released from β-cells may rise to 100μM within a 5μm radius to exert its effect on the regulation of insulin secretion. Actually, a dose-response curve for human islet insulin secretion when treated with exogenous DA correlates with this physiologic dose released of DA upon its storage inside β-pancreatic cells (Chaudhry et al., 2016; Shankar et al., 2006). It is described that through activation of D2R, DA inhibit insulin secretion constituting a negative feedback action on endocrine pancreas contradicting the effect of incretins (Maffei et al., 2015; Ustione et al., 2013).

Liver:

Given that about 50% of DA is produced in the gastrointestinal tract (GI) by enteric neurons and intestinal epithelial cells, high level of DA reaches the liver through the hepatic portal vein (Chaudhry et al., 2016; Xue et al., 2018). There were no proper studies showing the presence/ absence of dopaminergic synthesis machinery in the liver. Nevertheless, it is known from several studies that DA modulates cellular pathways in liver through the activation of dopamine receptors. For example, hepatocyte plasma membranes isolated from streptozotocin-induced (STZ) diabetic rats presented different composition from plasma membranes isolated from healthy animals (diminished cholesterol, sialic acid, lipids and glycoprotein content), resulting in a decreased dopamine-binding capacity to its receptors (Nassar et al., 1986).

Moreover, DA has shown to regulate hepatic inflammation: i) by suppressing autoimmune hepatitis through D1-dependent inhibition of IL4 and IFNγ production in iNKT cells, preventing

liver damage (Xue et al., 2018); ii) reducing liver lesions by lowering hepatic inflammatory and apoptotic markers and improving the survival rate of lipopolysaccharide (LPS)/D-galactosamine, in a (D-Gal)-induced acute liver injury mouse model (Zhou et al., 2018). Moreover, it has also been shown that experimental depletion of dopaminergic neurons in the liver using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) significantly increased the concanavalin A- induced hepatitis (Xue et al., 2018). Nevertheless, the role of DA in liver function specifically in what concerns it metabolic function, DA regulatory mechanisms are still unknow and need to be elucidated better in the future.

Adipose tissue:

Dopamine receptors were firstly identified at the mRNA and protein levels in human WAT by Borcherding et al., during her experiments aiming to study the regulation of AT prolactin and adipokines release. Dopamine receptors expression was demonstrated both in stromovascular cells (SVC) and mature adipocytes, being D1R more expressed in mature adipocytes than D2R (Borcherding et al., 2011). Moreover, the expression of active arylsulfatase (ARSA) that converts dopamine-sulphate into the active monoamine was confirmed both in SAT and adipocytes cell lines (Borcherding et al., 2011).

Moreover, in BAT, dopaminergic signalling machinery (D1 and D2 receptors) have been identified in homogenates obtained from rats, and DARPP-32 protein, an intracellular third messenger for dopamine activation of D1R subtype receptors have also been identified in pig's BAT (Kohlie et al., 2017). Dopamine actions in the BAT are supported by sympathetic nerve supplying (Bahler et al., 2017). It is well known that CA are critically involved in the regulation of BAT thermogenesis. Thus, it has been suggested that DA could have a homeostatic role similar to norepinephrine, which mediates BAT energy expenditure (Maxwell et al., 1985).

2.1.3 Dopamine as metabolic modulator

Central versus peripheral actions

Dopamine has been suggested to be a metabolic modulator due to its modulatory actions in the CNS. Alterations in endogenous dopaminergic and serotonergic rhythms have been implicated in the modulation of suprachiasmatic nuclei (SCN) and ventromedial hypothalamus (VMH) during the transition from the insulin-sensitive (IS) to insulin-resistant (IR) state. In fact, VMH connects with other hypothalamic nuclei paying a key role in the modulation of autonomic nervous system function, hormonal secretion, peripheral glucose/lipid metabolism, and feeding behaviour (DeFronzo, 2011; Lopez Vicchi et al., 2016; Pijl, 2003). Thus, central actions of DA system that may mediate its metabolic effects are: i) maintenance of the biological clock in the SCN of the hypothalamus and regulation of endocrine axes, namely prolactin secretion; ii) the regulation of hypothalamic noradrenaline output, by decreasing its levels; iii) participation in appetite control (Berinder et al., 2011; DeFronzo, 2011; Freyberg & McCarthy, 2017; Lopez Vicchi et al., 2016; Luque et al., 2016).

i. It is known that in seasonally-induced obese animals the role for circadian phase-dependent rhythms is important to increase hypothalamic dopaminergic tone for the maintenance of the lean and insulin sensitive conditions (DeFronzo, 2011; Ezrokhi et al., 2014; Freyberg & McCarthy, 2017; Lopez Vicchi et al., 2016; Pijl, 2003). Circadian rhythms appear to play a role in reversible insulin resistance, as a conserved adaptation and evolutionary advantage to food deficiency during winter and hibernation. Dopamine and serotonin are integral in modulating the circadian rhythm metabolic changes associated with hibernation (Ezrokhi et al., 2014; Freyberg & McCarthy, 2017; Lamos et al., 2016; Stoelzel et al., 2020). Moreover, IR animals, including genetically obese *ob/ob* mice, present decreased hypothalamic dopamine and increased norepinephrine levels,

with abnormal modulation of neuropeptide Y and corticotropin-releasing factor (Lamos et al., 2016).

ii. Within the VMH, it has been documented that in animals that undergo to seasonal changes, both serotoninergic and noradrenergic levels and activity increased during IR states and return to normal when restoring IS state (DeFronzo, 2011). Such changes are apparently correlated with increased lipolytic activity due to overstimulation of sympathetic activity (DeFronzo, 2011). On the other hand, DA levels are low during the IR state and increase to normal following the return of the IS state (DeFronzo, 2011; Grunberger, 2013). Accordingly, type 2 diabetic individuals are believed to have an early morning fall in dopaminergic tone, which leads to increased sympathetic activity (DeFronzo, 2011; Freyberg & McCarthy, 2017). In a study conducted by Ezrokhi et al., in spontaneous hypertensive rats (SHR), treatment with bromocriptine (10 mg/kg i.p. for 16 days) presented significantly reduced ventromedial hypothalamic norepinephrine and serotonin levels to the normal range, restoring VMH dopaminergic tone (Ezrokhi et al., 2014).

iii. Central dopaminergic 'reward' pathways regulate appetite control. Rhythmic dopamine signals that are originate in the Ventral Tegmental Area (VTA) and project to the ventral striatum [nucleus accumbens (NAc)] regulate the reward pathways that underlie motivation, food craving, and anticipation mechanisms (Freyberg & McCarthy, 2017; Lopez Vicchi et al., 2016).

In post-mortem studies, both humans and rodents' diabetic brains presented reduced endogenous dopamine synthesis in the ventral striatum. Additionally, positron emission tomography studies have shown decreased striatal dopamine D2R and D3R availability in patients with obesity

(Caravaggio et al., 2015; Lopez Vicchi et al., 2016). Thus, it has been suggested that decreased striatal and hypothalamic activity of dopamine receptors may constitute a neuroadaptive response to overconsumption of palatable food, leading to compensatory hyperphagia and excess weight gain (Lamos et al., 2016; Lopez Vicchi et al., 2016). Interestingly, it was also observed that striatal D2R density increased following weight loss after obese patients has been submitted to bariatric surgery (Lopez Vicchi et al., 2016; Steele et al., 2010).

Nonetheless, whether lower numbers of D2R and its availability is an inherent condition leading to overeating behaviour and weight gain, or obesity alter DA signalling and D2R expression remains to be elucidated (Davis et al., 2009).

Regulation of glucose-stimulated insulin secretion:

Despite the hypothesis that increase hypothalamic DA in CNS is the main mechanism of peripheral metabolic improvement and insulin sensitivity, increased evidences suggest that peripheral dopamine play important roles in modulating metabolic regulation in peripheral tissues. Human β-cells islets have shown to secrete DA in a glucose concentration-dependent manner. Additionally, DA release is coincident with insulin release. Besides, both rodents and humans treated with DA or its precursor L-DOPA presented decreased glucose-stimulated insulin secretion (GSIS) from isolated islets (Chaudhry et al., 2016; Farino et al., 2019). Such inhibition apparently involves D2R activation, which is expressed in both β and δ cells and are also found in the insulin granules (Chaudhry et al., 2016; Farino et al., 2019; Maffei et al., 2015). *In vitro,* stimulation of these receptors in pancreatic islets and cultured β-cells with either exogenous DA or D2R/D3R agonists inhibited GSIS. On the other hand, D2R antagonists, such raclopride and sulpiride, blocked the inhibitory effect of DA on insulin secretion from islets of wild-type mice (Bucolo et al., 2019; Farino et al., 2019; Garcia-Tornadú et al., 2010). Similarly,

an agonist for D2R and D3R, quinpirole, presented a dose-dependent inhibition of GSIS (Farino et al., 2019; Kostrzewa, 1995; Lopez Vicchi et al., 2016). To further investigative the singular contribution of each receptor (D2R and D3R) in modulating GSIS, new blocker compounds have been developed, thus the D3R-selective blocker R22 successfully reduced L-DOPA's inhibition of GSIS while the D2R-selective inhibitor ML321 partially reduced L-DOPA-induced GSIS inhibition. These results suggested that both D2R and D3R mediate GSIS inhibition although the joint action of these two receptors produce a greater inhibition (Cortés et al., 2016; Farino et al., 2019; Xiao et al., 2014). Moreover, a recently developed transgenic mice model, by Farino and collaborators the β-cell-specific D2R KO allows to investigate the specific role of D2R-GSIS inhibition in a tissue specific approach (Farino et al., 2019). This animal model presented marked postprandial hyperinsulinemia *in vivo* and lack of L-DOPA-induced GSIS inhibition in isolated β-cells islets. Treatment of islets from β-cell-specific D2R KO, global D3R KO and WT controls with increasing concentrations of exogenous DA have demonstrated that both β-cell-specific D2R KO and global D3R KO mice were less sensitive to DA inhibitory effects on GSIS compared to WT controls (Farino et al., 2019). This confirm that both receptors are important to modulate GSIS inhibition, ultimately regulating insulin secretion and promoting long-term β-cell function and glucose homeostasis (Chaudhry et al., 2016; de Leeuw van Weenen et al., 2010; Farino et al., 2019).

The global mutant mice lacking D2R has been extensively studied (Garcia-Tornadú et al., 2010) highlighting the pivotal role for D2R in insulin secretion and glucose homeostasis, since $DRD2^{-/-}$ male mice exhibited impairment of insulin response to glucose overload and elevated fasting glucose levels. Moreover, these mice were glucose intolerant, and presented reduced β-cell mass at 7 months of age. Glucose and insulin tolerance tests suggested that glucose intolerance is

mainly caused by a blunted insulin secretory response rather than an increase in peripheral insulin resistance, which was both confirmed by GSIS reduction *in vivo* and *in vitro* (Garcia-Tornadú et al., 2010).

In what concerns D1R role in this context, it has been found mainly expressed in β-cells where it stimulates insulin release, opposing D2R effects (Bucolo et al., 2019). VMAT2 appears to be critical in the dopaminergic control of GSIS in rodents; given that the administration of a specific VMAT2 inhibitor, tetrabenazine (TBZ), during intraperitoneal glucose tolerance testing (IPGTT) decreased glycemia and enhanced insulin secretion. This data suggests that vesicular transport and storage of DA in β-cells contributes to increase DA concentration responsible for regulating *in vivo* glucose homeostasis and insulin production, by reducing GSIS (Chaudhry et al., 2016).

Current body of evidence supports the idea of a dopaminergic negative feedback based on locally produced and possibly gut-derived DA which regulates insulin secretion from human and murine β-cells in a paracrine/autocrine way (Farino et al., 2019; Maffei et al., 2015; Ustione et al., 2013). Indeed, lack of dopaminergic inhibition in the $DRD2^{-/-}$ mouse model causes a gradual deteriorating effect on insulin homeostasis, leading to development of glucose intolerance (Garcia-Tornadú et al., 2010). Such process of β-cell exhaustion with depletion of insulin granules and absence of secretory pulses of insulin is consistent with what is observed in T2D. Thus, the dopaminergic negative feedback may be understood as a protective mechanism towards long-term β-cell survival and prevention of exhaustion (de Leeuw van Weenen et al., 2010; Maffei et al., 2015).

Regulation of insulin-sensitive tissues

There is few evidence that peripheral dopamine mediates the regulation of glucose metabolism. Nevertheless, it was shown that L-DOPA regulates glycogen concentration through the activity

of glycogen synthase and modulates insulin-stimulated glucose transport in rat muscle (Chaudhry et al., 2016). Additionally, haloperidol, a non-selective D2R antagonist, has shown to blunt the positive effects of caloric restriction in improving glucose tolerance and insulin sensitivity (Nash, 2017).

In adipose tissue, both DA and bromocriptine has shown to inhibit prolactin gene expression and secretion, which was reverted by the IBMX, a phosphodiesterase inhibitor, suggesting a role for cAMP system in the transcriptional control of prolactin in adipocytes (Borcherding et al., 2011). Regarding adipokines, leptin secretion was suggested to be supressed by DA in SAT explants, isolated mature adipocytes and differentiated primary adipocytes cell cultures. Such effects were observed after incubation with the D1R/D5R antagonist SKF38393, suggesting a role for D1R in the modulation of leptin secretion by adipocytes (Borcherding et al., 2011). Additionally, short-term treatment with bromocriptine, in obese women with standardized caloric intake, significantly reduced plasma circulating levels of leptin. Since there was no evidence indicating that bromocriptine alters leptin clearance from the circulation, this suggested that activation of D2R, modulates the decreased release of leptin levels which contradicts Borcherding finding (Kok, et al., 2006a). However, the direct effect of D1R/D5R activation deceasing leptin secretion from adipocytes does not retrieve the importance of D2R activation inducing the same result after *in vivo* treatment with bromocriptine in humans. Moreover, it suggests a possible modulation or balance of both receptor type regarding acute or systemic treatment with dopamine receptors agonists. Borcherding and co-workers also demonstrated that both dopamine and SKF38393 (D1R antagonist) increase adiponectin in the media of differentiated primary adipocytes and IL6 in proliferating primary adipocytes, although the exact mechanisms were not described (Borcherding et al., 2011).

Despite the effect of DA in adipokines release, the role of other catecholamines cannot be discarded. Thus, Than and co-workers, have shown that incubation of differentiated adipocytes from mouse 3T3-L1 cell line with 1μM of epinephrine, norepinephrine, or dopamine significantly inhibited the secretion of leptin, which was reversed by propranolol, a β-adrenergic receptor antagonist, but not by phentolamine or haloperidol (antagonist for D2, D3 and D4 dopaminergic receptors) or SCH-23390 (antagonist for D1 and D5 dopaminergic receptors) (Than et al., 2011). Therefore, the involvement of DA in regulating adipokines release by adipocytes and a possible crosstalk with other catecholamines needs to be further clarified in the future.

It has been found that after stimulation of SV-40 T immortalised brown adipocytes cell culture with DA, cAMP concentrations increased (Kohlie et al., 2017). Additionally, after 24h treatment either with DA or with D1-like receptor agonist (SCH 38393) oxygen consumption rates, mitochondrial membrane potential and uncoupling protein 1 (UCP1) levels have increased. Furthermore, with DA treatment, PPARα protein levels also increased, suggesting increased lipolysis in BAT, accompanied with increased p38 MAPK phosphorylation (involved in the activation of transcriptional factors controlling the expression levels of PGC1α and UCP1 (Bordicchia et al., 2012; Kohlie et al., 2017). Moreover, DA-mediated effects on oxygen consumption, mitochondrial membrane potential and UCP1 levels were reverted by D1-like receptor antagonist SCH 23390 which was not observed with the treatment of D2-like receptor antagonist raclopride (Kohlie et al., 2017). On the other hand, a study performed in both lean and obese diet-induced animals showed that a single intracerebroventricular bromocriptine injection increased BAT energy expenditure, interscapular temperature and glucose uptake, while no changes in body temperature or respiratory quotient were observed. Nevertheless, UCP1 protein

levels increased as well as animals' tolerance to cold exposure after the treatment. Moreover, lipid droplets in BAT adipocytes also reduced after bromocriptine treatment (Folgueira et al., 2019). It has also been documented that, cold exposure caused increased DA levels in rat BAT, as well as evidences for direct dopaminergic effects on mitochondrial mass and thermogenesis in murine brown adipocytes *in vitro* (Iwen et al., 2017). Interestingly, in a cohort study in young heathy volunteers, cold exposure protocol was used to increase BAT activity via stimulation of sympathetic nervous system (SNS). Indeed, sympathetic activation substantially increased upon cold exposure, which also resulted in higher circulating plasma concentrations of noradrenaline and DA, with no alteration on adrenaline levels (Iwen et al., 2017). Noradrenalin is well recognized to be key in the regulation of SNS-mediated BAT thermogenesis. In contrast, few information is given regarding DA in the context of BAT activation in human. BAT stimulation by cold exposure and consequently, SNS activation is associated with improved whole-body glucose metabolism in healthy lean subjects, a mechanism independent of pancreatic insulin secretion. The increased DA plasma levels upon cold exposure suggested a relevant dopaminergic impact on BAT metabolism in humans that needs to be further investigated in the future, since NE, cannot be excluded from BAT sympathetic activation in this study (Iwen et al., 2017).

In contrast, another study also performed in young lean healthy mean volunteers, treatment with bromocriptine (for 2 weeks (1.25mg/day in the first week and 2.5mg/day after) have shown no significant differences in BAT activity and volume. Nevertheless, subjects became less insulin-sensitive after bromocriptine treatment (Bahler et al., 2017). This is an important result showing an opposite effect of bromocriptine in lean healthy subjects to what is described in obese and insulin resistant patients, where bromocriptine is described to improve insulin sensitivity (Bahler

et al., 2017; DeFronzo, 2011; Lopez Vicchi et al., 2016; Roe et al., 2015). Therefore, it would be of great importance to address further studies on the negative impact of bromocriptine in healthy lean volunteers (Bahler et al., 2017).

2.1.4. Metabolic effects of dopamine-based therapeutic approaches

L-DOPA:

L-DOPA is the first-line treatment for Parkinson's disease that showed to reduce insulin secretion during the oral glucose tolerance test in Parkinson's patients. Similarly, in rodents, L-DOPA injection results in an inhibitory action on insulin response to different secretagogues and, as already mentioned, islets stimulation with L-DOPA reduced GSIS (Farino et al., 2019; Lopez Vicchi et al., 2016). On the other hand, dopamine antagonists (such as the antipsychotics) increase glucose and insulin levels in male rats, suggesting that even though insulin secretion is mainly regulated by glucose, it can be fine-tuned by autonomic input and also by the dopaminergic system (Lopez Vicchi et al., 2016).

Bromocriptine:

Bromocriptine was firstly prescribed for the treatment of Parkinson's disease, prolactinoma and and pituitary tumors and also to prevent post-partum lactation, since it reduces prolactin secretion (Berinder et al., 2011; Furigo et al., 2019; Lamos et al., 2016; Lopez Vicchi et al., 2016). Nevertheless, since 1980 it has been shown to exerts positive effects in patients with T2D, which ultimately led to the FDA approval, by 2009 for use "as an adjunct to diet and exercise" improving glycaemic control in adults suffering from T2D (Lamos et al., 2016; Lopez Vicchi et al., 2016).

Interestingly, by altering bromocriptine formulation to a quick release (Q-R) approach, instead of bromocriptine being administered 2 to 3 times daily in separated doses as usually occurred for

the treatment of Parkinson's and prolactinomas, its quick release formulation allowed a morning dosage only once a day to treat T2D (2- to 3-fold lower dosage than what is use for prolactinomas treatment and 10 to 20 times lower than what is used to Parkinson's treatment) (Via et al., 2010). Furthermore, bromocriptine-QR appears to function as an insulin sensitizer presenting a good safety profile, preventing weight gain, avoiding hypoglycaemic episodes and presenting lower cardiovascular risk (Grunberger, 2013; Lamos et al., 2016; Lopez Vicchi et al., 2016; Roe et al., 2015; Tillman & Kim, 2018; Via et al., 2010).

According to Roe et al., it has been suggested that bromocriptine-QR can induce an additive interaction with insulin therapy. Thus, it provides enhanced responsiveness to the exogenous insulin therapy, producing higher effectiveness in lowering postprandial blood glucose and also lowering the insulin requirement, enabling the reduction of its dosage (Roe et al., 2015).

Bromocriptine-QR is also associated with decreased fasting triglycerides, blood pressure, and heart rate. Furthermore, it also presents an effect on weight and glycemia control in obese non-diabetic and diabetic individuals (Lamos et al., 2016). Weight loss, body fat loss and improved glucose tolerance has also been reported in some cohorts of clinical trials in obese men treated with bromocriptine-QR (1.6-2.4 mg/day, for 18 weeks) (Cincotta & Meier, 1996; Lopez Vicchi et al., 2016; Via et al., 2010). Although not recommended for type 1 diabetes, some data suggested that bromocriptine may have a protective effect on the preservation of endogenous insulin secretory capacity of β-cells (Lopez Vicchi et al., 2016).

According to Kok et al., bromocriptine trough D2R activation promotes an improvement of several metabolic features in obese women (Kok, et al., 2006b) It was shown to significantly decrease systolic blood pressure and parameters of glucose metabolism in fasting conditions

(glucose, insulin, C-peptide). Furthermore, plasma prolactin concentrations were also significantly reduced (Kok, et al., 2006b).

Interestingly, in a study performed in SHR rats (model of genetic spontaneous hypertensive) the treatment with bromocritptine (10 mg/kg i.p. for 16 days) improved insulin sensitivity and significantly reduced hepatic levels of several pro-inflammatory factors and master transcriptional activators of lipogenesis, gluconeogenesis, and free fatty acid oxidation in comparison with control SHR rats (Ezrokhi et al., 2014).

Bromocriptine also seems to impact food intake. Bromocriptine treatment (i.p.10 mg/kg, for 4 weeks) in Zucker and diet induced-obesity rats reduced food intake (Davis et al., 2009). Moreover, bromocriptine impacted on fat deposition in diet-induced obesity rats, as epididymal fat depots were significantly decreased and retroperitoneal fat depot showed a trend to decrease. Also in this study, bromocriptine treatment promoted a decrease in plasma triglycerides and free fatty acids, suggesting a lipolytic effect of the treatment. Opposingly Zucker animals did not exhibit a significant reduction in body fat (Davis et al., 2009).

The peripheral mechanism of action of bromocriptine is still not fully understood, thus it has been suggested a CNS regulation with the involvement of circadian dopamine rhythm, where bromocriptine-QR administration was shown to restore the diminished circadian peak of dopaminergic activity, resulted in an improvement of systemic insulin sensitivity (DeFronzo, 2011; Roe et al., 2015). Preclinical studies suggested that such effects are mediated via a circadian-time-dependent effect to ameliorate hypothalamic activity that potentiate insulin sensitivity and improved fuel sensing mechanisms, which are lost during hibernating-insulin-resistant states where dopamine hypothalamic activity is diminished. Moreover, T2D patients

also present decreased central dopaminergic activity which peak is believed to occur in the morning awakening (Luo et al., 2018; Roe et al., 2015; Stoelzel et al., 2020).

Importantly, further studies should be conducted to better elucidate bromocriptine mechanism of action that may also occur peripherally in insulin sensitive tissues as the few available literature already suggested, that are consistently to the presence of its dopaminergic signalling machinery (Freyberg & McCarthy, 2017; Lopez Vicchi et al., 2016; Tavares et al., 2021).

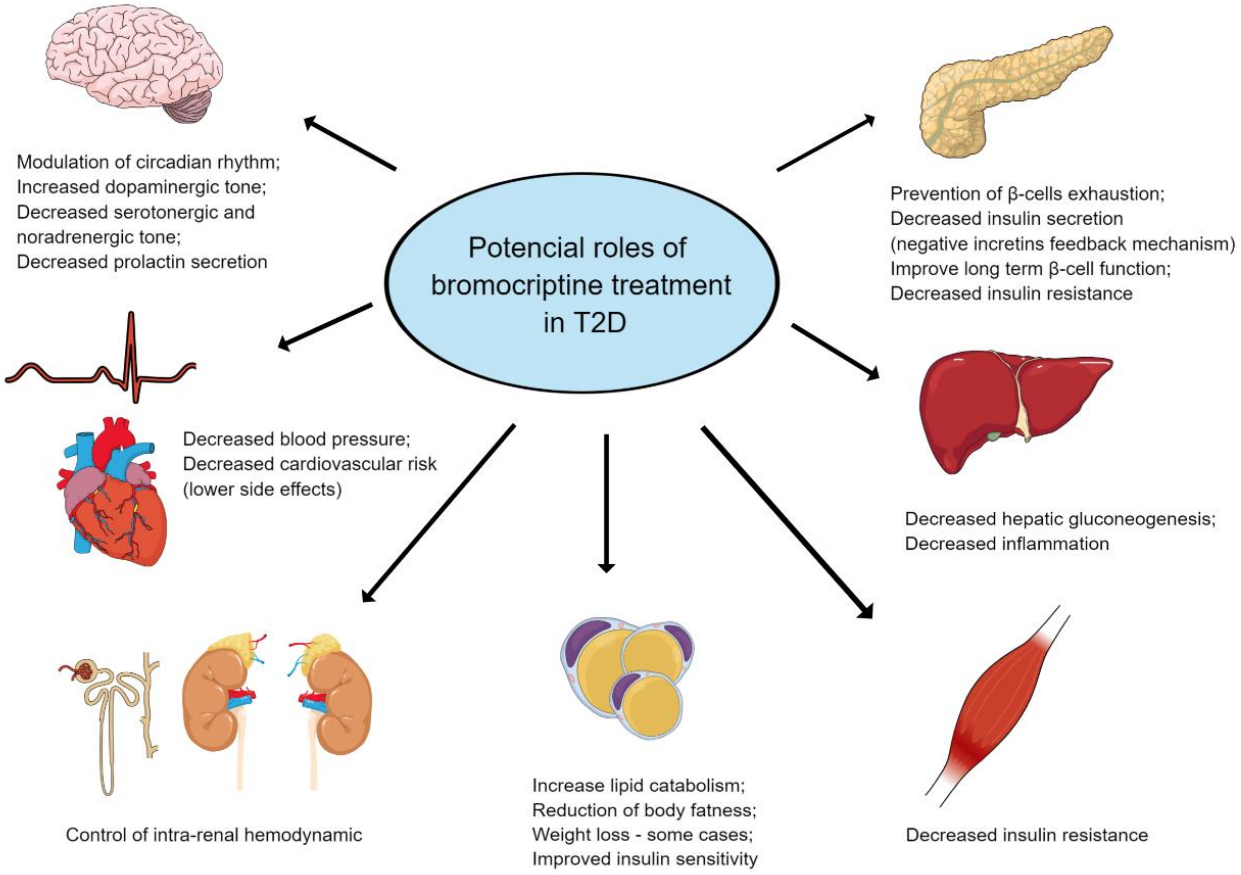


Figure 6 – Schematic figure of central and peripheral actions of bromocriptine.

Centrally bromocriptine has shown to modulate dopamine (DA) circadian rhythm, increasing hypothalamic DA tone, leading to peripheral metabolic improvement. On the other hand, peripherally, bromocriptine improves blood pressure and function as natriuretic hormone, increasing NaCl renal

excretion. Moreover, it improves peripheral insulin sensitivity in adipose tissue, skeletal muscle and liver. Additionally, both dopamine and bromocriptine regulate via D2R activation, insulin secretion, preventing pancreatic β-cell exhaustion. Adapted from Roe et al., 2015.

Lessons from antipsychotic drugs and their effects on peripheral metabolism:

Antipsychotic drugs (APDs) are commonly prescribed for the treatment of high range of psychiatric disorders including: schizophrenia, major depression, bipolar disorder, aggressive behaviours, dementia, anxiety, and post-traumatic stress (Freyberg & McCarthy, 2017; Lopez Vicchi et al., 2016). Consequently, they are some of the most widely psychotropic medications used today. Despite their many uses, APDs present significant metabolic side effects, causing weight gain, increased risk of T2D development, cardiovascular disease and metabolic disturbances characterized by abdominal obesity, glucose intolerance, insulin resistance, hypertension, and dyslipidaemia (Freyberg & McCarthy, 2017; Lopez Vicchi et al., 2016; Via et al., 2010). First generation APDs such as haloperidol, commonly considered safer from a metabolic perspective, may however, produce significant metabolic disturbances, while second-generation APDs like olanzapine (D2R antagonist) are well-known to cause substantial weight gain and metabolic dysfunction (Freyberg & McCarthy, 2017; Lopez Vicchi et al., 2016; Nash, 2017).

Knowing that APDs are effective on modulating dopamine D2-like receptors, mainly D2 and D3 receptors, this suggests a critical role for DA in promoting not only the therapeutic actions of these drugs, but also their metabolic side effects (Farino et al., 2019; Freyberg & McCarthy, 2017). APDs achieve their effects as both antagonists and inverse agonists of D2R and D3R, being able to inhibit cellular signalling even in the absence of endogenous DA (Farino et al.,

2019; Nash, 2017). However, APDs interact with numerous G protein-coupled receptors including dopaminergic, serotonergic, adrenergic, muscarinic, and histaminergic receptors (Freyberg & McCarthy, 2017; Lopez Vicchi et al., 2016). The pleiotropic nature of APD-receptor interactions may also contribute to their numerous side effects (Farino et al., 2019; Nash, 2017).

As mentioned before, pancreatic DA inhibits GSIS in β-cell via D2R/D3R activation acting by an autocrine/paracrine mechanism. Accordingly, APD-induced blockade of D2R/D3R signalling significantly enhances GSIS, resembling aspects of the chronic hyperinsulinemia found in T2D (Freyberg & McCarthy, 2017). Indeed, rodent models of APD-administration recapitulate the above-mentioned findings, as weight gain and peripheral insulin resistance, pointing to a direct action of APDs on peripheral DA targets, including pancreatic β-cells and adipose tissue (Borcherding et al., 2011; Freyberg & McCarthy, 2017; Nash, 2017).

The blockade of peripheral D2-like receptors with antipsychotic medications may significantly contribute to metabolic alterations clinically observed (Farino et al., 2019; Freyberg & McCarthy, 2017). Therefore, understanding the molecular pathways involved in APDs-induced metabolic disturbances may help to identify new therapeutic opportunities to metabolic syndrome and T2D.

CHAPTER 2 - HYPOTESIS AND AIMS

Besides CB role as chemosensory organ of arterial blood, it has also been described as a metabolic sensor, being responsive to hormones like insulin and leptin (Conde et al., 2014; Shirahata et al., 2015). Additionally, it has been demonstrated that overstimulation of CB is involved in the aetiology of insulin resistance and hypertension in animal models of metabolic syndrome, which may be related to insulin and leptin resistance (Ribeiro et al., 2013; Sacramento et al., 2017). Interestingly it appears that insulin stimulation in the peripheral chemoreceptors in CBs (in hyperinsulinemia state) triggers CB sympathoadrenal overstimulation, contributing to a vicious cycle leading to insulin resistance (Leonard et al., 2018). Moreover, clinical data from T2D patients have shown that upon functional suppression of CB activity via hyperbaric oxygen therapy, plasma glucose levels were significantly reduced (Sacramento et al., 2017; Vera-Cruz et al., 2015; Wilkinson et al., 2012). Additionally, oxygen therapy in both hypertensive and type 2 diabetic patients have shown to decrease systolic blood pressure and improve insulin sensitivity (Sacramento et al., 2017).

CB denervation was shown to prevent the development of insulin resistance induced by hypercaloric diets (Ribeiro et al., 2013). Furthermore, functional abolition of CB activity restored insulin sensitivity in mesenteric adipose tissue. Moreover, tissue glucose uptake increased both in mesenteric adipose tissue and liver as well as GLUT2 levels, which improved systemic glucose homeostasis in rats with high-caloric diet-induced metabolic syndrome (Sacramento et al., 2017). Such results suggested that CB may play a direct role in the metabolic modulation of peripheral insulin sensitive tissues, such as liver, skeletal muscle, and adipose tissue.

CB is also one of the main dopamine-producing organs (as other CA's), which are released mostly by type I cells in the glomerulus, and partially reach the circulation, contributing to the DA circulating levels (Leonard et al., 2018; Wakai et al., 2015). Dopamine receptors has been

reported to be expressed in the CB, namely D2R, which are expressed at petrosal ganglions in rats (Wakai et al., 2015). D2R stimulation on CB chemosensory-petrosal neurons synapse mediates inhibitory mechanisms that are important to regulate the CB sensory discharge (Benot & Lopez-Barneo, 1990; Leonard et al., 2018). However, it is possible that during CB chronic overstimulation the D2R-mediated inhibitory action of DA is not enough to revert this phenotype. Moreover, hypothalamic DA levels are decreased during insulin resistant states, and the FDA-approved drug for the treatment of T2D, the D2R agonist, bromocriptine appears to regulate hypothalamic DA tonus to basal levels, promoting peripheral metabolic improvements (glucose tolerance and insulin sensitivity) (DeFronzo, 2011; Lopez Vicchi et al., 2016; Via et al., 2010).

Thus, addressing the fact that dopaminergic modulation could emerge as a strategy to treat pathologies associated with CB dysfunction, namely insulin resistance, T2D and obesity, we established as main objective of this work to.

Specifically, we aim to:

1. Investigate the role of peripheral DA and its receptors on glucose uptake and phosphorylation of insulin receptor and AMPK in insulin sensitive tissues, namely liver, skeletal muscle, white and brown adipose tissues. evaluate the role of peripheral DA and the contribution of its receptors to the modulation of insulin sensitivity, glucose uptake and metabolic activity in insulin-sensitive tissues, as well as the mechanisms behind these effects
2. Study the expression of dopamine receptors in patients with metabolic disorders and its correlation with markers of adipose tissue function.

3. Assess adipose tissue metabolic function and insulin sensitivity after dopaminergic signalling modulation through bromocriptine in an animal model of type 2 diabetes and obesity.

In order to achieve our first aim, the role of peripheral dopamine in regulating tissue glucose uptake, DA was administered *in vivo* to normal rats before an oral glucose load. Also, in tissues isolated from normal rats, a similar experiment was performed *ex vivo* in the presence of DA or bromocriptine. In both experiments, a radiolabeled tracer (tritium) conjugated to glucose was used and its uptake quantified using a scintillation counter.

Moreover, in order to disclose modulation of peripheral metabolism by DA and the contribution of each dopamine receptor, our next aim was to treat tissue explants (liver, soleus muscle, WAT and BAT) with DA or bromocriptine along with antagonists of dopamine receptors (domperidone- selective D2R antagonist and haloperidol non-selective D2R and D1R antagonist). Such explants were than used to evaluate insulin signalling and AMPK activation, as well as lipid metabolism pathways in white adipose tissue.

Secondly, by performing a gene expression analysis, we aimed to understand if peripheral dopaminergic signaling, namely dopamine receptors (D1R and D5R – type 1; D2R, D3R and D4R – type 2 receptors family) could be impaired in VAT samples from patients with metabolic disorders (obese-insulin sensitive and obese insulin-resistant patients). This study was performed in collaboration with Hospital Geral de Coimbra (Covões) - Centro Hospitalar Universitário de Coimbra, where patients with metabolic disorders were selected for metabolic surgery and VAT samples were collected previous the procedure.

Finally, we aimed to understand if the modulation of peripheral dopaminergic signalling with bromocriptine treatment could improve adipose tissue function by modulation adipose tissue metabolic pathways (lipolysis, lipogeneses and lipid oxidation) as well as glucose uptake and insulin signalling, using an animal model of spontaneous T2D (Goto-Kakizaki (GK) rats) fed a high-caloric diet to induce obesity. Moreover, we also aim to be able to disclose bromocriptine mechanisms of action in other insulin-sensitive peripheral organs and overall glucose and lipid metabolism.

CHAPTER 3- MATERIALS AND METHODS

3. Materials and methods

3.1 Animal study (healthy lean Wistar rats)

Experiments were performed in male Wistar rats (200–300 g) with 8-10 weeks old obtained from the vivarium of the Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisboa, Portugal. Animals were kept under controlled temperature and humidity (21 ±1°C; 55 ± 10% humidity) with a 12h light/ dark cycle and ad libitum access to food and water. All animal experimental and care procedures were approved by the Ethics Committee of Faculdade de Ciências Médicas|Nova Medical School and by Direcção-Geral de Alimentação e Veterinária (DGAV), the Portuguese National Authority for Animal Health Animal Care. Principles of laboratory care were followed in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Our work complies with the animal ethics guidelines as outlined in the editorial by Grundy (2015).

3.2 *In vivo* tissue-specific glucose uptake.

Animals were fasted overnight and randomly divided into control (0.9% NaCl) versus dopamine (Medopa, Medinfar) injected group (100nmol). Dopamine or saline were administered in the tail vein in a bolus of 0.1 ml. Immediately after dopamine or saline administration, an oral glucose tolerance test (OGTT) with a bolus of 2-deoxy-D-[1,2-^{3}H]-glucose (2-DG, 1mCi/ml; specific activity: 8 Ci/mmol; PerkinElmer, Madrid, Spain) mixed with glucose (100µCi/kg body weight; 2g/kg body weight) was performed by gavage administration, to better mimic food ingestion and all the molecular, anatomic, histologic, and immunologic characteristics of food ingestion. Glycemia was measured at baseline and 1 hour after glucose bolus administration with a glucometer (Precision Xtra Meter, Abbott Diabetes Care, Portugal) and test strips (Abbott

Diabetes Care, Portugal). In order to determine glucose-specific activity, 20µl plasma (collected at baseline and 1 hour after glucose administration) was deproteinized with 200µl ice-cold perchloric acid (0.4N), centrifuged and its radioactivity measured in a scintillation counter (Tri-Carb 2800TR, Perkin-Elmer, Madrid, Spain). Animals were euthanized one-hour post glucose bolus administration with sodium pentobarbital (60mg/kg, i.p) and tissues, such as liver, soleus muscle, and the white (WAT: mesenteric, mWAT; epididymal, eWAT) and brown adipose tissues (BAT) were collected (50-200mg) and homogenized in 1ml ice cold perchloric acid (0.4N). Tissue samples were centrifuged and 2-deoxy-D-[^{3}H] glucose incorporation was measured in the supernatants by scintillation counting. Tissue glucose uptake (defined as the glucose metabolic index, Rg´) was calculated using the equation described by Sacramento *et al.* (2017): ($Rg' = (CpCm*)/(\int Cp* \, t \, dt)$), where Cp is the plasma glucose concentration at steady state over the 60-minute period (mmol.L^{-1}); Cm* is tissue accumulation of 2-deoxy-D-[1,2-^{3}H]-glucose per mass units at 60 minutes (dpm.mg tissue^{-1}); Cp* is the 2-deoxy-D-[1,2-^{3}H]-glucose concentration (dpm.ml^{-1}); and t equals 0 when the tracer is administered as a bolus. Therefore, Rg' is expressed in mmol.mg tissue^{-1} (Sacramento et al., 2017).

3.3 *Ex vivo* tissue-specific uptake:

After an overnight fast, the animals were euthanized with sodium pentobarbital (60mg/kg, i.p.) and the liver, soleus, WAT (mesenteric, mWAT and epididymal, eWAT) (50-150mg) were rapidly collected to ice-cold 20%O_2 5%CO_2-equilibrated Tyrode solution (in mM: 140 NaCl, 5 KCl, 2 CaCl2, 1.1 MgCl2, 10 HEPES, 5.5 glucose, pH 7.40). Three to four pieces of tissue per Eppendorf tube (2ml) were then incubated at 37ºC during 15 minutes for stabilization and afterwards moved to other tubes for incubation during 15 min each piece in different experimental conditions. Different tissues samples were incubated in the presence and absence of

insulin (10mU.ml^{-1}) together with: dopamine (10µM); dopamine plus domperidone (50nM); dopamine plus haloperidol (500nM), bromocriptine (10µM); bromocriptine plus domperidone and bromocriptine plus haloperidol. Samples were then incubated with 2-deoxy-D-[1,2-^{3}H]-glucose for 30 minutes and homogenized. Uptake of 2-deoxy-D-[1,2-^{3}H]-glucose was performed as described in the previous section. Results were expressed in cpm.mg tissue^{-1}.

Drug concentrations were chosen aiming to achieve the complete activation of receptors, and therefore 100x higher than the respective EC50 for agonists and IC50 described to antagonists. Bromocriptine was used in a concentration that mainly affects D2R, and haloperidol in a dose that blocks both D1R and D2R (Beaulieu et al., 2019).

3.4 Drugs

All drugs were prepared on the day of each experiment. Dopamine (Medopa) was obtained from Medinfar (Portugal), bromocriptine was kindly provided by Generis (Portugal), domperidone and haloperidol were obtained from Sigma-Aldrich (Portugal). The working solutions were prepared in Tyrode solution at the following concentrations: dopamine at 10µM, bromocriptine at10µM, domperidone at 50nM and haloperidol at 500nM. All dopamine receptors agonists and antagonists' doses were chosen based on the specificity characteristics for D1R and D2R provided on the IUPHAR database (Beaulieu et al., 2019). Sodium pentobarbitone (Eutasil) was obtained from Sanofi-Veterinária (Miraflores, Portugal).

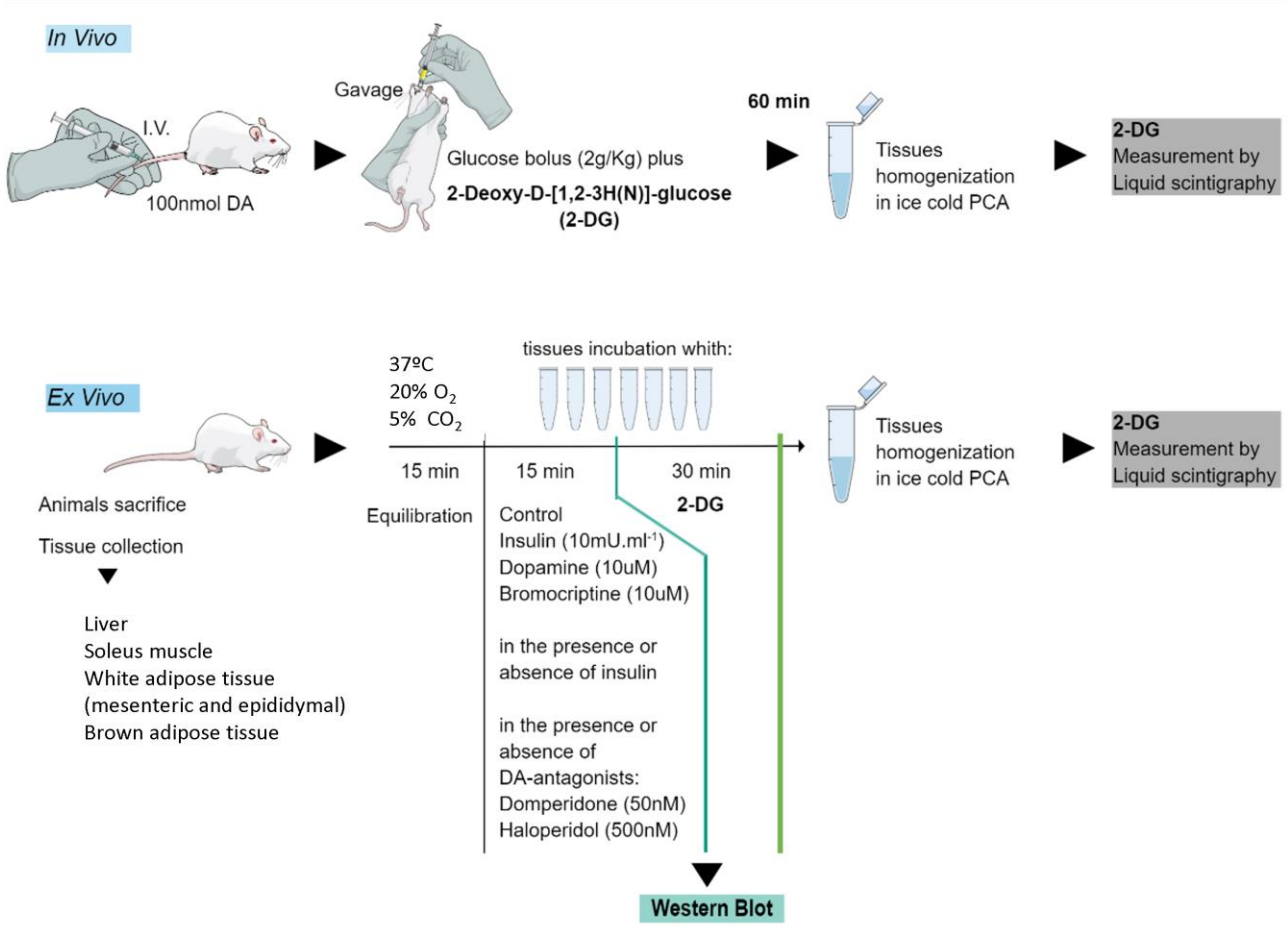


Figure 7 - Schematic representation of the experimental design of *in vivo* and *ex vivo* tissue glucose uptake procedure.

In *in vivo* experiments dopamine (100 nmol) was intra-venous (i.v.) administered follow a bolus of oral gavage glucose (2g/Kg) with radiolabeled glucose 2-Deoxy-D-[1,2-3H(N)]-glucose (2-DG). Tissue glucose uptake, in tissues homogenates, were assessed 1 hour after by liquid scintigraphy. In e*x vivo* experiments collected tissues (liver, soleus muscle, mesenteric and epididymal white adipose tissue and brown adipose tissue) were incubated during 15 minutes with control media, insulin ($10mU.ml^{-1}$, positive control), dopamine (10µM) or bromocriptine (10µM) separately or in combination with insulin and or dopamine receptors antagonists: domperidone (50nM selective D2R antagonist) or haloperidol (500nM, non-selective D1R and D2R antagonist). For tissue glucose uptake, tissues were 30 minutes incubated with 2-DG and then homogenate in ice cold perchloric acid (PCA) to quantify by liquid scintigraphy the amount of tissue glucose uptake. For western blot analysis: after 15 minutes of tissues incubation with the defined above-mentioned conditions, tissues were frozen in liquid nitrogen and kept until its utilization.

3.5 Human study:

A cohort of obese patients in a total of 94 subjects (77 women and 15 men; aged between 25 to 65 years) was selected at the obesity surgery consultation at the Hospital Geral de Coimbra (Covões) - Centro Hospitalar Universitário de Coimbra to perform metabolic surgery. All subjects signed the informed consent and the study was approved by the institutional ethics committee (Ethics Committee of the Coimbra University Hospital Center), according to the principles outlined in the Declaration of Helsinki. Patients were divided in groups according to glycaemic profile: fasting glucose levels, HbA1c levels and Ox-HOMA2IR. Subject characterization resulted in four different groups: 1 – insulin sensitive group (IS) (n=17), composed by individuals that were both IS and normoglycemic (NG) (Ox-HOMA2IR<1); 2 – insulin resistant (IR) and NG group (n=29), with insulin resistant patients (Ox-HOMA2IR>1) that were normoglycemic (fasting glucose<100 mg/dL and HbA1c<5.7%); 3 – prediabetic group (n=28), that allocated IR patients with fasting glucose levels from 100 to 125mg/dL or HbA1c between 5.7 and 6.4%; 4 – T2D group (n=18), constituted by IR subjects diagnosed with T2D (fasting blood glucose>125mg/dL or HbA1c>6.4%) (Rodrigues, 2020).

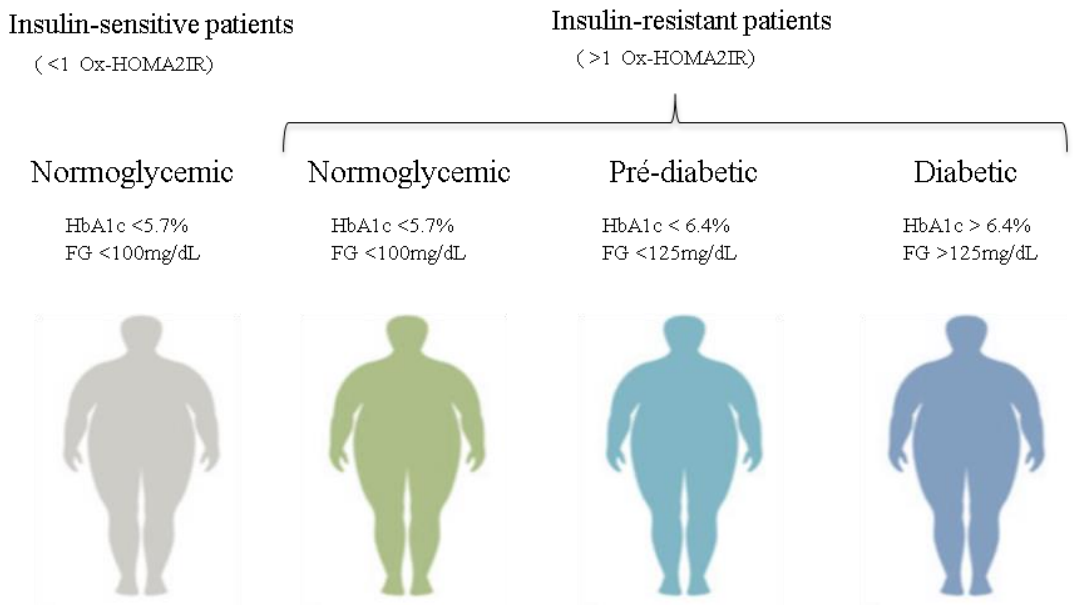


Figure 8 - Schematic representation of patients characterization and division according to its glycemic profile: fasting glucose levels, HbA1c and insulin resistance index Ox-HOMA2IR.
These resulted in: 1- Insulin sensitive, normoglycemic (IS); 2- Insulin resistant, normoglycemic (IR NG); 3- Insulin resistant pre-diabetic (IR PD) and insulin resistant diabetic patients (IR T2D). All patients presented a BMI > 30 kg/m^2.

3.6 RNA extraction:

Total RNA was extracted from VAT human biopsies (100mg) using a RNeasy Lipid Tissue Mini Kit (Qiagen, Germany). RNA samples were analysed by NanoDrop One/One spectrophotometer (ThermoFisher, Waltham, MA, USA) at 260nm to evaluate its concentration. RNA integrity was also analysed through capillary electrophoresis with an Agilent RNA 6000 Nano Kit and the results were obtained with the Agilent 2100 Bioanalyser (Agilent Technologies, CA, USA).

3.7 Quantitative real-time polymerase chain reaction using the high throughput platform biomark$_{tm}$ HD system:

RNA sample was diluted into the same concentration (25 ng/µL). Reverse transcriptase enzyme [qScript cDNA super mix (Quanta BioSciences)] was added to each RNA sample to obtain cDNA. To amplify cDNA samples, they were mixed with a mix of pooled primers (500 nM final

concentration each), with a PreAmp Master Mix enzyme (Fluidigm), following 12 cycles of thermal cycling according to the enzyme manufacturer. Samples were then treated with Exonuclease I (New England Biolabs) to remove unincorporated primers and were diluted 5x in TE buffer (10 mM Tris-HCl, 1 mM EDTA). For each sample a Pre-Mix was prepared (SsoFast Eva Green Supermix, BioRad), with DNA binding dye sample reagent at 20x (Fluidigm). Thereafter, samples were pipetted into the respective inlet of a Fluidigm® 96.96 Gene expression IFC. For each gene assay, a mix with 2x Assay loading reagent (Fluidigm), forward and reverse primers (50μM stock) diluted TE buffer was individually prepared. Primers were obtained by Sigma Aldrich, (USA) and reconstituted in water to a final concentration of 100 μM. There sequences were decried bellow at table 1. Then, each gene assay was pipetted into their respective assay inlets on the chip and loaded with the Load mix (136x) script of the HX controller (HD Biomark), using the BioMark HDTM, accordingly to the cycling parameters recommended by Fluidigm® for 96.96 Gene expression Integrated Fluidic Circuit. Then data were collected with Data Collection Software and analysed using Fluidigm® Real Time PCR Analysis v2.1 software. All data were normalized for the reference gene *ATCB*.

Gene	**Forward**	**Reverse**
DRD1	ACACAATTAACTCCGTTTCC	GTAGTGTCCCTGTTTGATTG
DRD2	TCCACTAAAGGGCAACTG	GGAAACTCCCATTAGACTTC
DRD4	CATCTACACTGTCTTCAACG	ATTAACGTACAAAAGCGCC
UCP-1	ACAGCACCTAGTTTAGGAAG	CTGTACGCATTATAAGTCCC
PPARA	CCTAAAAAGCCTAAGGAAACC	GATCTCCACAGCAAATGATAG
INSR	GATCCAATCTCAGTGTCTAAC	CCTTTGAGGCAATAATCCAG

Table 1– Primers sequences of evaluated genes

3.8 Animal study (T2D diet induced obesity animal model):

Wistar rats from our breeding colonies (Faculty of Medicine, University of Coimbra) were kept under standard conditions (Matafome et al., 2012; Rodrigues et al., 2013). The experimental protocol was approved by the local Institutional Animal Care and Use Committee (ORBEA 04-2015) 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). At 1 month old, type 2 diabetic GK rats were divided into two groups, the first maintained with the same standard diet until 6 months-old (A03; 5% triglycerides and 45% carbohydrates, SAFE, France) (GK group, n=33), while the other was fed a high-caloric diet (HCD) in the same period (customized A03 high-caloric diet with 20% fat plus 20% sucrose, SAFE, France) to induce weight gain. The HCD-fed group was randomly divided into three groups: the first without further treatment apart from the diet (GKHCD, n=20), the second with bromocriptine treatment in the last month (GKHCDBr, n=17) and the third with vehicle administration during the same period (GKHCDVh, n=15). Male Wistar rats fed with a standard diet for 6 months were used as control (n=22). Bromocriptine gently supplied by Generis®, (Amadora, Portugal) was diluted 1:4 DMSO/H_2O and daily administered by intraperitoneal (i.p) injection (10mg/Kg/day) during the last month. In the vehicle group, the same volume (100ul) of the vehicle 1:4 DMSO/H_2O was administered i.p. during the same period.

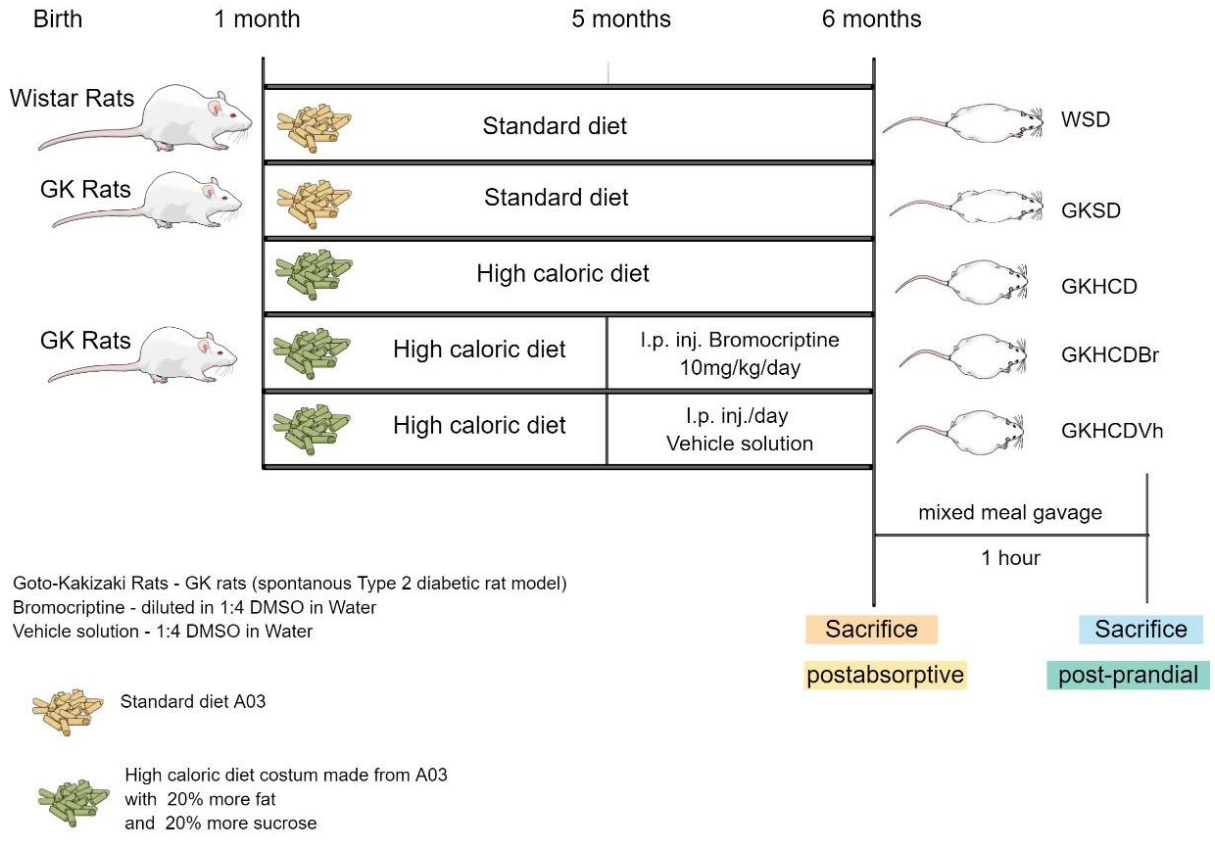


Figure 9 - Schematic representation of the experimental design and animals group division according to its diet and treatment.

1- Wistar rats fed a standard diet WSD (healthy control groups) T2D animals (GK rats) divided into: 2- GKSD, fed a standard diet; 3-GKHCD fed a high caloric diet; 4- GKHCDBr, GK fed a high caloric diet and treated with bromocriptine for 4 weeks and 5- GKHCDVh, GK fed a high caloric diet and treated with vehicle solution for 4 weeks.

3.9 Insulin tolerance teste:

Two days previous the sacrifice, an intraperitoneal insulin tolerance test (IPITT) was performed. Fasted (6h) animals were injected with insulin (0.25U/Kg, Lilly, Portugal) and glucose was measured before and after 15, 30, 60 and 120 minutes using a Glucometer (Bayer, Germany). The area under the curve was then calculated.

3.10 Blood sampling and tissue collection:

On the day of the sacrifice, fasting (6 h) glycemia, triglyceridemia and cholesterol levels were measured in the tail vein using a Glucometer (Bayer, Germany) and the Accutrend system (Roche, Germany) with the respective reactive test stipes. Before sacrifice, animals were divided into 2 subgroups, those sacrificed after 6h fasting – "postabsorptive" (WSD = 15, GKSD= 21, GKHCD= 13, GKHCDBr= 11, GKHCDVh= 9) and those sacrificed 1h after a mixed meal ingestion – "post-prandial" (WSD= 7, GKSD= 12, GKHCD= 7, GKHCDBr= 6, GKHCDVh= 6). Then, animals were anesthetized with ketamine chloride (75 mg/kg, Nimatek, Dechra, UK) and chlorpromazine chloride (2.65 mg/kg, Lab. Vitória, Portugal).

In the set of animal sacrificed after 6h fasting, plasma and serum samples were collected by cardiac puncture as described before (Matafome et al., 2012; Rodrigues, 2020) to further evaluation of insulin, glucagon, leptin, adiponectin and free fatty acid levels. Animals were sacrificed by cervical displacement, and peri-epididymal adipose tissue (pEWAT), brown adipose tissue (BAT), muscle and liver were collected, weighted and stored in 4% of formalin solution or at -80ºC. In the set of animals was administered through gavage a mixed meal, 3ml of Nutricia, Fortimel (Nestle, Switzerland) and 1h later were sacrificed for tissue collection.

3.11 Reagents and ELISA kits:

Salts and organic solvents used in solution preparations were purchased to Fisher scientific (Leicestershire, UK), Sigma Chemicals (United States of America - USA) or Merck Darmstad (Germany), with the highest grade of purity commercially available. Plasma insulin levels were assessed through the Rat Insulin ELISA Kit, (Mercodia, Sweden), plasma glucagon using the ELISA Kit (Wako, Germany) and plasma levels of leptin and adiponectin were measure using the Mouse/Rat Leptin Quantikine ELISA Kit and Rat Total Adiponectin/Acrp30 Immunoassay

(R&D system, USA). Free fatty acids were assessed using the FFA Assay Kit (ZenBio, NC, USA). Furthermore, 100 mg of liver samples were homogenised in the lysis buffer (1mL of 5% NP-40/ddH2O solution) and hepatic triglycerides content was quantified by colorimetric Triglyceride Quantification Assay Kit (Abcam 65336, UK). Moreover, 30mg of pEWAT were homogenised in PBS 0.02M, pH7.2 (BAM R61, FDA) to quantify β-Hydroxybutyrate by a colorimetric assay (Biovison K632-100, USA).

3.12 Western blot

For Western Blot analyses, tissue both explants were collected and immediately stored at -80ºC after the 15 minutes incubation under the different experimental conditions, while tissues from GK-T2D diet-induced obesity animal model and respective healthy Wistar and GK controls were immediately stored at -80ºC after animal's sacrifice. Tissues (100mg/ml, n=5-9) were homogenized as previously described by Matafome *et al.* (2012). Denatured samples were loaded in 8% polyacrylamide gels, separated by SDS-page and transferred to a PVDF membrane (Advansta, USA).

Membranes were incubated with the specific primary antibodies overnight at 4ºC and then incubated 2h at room temperature with secondary antibodies anti-rabbit and anti-goat were obtained from Bio-Rad (Spain) and Invitrogen ThermoFisher Scientific (USA), respectively. Calnexin (AB0041-200) from SICGEN (Cantanhede, Portugal) was used as loading control. Membranes were revealed using ECL substrate (Advansta, USA) in a VersaDoc system (Bio-Rad, USA) and analyzed with ImageQuant® (Molecular Dynamics, USA) (Matafome et al., 2012).

Primary antibodies to the phosphorylated forms of: insulin receptor (InsR, Tyr972, ab5678, and InsR, Tyr 1361, ab60946) obtained from Abcam, UK, while protein kinase B (PKB), also known

as Akt (Akt, Ser473, #4058S), adenosine monophosphate kinase (AMPK, Thr172, #2535S), hormone sensitive lipase (HSL, Ser563, #4139S), acetyl CoA carboxylase (ACC, Ser79, #11818P) and ATP citrate lyase (ACL, Ser455 #4331P) were obtained Cell Signalling, USA.

Primary antibodies used for total form of: AMPK GLUT 4 and PPARγ (#2532, (#2213S and #2443S, Cell Signaling, USA), GLUT2 (ab54460, UK). InsRβ (sc-57342, Santa Cruz Biotechnology, USA). D1R, D2R, DARPP32, DARPP32-Thr34 and tyrosine hydroxylase (TH) were from (ab81296, ab85367, ab40801 and ab51114 respectively, Abcam, UK; T1299, Sigma Aldrich, USA).

3.13 Statistical analysis:

In glucose uptake experiments, a piece of each evaluated tissue (from a minimum of 5 animals per condition) was used as individual points for the conditions incubated with dopamine or its agonist and antagonists. For statistical analyses all data was evaluated using GraphPad Prism Software, version 8 (GraphPad Software Inc., USA) and presented as scatter plots or mean values with standard deviation (SD). The significance of the differences between the mean values was calculated by one-way ANOVA with Dunnets's multiple comparison tests. Differences were considered significant at $p<0.05$.

In the clinical human data non-parametric tests were performed (sample size < 30/group) and results were presented as median and interquartile range. Kruskal-Wallis and Mann Whitney testes were applied to compare gene expression between groups according to its division. Spearman correlation test was performed to assess correlation between variables. In the animal study, results were presented as mean ± SD. One-way ANOVA test with Tukey multiple comparisons was used to performed systemic parameters analysis and total proteins quantification. For phosphorylated proteins Kruskal-Wallis test (all pairwise multiple

comparisons) was applied to determine statistical differences between the groups. Differences were considered significant at $p<0.05$. All computation analysis was performed using GraphPad Prism (6.0 version, USA).

CHAPTER 4 - RESULTS

4.1 Peripheral dopamine directly acts on insulin-sensitive tissues to regulate insulin signalling and metabolic function

4.1.1 Peripheral dopamine mediates in vivo tissue glucose uptake

Dopamine (100 nmol) was i.v. administered immediately before oral glucose administration. Figure 10 presents the effect of dopamine on glucose uptake in insulin-sensitive tissues. The soleus muscle and the BAT exhibited a higher glucose uptake in relation to the rest of the tissues showing that these are tissues with high rate of glucose uptake (baseline liver = 2.03; mWAT = 2.18; eWAT = 1.30; Soleus = 3.73; BAT = 4.73 mmol/mg tissue). Dopamine significantly increases glucose uptake in all tissues tested, except mWAT, producing an increase of 49% in the liver, 88% in the eWAT, 70% in soleus muscle and 88% in the BAT.

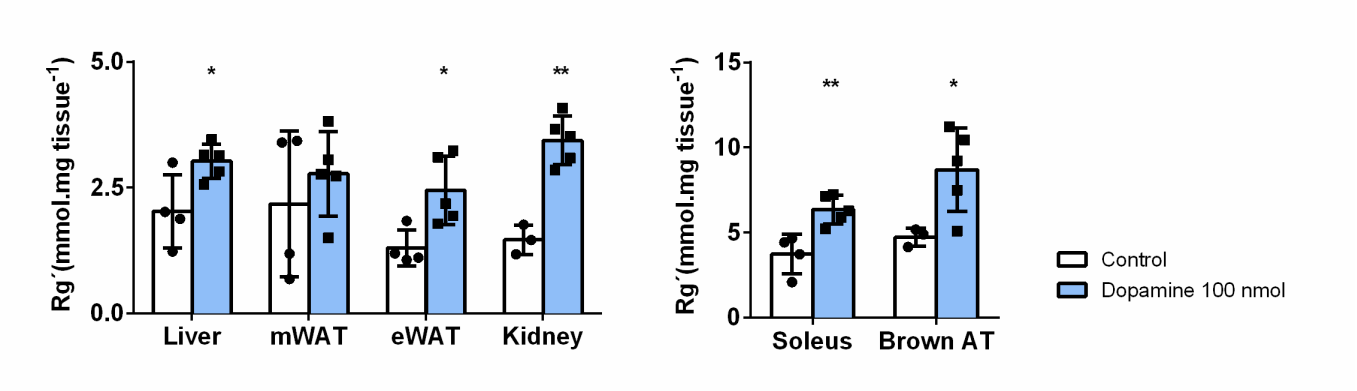


Figure 10 - Effect of in vivo dopamine administration on glucose uptake in insulin-sensitive tissue.

Exogenous dopamine administration at 100 nmol enhances glucose uptake significantly in all tissues except for mWAT. Values and bars represent means ± SD. One-way ANOVA test with Dunnet's post-hoc comparisons test was performed, where * - different from control. Levels of significance: *p<0.05; **p<0.01; ***p<0.001. mWAT- mesenteric white adipose tissue; eWAT – epididymal white adipose tissue; BAT- brown adipose tissue.

4.1.2 Dopamine directly acts in insulin-sensitive tissues through different receptors to regulate glucose uptake.

Dopamine directly induces glucose uptake in the liver via D2R: Insulin significantly increased glucose uptake by 37% in the liver (control = 31cpm.min^{-1}) (fig. 11A). Dopamine alone or in the presence of domperidone did not change glucose uptake in the liver (fig. 11A). However, dopamine+insulin-induced increase of glucose uptake was abolished by domperidone (26% decrease, fig. 11A), suggesting that D2R has a role on insulin effect on glucose uptake in the liver. This was further suggested by the significant increase in glucose uptake by 48% in response to bromocriptine, an effect that was independent and additive to insulin-induced glucose uptake (fig. 11A). Domperidone inhibited this effect of bromocriptine, confirming the D2R-mediated effect.

Dopamine directly induces glucose uptake in the skeletal muscle via D1R: The skeletal muscle is a major organ involved in glucose homeostasis and therefore herein we evaluated the role of dopamine signalling in skeletal muscle glucose uptake. Similar to insulin, dopamine applied alone significantly increased glucose uptake by 47%, an effect not mimicked by bromocriptine stimulation, suggesting a D1R-mediated mechanism (fig. 11B). This was confirmed using haloperidol, in a dose that blocks both D1 and D2 receptors, as it inhibits the effect of dopamine on glucose uptake.

Dopamine potentiates-insulin-induced glucose uptake in the mWAT via D2R: Finally, to understand the role of dopaminergic signalling in WAT depots with different metabolic functions, the mWAT and eWAT were also studied *ex vivo*. Dopamine or bromocriptine did not alter glucose uptake in both tissues (fig. 2C and D). Nevertheless, in mWAT but not in eWAT, both dopamine and bromocriptine potentiated insulin-mediated glucose uptake (162% and 185%

respectively) in comparison with control (35cpm/mg tissue, fig. 11C). This last effect was blocked in the presence of domperidone, confirming a D2R dependent mechanism.

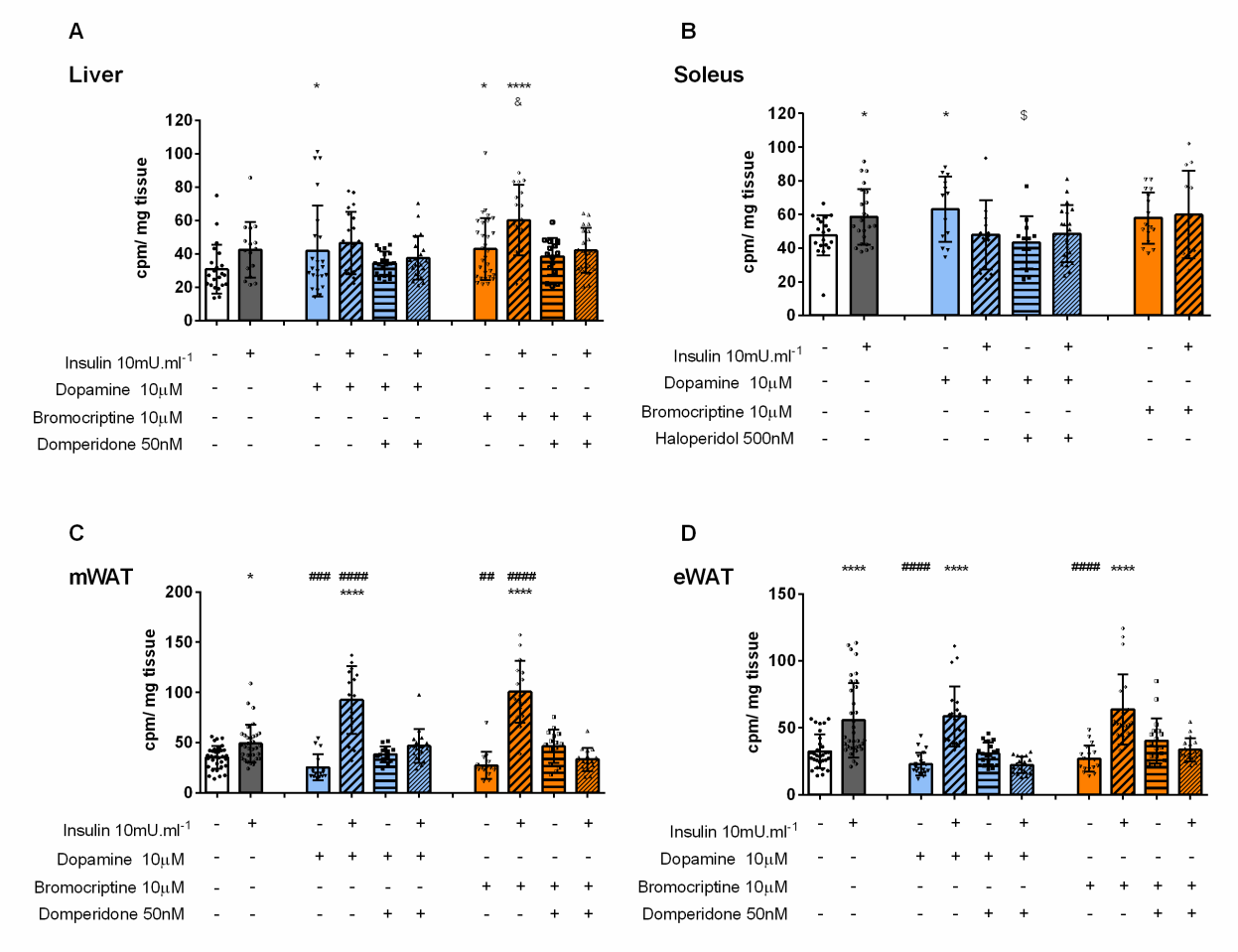


Figure 11 - Role of dopamine and the D2R agonist, bromocriptine, on ex vivo glucose uptake in the liver (A), mesenteric (B) and epididymal (C) adipose tissue, brown adipose tissue (D) and soleus muscle (E).

Effect of dopamine (10 µM, blue bars) and bromocriptine, a D2R agonist, (10 µM, orange bars) on 2-deoxy-D-[1,2-3H]-glucose uptake in the presence and absence of insulin (10mU.ml^{-1}) and its blockade by domperidone, a D2R antagonist (50 nM) and/or and haloperidol (500 nM) a D1R and D2R antagonist in the liver, soleus muscle, mWAT and eWAT. Bars represent means ± SD. One-way ANOVA with Dunnet's post-hoc comparison test * vs control; # vs insulin; $ vs dopamine; § vs dopamine plus insulin, & vs bromocriptine; £ vs bromocriptine plus insulin. Levels of significance: * $p<0.05$; **$p<0.01$;

***p<0.001; **** p<0.0001. mWAT- mesenteric white adipose tissue; eWAT – epididymal white adipose tissue; D1R – dopamine receptor 1; D2R– dopamine receptor type 2.

4.1.3. Dopamine differently regulates insulin signalling in insulin-sensitive tissues.

Bromocriptine-induced glucose uptake in the liver is independent of the InsR: To disclose the role of dopamine in insulin signalling, the levels of InsR phosphorylated at Tyr972 (InsR-Tyr972) were determined by western blot. As depicted in figure 12A, incubation with dopamine and bromocriptine did not change liver InsR-Tyr972 levels. However, since domperidone in the presence of bromocriptine and insulin increased the phosphorylation of InsR by 58% in comparison with control and 51% in comparison with bromocriptine (fig. 12A), we can speculate that D2R is involved in other InsR-induced mechanisms different from glucose uptake regulation.

Dopamine induces InsR phosphorylation in the skeletal muscle via D1R: In skeletal muscle, insulin and dopamine applied separately were able to induce InsR phosphorylation, by 8% and 18%, but when applied together the effect was not modified, suggesting that they act on the same signalling pathway (fig. 3B). Haloperidol decreased dopamine and dopamine plus insulin effects on InsR-Tyr972 levels by 22% and 38%, respectively (fig. 3B), an effect mimicked by domperidone, a selective D2R agonist (fig. 12B).

Dopamine-induced potentiation of insulin action in WAT is independent of the InsR: Insulin significantly increased InsR phosphorylation by 24% in the mWAT and by 46% in eWAT (fig. 12C and D). Dopamine or bromocriptine alone or in the presence of insulin did not alter InsR-Tyr972 phosphorylation in both tissues (fig. 12C and D). Nevertheless, an increase in the InsR-Tyr972 levels in mWAT and eWAT was observed when domperidone was added to the condition dopamine+insulin (82% and 274%) and bromocriptine+insulin (49% and 81%) (fig.

12C and D), suggesting that, as observed in the liver, D2R plays a role on other InsR-induced mechanisms than glucose uptake.

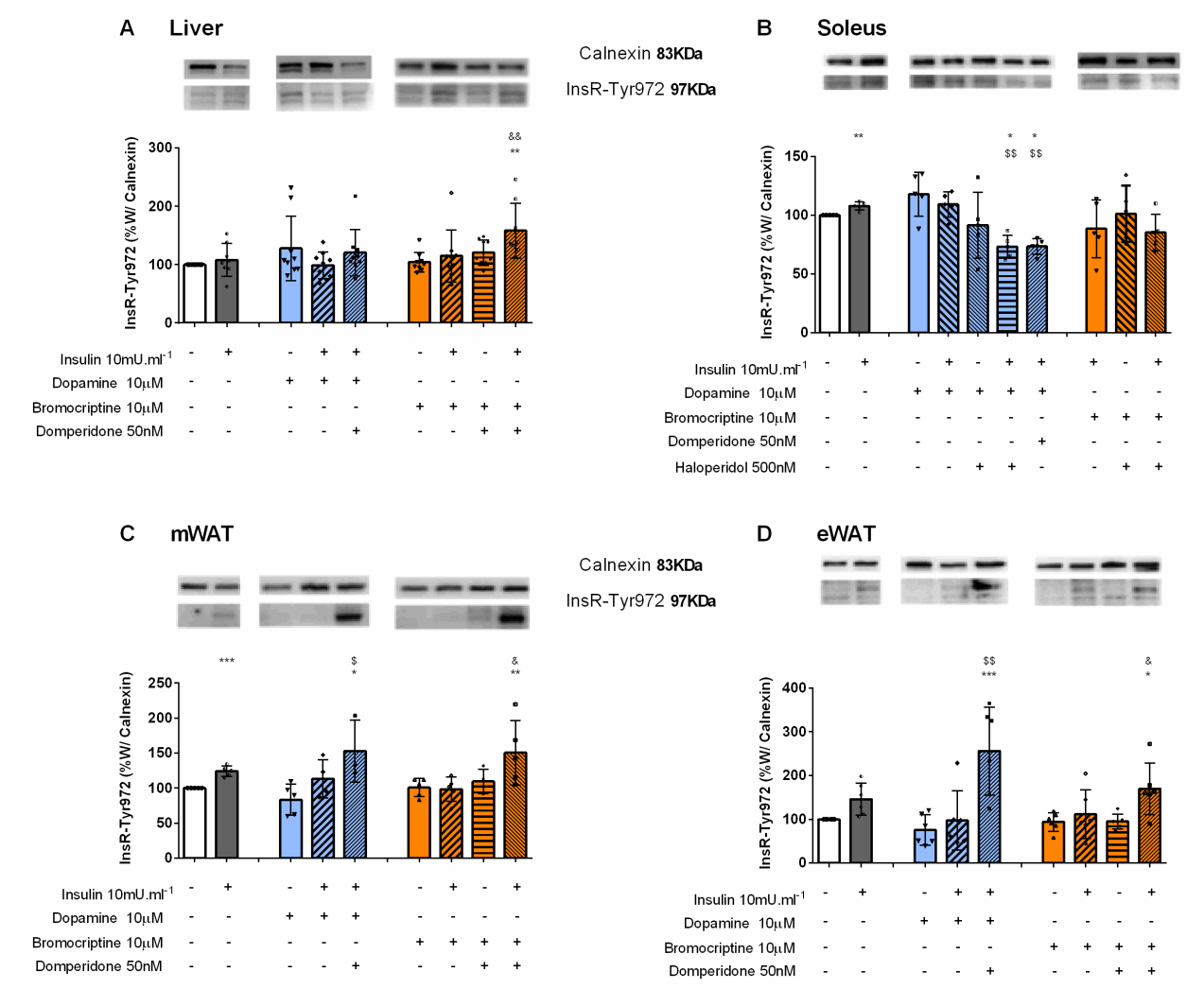


Figure 12 – effect of dopamine and D2R agonist, bromocriptine, on insulin receptor phosphorylation in the liver (A), soleus muscle (B), mesenteric (C) and epididymal adipose tissue (D).

Effect of dopamine (10 µM, blue bars) and bromocriptine, a D2R agonist, (10 µM, orange bars) on insulin receptor phosphorylation (InsR) in the presence and absence of insulin (10mU.ml^{-1}) and its blockade by domperidone, a D2R antagonist (50 nM) and/or and haloperidol (500 nM) a D1R and D2R antagonist in the liver, soleus muscle mWAT and eWAT. The top of figures shows representative western blots for the

effect of dopamine and bromocriptine on InsR-Tyr972 (97 kDa band) phosphorylation, in the presence and absence of insulin and its blockade by domperidone and/or haloperidol. Calnexin was used as loading control (83 kDa band). Bars represent means ± SD. One-way ANOVA with Dunnet's post-hoc comparison test * vs control; # vs insulin; $ vs dopamine; & vs bromocriptine. Levels of significance: * p<0.05; **p<0.01; ***p<0.001; **** p<0.0001. mWAT- mesenteric white adipose tissue; eWAT – epididymal white adipose tissue; D1R – dopamine receptor type 1; D2R – dopamine receptor type 2.

4.1.4 Dopamine regulates AMPK activation in insulin-sensitive tissues.

D2R is necessary for AMPK function in the liver: We also evaluated the impact of dopaminergic signalling on AMPK activation, an energy sensor that has a key role in the control of energy homeostasis and metabolism, by regulating hepatic glucose and lipid metabolism, through Thr172 phosphorylation. In the liver, a significant reduction of 26% and 21% was observed when domperidone was added to bromocriptine and to bromocriptine+insulin, respectively, in comparison with the control. This suggests that D2R activation is necessary for AMPK function in the liver (fig. 13A).

Dopamine activates AMPK in the skeletal muscle via D2R: Insulin significantly increased AMPK phosphorylation by 29% in the soleus muscle (fig. 13B). Dopamine and bromocriptine alone increased by 47% and 73% AMPK phosphorylation levels, effects that were not modified in the presence of insulin (fig. 13B). These effects were blocked by haloperidol and domperidone – D1R+D2R and selective D2R antagonists, respectively - suggesting that the effects of dopamine and bromocriptine on AMPK phosphorylation are mediated by D2 receptors.

D2R inhibits AMPK activation in WAT: In the mWAT dopamine and bromocriptine did not alter AMPK-Thr172 levels compared to the control group (fig. 13C). On the other hand, in eWAT, while dopamine did not affect AMPK-Thr172 phosphorylation, bromocriptine decreased AMPK-Thr172 phosphorylation by 44% when compared to control (fig. 13D). Incubation with dopamine

or bromocriptine in the presence of domperidone+insulin significantly increased the levels of AMPK-Thr172 in eWAT by 48% and 90% respectively (fig. 13D) and a similar trend was observed in mWAT (fig. 13C). Such results suggest an inhibitory effect of D2R on AMPK phosphorylation on the adipose tissue.

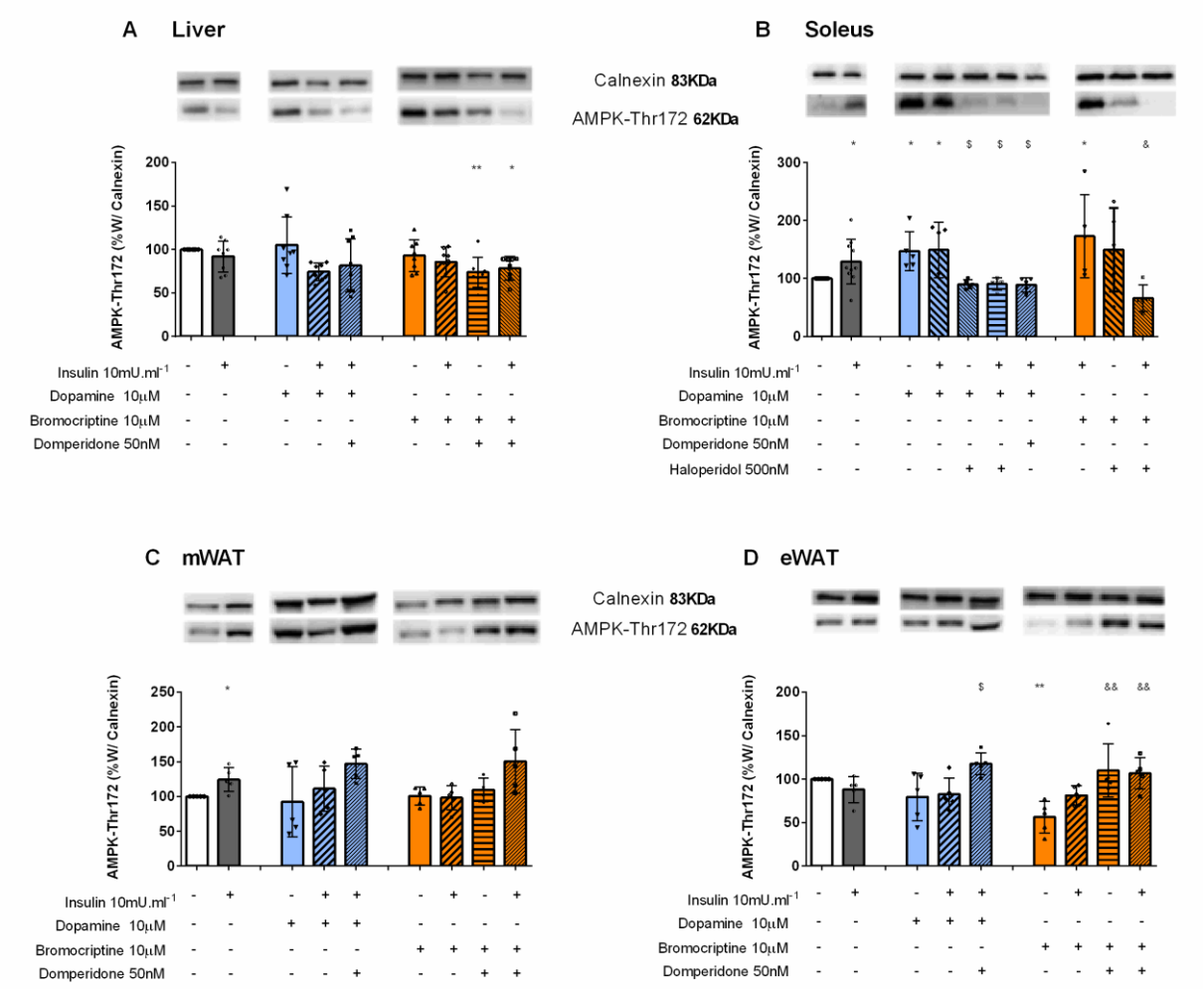


Figure 13 - Involvement of dopamine and bromocriptine on AMPK phosphorylation in the liver (A), soleus muscle (B) mWAT (C) and eWAT (D).

Effect of dopamine (10 µM, blue bars) and bromocriptine (10 µM, orange bars) on AMPK phosphorylation in the presence and absence of insulin (10mU.ml^{-1}) and its blockade by domperidone, a D2R antagonist (50 nM) and/or and haloperidol (500 nM) a D1R and D2R antagonist in the liver, soleus

muscle, mesenteric and epididymal adipose tissue. The top of figures shows representative western blots for the effect of dopamine and bromocriptine on AMPK-Thr172 (62 kDa band) phosphorylation, in the presence and absence of insulin and its blockade by domperidone and/or haloperidol. Calnexin was used as loading control (83 kDa band). Bars represent means ± SD. One-way ANOVA with Dunnet's post-hoc comparison test * vs control; # vs insulin; $ vs dopamine; & vs bromocriptine. Levels of significance: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. mWAT- mesenteric white adipose tissue; eWAT – epididymal white adipose tissue; D1R – dopamine receptor type 1; D2R- dopamine receptor type 2.

4.1.5 Inhibition of D2 receptor increases dopamine-mediated stimulation of adipose tissue lipid catabolic pathways.

As expected, Akt-Ser473 phosphorylation increased with insulin incubation in both mWAT and eWAT (73% and 152%, respectively) compared to control condition (fig. 14A-B, respectively). Dopamine or the D2R agonist, bromocriptine applied alone did not increase Akt-Ser473 phosphorylation levels in both WAT depots neither changed insulin-mediated increase of Akt-Ser473 levels (fig. 14A-B). These results suggest that both dopamine and bromocriptine exert their actions independent of insulin receptor pathway activation, since Akt-Ser473 is a downstream modulator of InsR signalling (fig. 14A-B).

In order to understand the implications of dopaminergic signalling on lipid metabolism pathways, we evaluated HSL, ACC and ACL activation through the measurement of their phosphorylated forms: HSL-Ser563, ACC-Ser79 (inhibitory) and ACL-Ser455. Insulin increased by 35% and by 73% HSL-Ser563 levels in mWAT and eWAT depots, respectively (fig. 14C-D). Interestingly, in both fat depots, dopamine or bromocriptine alone did not change HSL-Ser563 levels fig. 14C-D) but in the presence of domperidone+insulin HSL-Ser563 levels significantly increased in mWAT and eWAT comparing to dopamine (114% and 148%) or to bromocriptine (73%, and 217%, respectively (fig. 14C-D).

Interestingly, both dopamine and bromocriptine directly decreased by 50% and 66%, respectively, ACC-Ser79 phosphorylation levels in eWAT, meaning that these substances decreased ACC activity (fig. 14E-F). This effect was not observed in mWAT (fig. 14E-F). ACC-Ser79 levels were significantly increased in mWAT and eWAT in the presence of domperidone in combination with all the other tested conditions (fig. 14E-F), which is consistent with increased AMPK activation in the same condition, a known inhibitor of ACC. Dopamine and bromocriptine did not present a direct effect on ACL phosphorylation, but domperidone in presence of insulin significantly increased ACL-Ser455 levels in mWAT and eWAT by 298% and 156%, respectively, when compared to dopamine, and by 304% and 390% respectively when compared to bromocriptine (fig. 14G-H).

Altogether, our data suggest that D2R is involved in glucose uptake-independent activation of InsR and in the regulation of pathways associated with lipid metabolism in WAT, by impacting on the expression of phosphorylated forms of AMPK, HSL, ACC and ACL in both WAT depots.

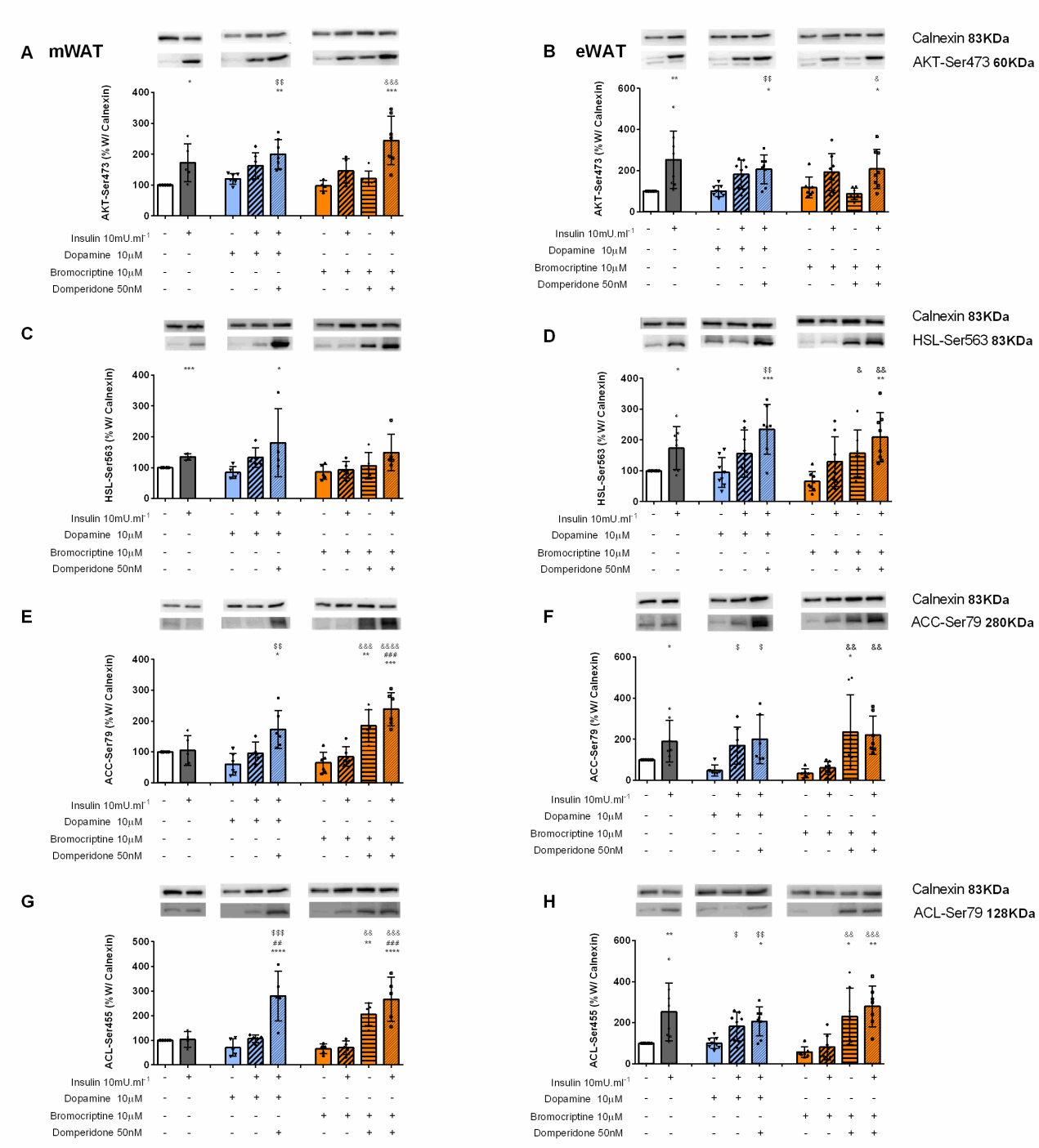


Figure 14 - Involvement of dopamine and bromocriptine on Akt (A, B), HSL (C, D), ACC (E, F) and ACL (G, H) phosphorylation in mWAT and eWAT, respectively.

Figures show the effect of dopamine (10 µM, blue bars) and the effect of bromocriptine (10 µM, orange bars) on mWAT and eWAT Akt (A, B), HSL (C, D), ACC (E, F) and ACL (G, H) phosphorylation, and

its blockade by domperidone (50 nM). Insulin dose used was 10mU.ml^{-}1. The top of figures shows representative western blots for the effect of insulin, dopamine and bromocriptine in the presence or absence of domperidone, a D2R antagonist, on Akt-Ser743 (A, B), HSL-Ser563 (C, D), ACC-Ser79 (E, F) and ACL-Ser455 (G, H) phosphorylation, in where the bands on top represent calnexin (83 kDa band), the loading control, and the bands below show the protein of interest – Akt-Ser743, HSL-Ser563, ACC-Ser79 and ACL-Ser455. Bars represent means ± SD. One-way ANOVA with Dunnet's post-hoc comparison test * vs control; # vs insulin; $ vs dopamine; § vs dopamine plus insulin; & vs bromocriptine; £ vs bromocriptine plus insulin. Levels of significance: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. mWAT- mesenteric white adipose tissue; eWAT – epididymal white adipose tissue; D2R – dopamine receptor type 2.

4.1.6 Discussion

Herein we described for the first time that peripheral dopamine stimulates glucose uptake in several tissues, in part through direct actions in liver, skeletal muscle and WAT. Moreover, dopamine receptors, especially D2R, play a role in these tissues on the regulation of InsR, Akt and AMPK phosphorylation. Also, D2R is involved in the regulation of lipid metabolism pathways in WAT, which could be relevant for unravel new therapeutic approaches for obesity-related diseases. Herein we also show that dopamine effects on glucose uptake occur preferentially through D1R in the skeletal muscle and through D2R in the liver and WAT (Table 2).

Dopamine effects on glucose metabolism were for many years attributed to the regulation of the CNS (DeFronzo, 2011; Lopez Vicchi et al., 2016), supported by the alterations in peripheral metabolism produced by antipsychotics (Freyberg & McCarthy, 2017; Lopez Vicchi et al., 2016). In agreement, the effects of bromocriptine, a D2 receptor agonist, on glucose metabolism have been attributed to the central regulation of sympathetic nervous system activity (DeFronzo, 2011). However, with the identification of dopamine receptors in the pancreas and in the adipose tissue and the description of its involvement in the regulation of insulin and adipokines secretion

(Borcherding et al., 2011; Chaudhry et al., 2016; Farino et al., 2019; Ustione et al., 2013), a peripheral role of dopamine has been considered. In other to increase the support for the role of peripheral dopamine on glucose metabolism, herein we show that the direct effect of dopamine on tissues outside the CNS is involved in the regulation of glucose and lipid metabolic pathways. Moreover, both dopamine receptors participate in this modulation playing distinct roles in the different insulin-sensitive tissues.

We showed *in vivo* that peripheral dopamine administration during an OGTT produces an overall enhancement of tissue glucose uptake by the insulin-sensitive tissues: in the liver, WAT (mesenteric and epididymal), soleus muscle and BAT suggesting that dopamine is involved in the peripheral glucose homeostasis. We also found that dopamine directly promotes peripheral glucose uptake in insulin sensitive tissues *ex vivo*, via its action on different dopamine receptor subtypes. Interestingly, increased *in vivo* glucose uptake in each tissue after dopamine administration does not resemble the *ex vivo* glucose uptake in the same tissues upon direct dopamine or bromocriptine stimulation, showing that dopamine may have direct and indirect effects on each of these tissues. For example, we can postulate that when administered systemically *in vivo*, dopamine may act to decrease sympathetic activation to efferent organs changing the metabolic turnover and the release of signalling molecules that will act distant from the releasing cells, as seen previously with other mediators (Guilherme et al., 2019). An example of this kind of mechanism is the regulation of adipokines secretion by WAT that will act on BAT to change its metabolic status (Guilherme et al., 2019). Another counter-regulatory mechanism that we can postulate to be involved in the regulation of peripheral dopamine effects could be the modulation of parasympathetic activity through the vagus nerve. It is known that the parasympathetic vagus nerve mediate changes in BAT sympathetic activity and energy

expenditure in response to altered afferent signalling coming from tissues such as the liver and WAT (Madden & Morrison, 2016). Finally, we can speculate that the use of alternative fuels in the other tissues in the *in vivo* experiments can be responsible for the forwarding of glucose to BAT and consequent increase of glucose uptake (Stanley et al., 2019).

Herein we also found for the first-time differences on the dopamine receptors involved in the regulation of glucose uptake measured *ex vivo*. D2R agonist directly stimulated glucose uptake in the liver, while in the soleus, even though dopamine stimulates glucose uptake, we cannot find any alteration with bromocriptine incubation, pointing out an involvement of D1 receptors in glucose uptake in the skeletal muscle. In the adipose tissue, D2R stimulation only potentiated the effect of insulin on glucose uptake, independently of InsR activation. Our results suggest an important and tissue-specific role of dopamine in the regulation of glucose uptake. Moreover, our results raised some questions or hypotheses regarding the involvement of dopamine receptors in the regulation of glucose uptake and glucose transporters, GLUTs. Given the difference between glucose transporters in the liver (insulin-independent GLUT2) and muscle and adipose tissue (insulin-dependent GLUT4) (Navale & Paranjape, 2016), it seems expectable that dopamine-mediated glucose uptake may be dependent on the type of GLUT translocated to the plasma membrane.

Interestingly, domperidone induced InsR-Tyr972 phosphorylation in liver and InsR-Tyr972 and Akt-Ser473 phosphorylation in WAT without any evident increase of glucose uptake, suggesting that D2R impact on insulin signalling on these tissues exerts other metabolic effects in cells apart from glucose uptake. Altogether, the results of dopamine receptors modulation herein described point towards a major role of D2R in the peripheral control of glucose metabolism in the insulin-

sensitive tissues with the exception for the soleus muscle where D1R appears to be a modulator of glucose uptake.

Additionally, from our results, it is clear that D2R potentiate insulin-glucose uptake at the mWAT, suggesting an interaction between insulin and dopamine signalling pathways to control glucose uptake in this tissue, while in the soleus muscle and the liver the effect seems to be additive. This is corroborated by the fact that dopamine- and bromocriptine-mediated glucose uptake in WAT is independent of InsR and Akt activation (downstream regulator of InsR pathway signalling) since phosphorylation of both proteins is only stimulated by insulin. Dopamine is known to act on D1R and D2R to regulate cAMP levels inside the cell, regulating neuronal excitability through membrane availability of ion channels (Savica & Benarroch, 2014). Apart from the classical activation of the Gαi/o protein coupled to the D2R and thereby the inhibition of adenylate cyclase, it is known that D2R has several effectors (Thummel, 2005) that can account for the effects herein observed for dopamine in WAT.

In the present manuscript we also describe for the first time that dopamine receptors regulate AMPK phosphorylation in eWAT and in the skeletal muscle. Interestingly, these effects were opposite, since dopamine decreased AMPK phosphorylation in eWAT via D2R, while enhanced AMPK phosphorylation in the soleus muscle upon stimulation with dopamine and bromocriptine. Confirming the D2R modulation, the effects of D2R agonism on soleus muscle AMPK phosphorylation were reversed by D2R antagonism.

We also show that D1 receptors modulate glucose uptake in the skeletal muscle, suggesting different pathways mediating metabolism in the soleus muscle. Nevertheless, we cannot forget that AMPK also facilitates GLUT4 translocation to the plasma membrane (Mihaylova & Shaw, 2011; Saha et al., 2014; Wolfe, 1998) and therefore we can speculate that D2R modulation might

contribute indirectly to glucose uptake as well. The exact mechanism by which D2R increases AMPK-Thr172 levels in skeletal muscle is not clearly understood and therefore more experiments will be needed in the future to clarify this.

On the other hand, D2R inhibition by domperidone in the presence of dopamine increased HSL, ACC and ACL phosphorylation, and therefore activates HSL and ACL pathways and inactivates ACC pathway, in both mWAT and eWAT showing dopamine involvement in lipid metabolism pathways modulation in adipose tissue. HSL and ACL are involved in lipid catabolism pathways, so we can speculate that D2R inhibition promotes lipid oxidation in the adipose tissue. Regarding ACC, its main function is to catalyze lipogenesis, and therefore domperidone by inactivating this enzyme will promote a decrease in fatty acid synthesis and a shift of lipid metabolism to lipolytic pathways. This, together with HSL and ACL activation, will improve lipid metabolism in the adipose tissue and therefore will benefit metabolic diseases treatment. Moreover, we recently described that bromocriptine treatment in GK rats, a type 2 diabetes rat model, upregulates catabolic pathways, improving metabolic profile (Tavares et al., 2021). All together we can clearly say that dopamine receptors modulate lipid metabolism in the adipose tissue, although one might find these results contradictory. However, we have to take into account that in the present work we are evaluating the acute effects of dopamine and the modulation of its receptors and also that we are in the absence of metabolic disease, which is described to alter dopamine receptors expression in the adipose tissue (Tavares et al., 2021).

Overall, we found that peripheral dopamine modulates glucose uptake through direct and indirect actions in insulin-sensitive tissues. D1R and D2R are differentially involved in glucose uptake and regulation of insulin signalling pathway and AMPK phosphorylation in insulin-sensitive tissues. Particularly, dopamine via D1R regulates glucose uptake in soleus muscle while via D2R

regulates glucose uptake in the liver and WAT. Importantly, we found that dopamine also impacts lipid metabolism pathways in WAT showing its importance for obesity. Nevertheless, the direct effects of dopamine only partially explain the tissue glucose uptake observed after its *in vivo* peripheral administration. Given that dopamine is not able to cross the blood-brain-barrier, other peripheral indirect mechanisms of glucose homeostasis regulation by dopamine may be involved and should be addressed in the future.

Tissue	Parameter	Dopamine direct effect	D1 effect	D2 effect
mWAT	Glucose uptake Insulin signaling AMPK signaling Lipid storage	=	=	⬆ = = ⬆
eWAT	Glucose uptake Insulin signaling AMPK signaling Lipid storage	=	=	= = ⬇ ⬆
Liver	Glucose uptake Insulin signaling AMPK signaling	= = ⬆	=	⬆⬆ = ⬆
Skeletal muscle	Glucose uptake Insulin signaling AMPK signaling	⬆ ⬆ ⬆	⬆⬆ = =	= ⬆ ⬆

Table 2 - Summary of the findings supporting the involvement of peripheral dopamine and its receptors, on glucose uptake, insulin action, AMPK signalling and lipid storage.

Peripheral dopamine stimulates glucose uptake in the liver, skeletal muscle and white adipose tissue (WAT). Dopamine directly acts in the same tissues to regulate InsR, Akt and AMPK phosphorylation, being also involved in the regulation of metabolic functions in WAT, which can be an important fact for obesity-related diseases. Dopamine direct effects in insulin-sensitive tissues are differentially modulated by D1 and D2 dopamine receptors.

4.2 Dopamine D2 receptor agonist, bromocriptine, remodels adipose tissue dopaminergic signalling and upregulates catabolic pathways, improving metabolic profile in type 2 diabetes.

4.2.1 Expression of dopamine receptors is impaired in the visceral adipose tissue of patients with insulin resistance

Patients included in the diabetic group (fasting blood glucose>125mg/dL or HbA1c>6.4%), were previously shown to have a failure of beta-cell function, and lower levels of HDL cholesterol and adiponectin, markers of adipose tissue dysfunction. Detailed clinical characterization of the groups is described in table 3 (Rodrigues et al, 2020).

PARAMETER	GROUP	GENDER	MEDIAN	IQR (Q1; Q3)	P VALUE
AGE (Y)	IS **(1)**	F (14); M (3)	45	36;55	n.s.
	IR_NG **(2)**	F (24); M (5)	39	32.5;49	
	IR_PD **(3)**	F (22); M (6)	47	39.3; 54.5	
	IR_D **(4)**	F (17); M (1)	48	43.5;56.5	
BMI (Kg/M^2)	IS **(1)**		41	37.38; 45.75	n.s.
	IR NG **(2)**		41.95	38.63; 46.65	
	IR PD **(3)**		44.90	40.60; 48.50	
	IR D **(4)**		44.60	41.53; 52.78	
HOMA2IR	IS **(1)**		0.535	0.40; 0.75	
	IR_NG **(2)**		2.360	1.86; 3.44	p<0.001 vs **1**
	IR_PD **(3)**		1.78	1.68; 2.66	p<0.001 vs **1**
	IR_D **(4)**		2.20	1.64; 4.80	p<0.001 vs **1**
HbA1C (mmol/ mol %)	IS **(1)**		5.5	5.25; 5.60	
	IR NG **(2)**		5.4	5.30; 5.50	
	IR_PD **(3)**		5.9	5.70; 6.05	p<0.05 vs **1;** p<0.01 vs **2**
	IR_D **(4)**		7.05	6.60; 7.70	p<0.001 vs **1** and **2;** p<0.01 vs **3**
FASTING GLYCEMIA (Mg/ dL)	IS **(1)**		79	76; 91	
	IR NG **(2)**		88	85; 92	
	IR PD **(3)**		92	88; 105	p<0.05 vs **1**
	IR_D **(4)**		114	102; 149	p<0.001 vs **1** and **2;** p<0.01 vs **3**

Table 3 - Patient characterization and division according to its glycemic profile: fasting glucose levels, HbA1c and insulin resistance index Ox-HOMA2IR.

Patient characterization resulted in four different groups: 1 – insulin sensitive group (IS); 2 – insulin resistant normoglycemic group (IR_NG); 3 – Insulin resistant and prediabetic group (IR_PD); 4 – Insulin resistant and T2D group (IR_D). Characterization and division of patients according also to its age, gender an BMI.

Here, we have investigated the gene expression of dopamine receptors in the adipose tissue of these patients and correlated their expression with markers of adipocyte metabolic function. Expression of *DRD1, DRD2* and *DRD4* was evaluated in the four groups of patients, showing decreased *DRD4* expression in patients with prediabetes and T2D when compared to IS patients ($p<0.05$, figure 15C). No significant differences were observed for *DRD1* and *DRD2* expression (fig. 15A-C), and no expression was detected for *DRD3* and *DRD5*. Importantly, *DRD1* expression was 10-fold higher than the other dopamine receptors (fig. 15A). In order to evaluate the relation between insulin resistance (IR) and dopamine receptor expression, all IR patients (groups 2, 3 and 4) were compared to IS, as depicted in figure 15 (D, E, F). Decreased *DRD4* expression in patients with IR was also observed when comparing to IS patients (fig. 15F, $p<0.05$). Despite no differences were observed for *DRD2* expression, a trend to decreased *DRD1* expression ($p=0.06$) was observed in IR patients when compared to IS individuals. In order to understand the possible involvement of dopamine receptors in regulating adipocyte metabolism, the correlation between *DRD1* expression with *INSR* and genes involved in lipid metabolism was determined. Figure 15 shows a positive correlation between *DRD1* expression and *UCP-1* ($p<0.001$, $\rho= 0.85$) (G), *PPARA* ($p<0.001$, $\rho= 0.55$) (H) and *INSR* expression ($p<0.001$, $\rho= 0.52$) (I). Importantly, expression of *UCP-1*, *PPARA* and *INSR* was also decreased in diabetic patients, suggesting that the impairment of adipose tissue dopaminergic signalling is correlated with impaired insulin sensitivity and metabolic function (data not shown). Multiple regression

analysis has shown that the correlations of *D1DR* with *UCP-1*, *PPAR* and *INSR* are independent of insulin resistance and BMI (figure 16).

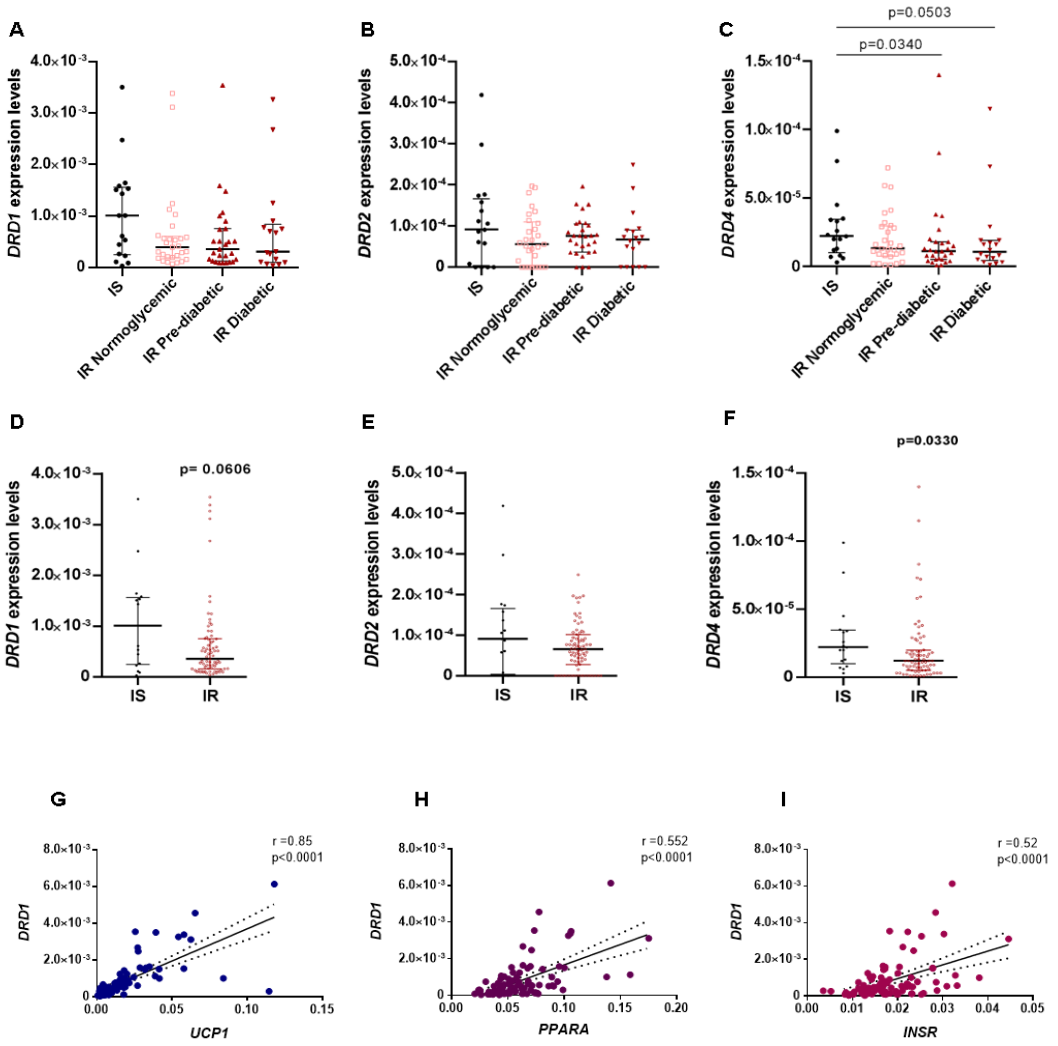


Figure 15 - Expression of dopamine receptor in visceral adipose tissue (VAT) of patients with metabolic syndrome.

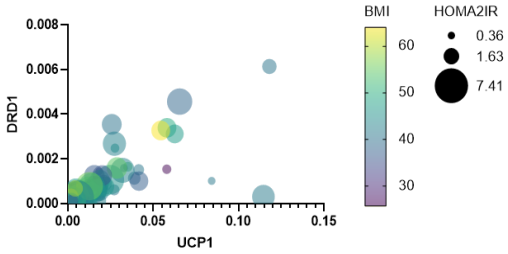
Upper line shows VAT expression of *DRD1* (A), *DRD2* (B) and *DRD4* (C) in each group of patients: insulin sensitive (IS), and insulin resistant (IR) normoglycemic, prediabetic, and type 2 diabetic patients. All IR patients were grouped and compared with IS patients for *DRD1* (D), *DRD2* (E) and *DRD4* (F). Bottom line sown the correlations between *DRD1* expression and *UCP-1*(G) and *PPARA* (H) and the insulin receptor *INSR* (I). Values are median ± interquartile range. Kruskal Wallis and Mann Whitney

tests were used to analyse gene expression data according to the patient's division. Spearman correlation teste was used to performed correlation analysis.

UCP1:

Model		
Analysis of variances	**F (DFn, DFd)**	**P value**
Regression	F (3, 83) = 32.76	p< 0.0001
DRD1	F (1, 83) = 93.26	p< 0.0001
BMI	F (1, 83) = 2.153	p= 0.1461
HOMA2IR	F (1, 83) = 0.4427	p= 0.5077

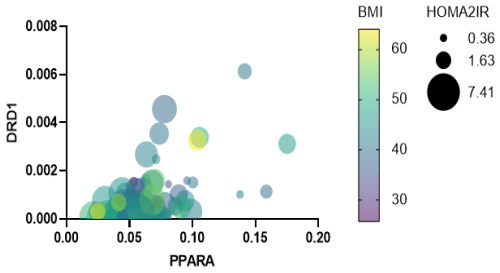
Parameter estimates	Variable	P value	Summary p value
β0	Intercept	0.0419	*
β1	DRD1	<0.0001	****
β2	BMI	0.1461	n.s.
β3	HOMA2IR	0.5077	n.s.
Goodness of fit			
R squared		0.5422	



PPARA:

Model		
Analysis of variances	**F (DFn, DFd)**	**P value**
Regression	F (3, 83) = 15.72	p< 0.0001
DRD1	F (1, 83) = 40.20	p< 0.0001
BMI	F (1, 83) = 1.326	p= 0.2528
HOMA2IR	F (1, 83) = 3.227	p= 0.0761

Parameter estimates	Variable	P value	Summary p value
β0	Intercept	<0.0001	****
β1	DRD1	<0.0001	****
β2	BMI	0.2528	n.s.
β3	HOMA2IR	0.0761	n.s.
Goodness of fit			
R squared		0.3624	



INSR:

Model		
Analysis of variances	**F (DFn, DFd)**	**P value**
Regression	F (3, 83) = 10.83	p< 0.0001
DRD1	F (1, 83) = 28.43	p< 0.0001
BMI	F (1, 83) = 1.316	p= 0.2547
HOMA2IR	F (1, 83) = 1.2191	p= 0.2783

Parameter estimates	Variable	P value	Summary p value
β0	Intercept	<0.0001	****
β1	DRD1	<0.0001	****
β2	BMI	0.2547	n.s.
β3	HOMA2IR	0.2783	n.s.
Goodness of fit			
R squared		0.2812	

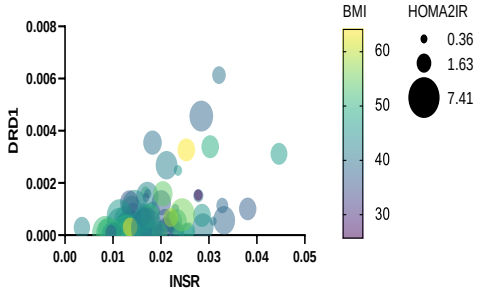
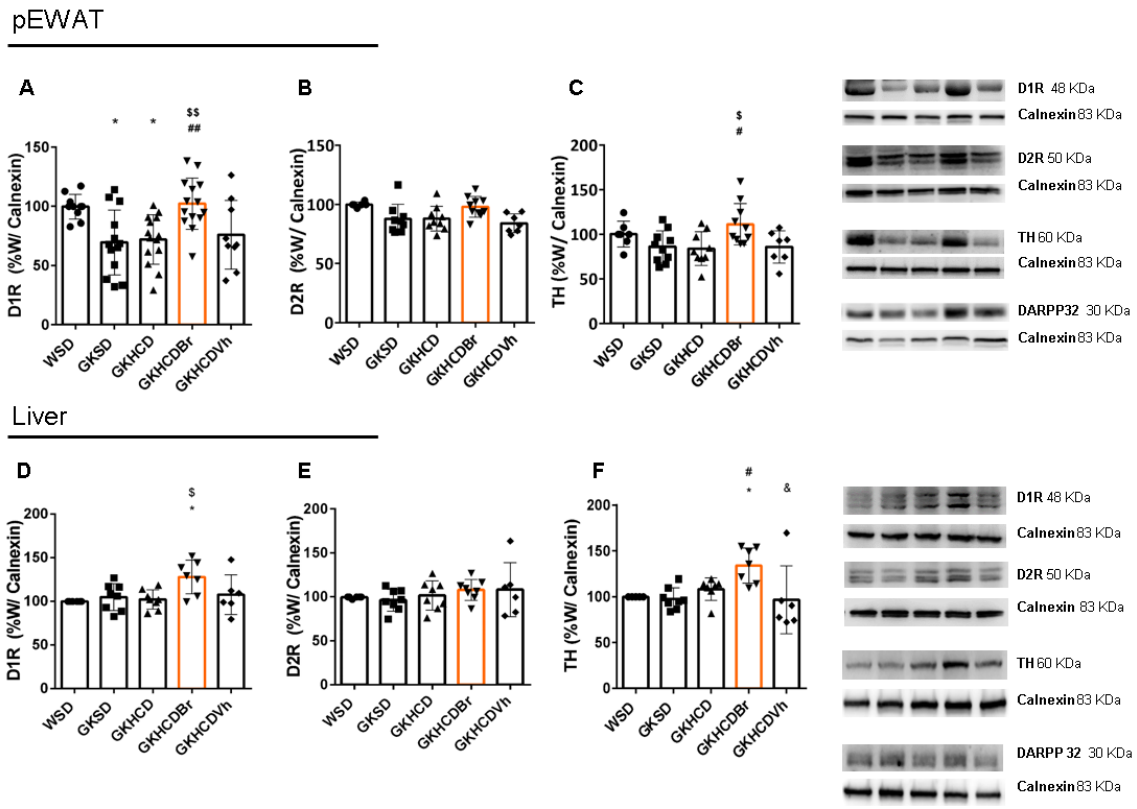


Figure 16 - Multiple linear regression showing that lower expression levels of *UCP1*, *PPARA* and *INSR* are positively correlated with lower *DRD1* expression regardless of patients insulin resistance and BMI.

4.2.2 Bromocriptine treatment increases D1R expression in pEWAT and liver in an animal model of type 2 diabetes and obesity

Although previous evidences point out to the central actions of bromocriptine, recent findings suggest a direct action of dopamine and bromocriptine in peripheral insulin-sensitive tissues (Tavares et al., 2020, 56th EASD Annual Meeting of the European Association for the Study of Diabetes). In order to understand the role of bromocriptine treatment in regulating dopaminergic signalling in insulin-sensitive tissues, the protein levels of D1R, D2R, the downstream effector DARPP32 and tyrosine hydroxylase (TH) were determined in pEWAT, liver, BAT and soleus muscle. Figure 17A shows decreased D1R levels in pEWAT of GK rats feeding a standard or a hypercaloric diet (GKHCD) ($p<0.05$), similarly to what is observed in human adipose tissue in figure 15. Bromocriptine treatment significantly improved D1R levels ($p<0.01$ vs GKSD and vs

GKHCD) to levels similar to Wistar control animals. TH levels were also increased in treated animals, suggesting higher local dopamine production (fig 17C, $p<0.05$ vs GK and GKHCD). Similarly, in the liver, D1R (fig. 17D, $p<0.05$ vs GK and GKHCD) and TH levels (17F, $p<0.05$ vs GK, GKHCD and GKHCDVh) were increased after bromocriptine treatment. On the other hand, D2R and DARPP32 levels were not altered among groups in both tissues (fig. 17B and E, representative Western blot images in the right panel). No significant changes in dopaminergic receptors and TH levels were observed in BAT and skeletal muscle (representative Western blot images at figure 17 G and H).



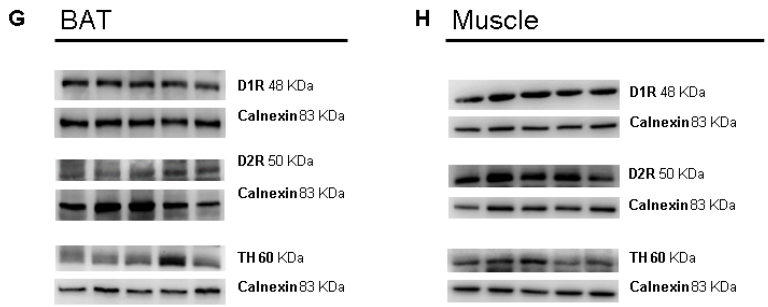


Figure 17– Alterations of dopaminergic signalling in peri-epididymal adipose tissue (pEWAT) and liver after bromocriptine treatment I an animal model of Type 2 diabetes and obesity.

Increased dopamine D1 receptor (D1R) (A) and tyrosine hydroxylase (TH) (C) in the pEWAT, while no differences were observed for dopamine D2 receptor (D2R) (B). Similar results were observed for D1R (D), D2R (E) and TH (F) in the liver. Representative Western blot images are shown in the right panel. The bottom line shows representative Western blot images from dopaminergic signalling in the brown adipose tissue (BAT) (G) and the skeletal muscle (H), where no differences were found between groups. Bars represent means ± SD. Differences between groups were assessed through the One-way ANOVA test with Tukey multiple comparisons. * Different from WSD; # different from GKSD; $ different from GKHCD; & different from GKHCDBr. Level of significance: * p<0,05; **p<0,01; ***p<0,001.

4.2.3 Bromocriptine increases insulin sensitivity and drives metabolic pathways towards lipid oxidation in pEWAT.

In order to understand the impact of dopaminergic system remodelling induced by bromocriptine in pEWAT, markers for the endocrine and metabolic function of adipose tissue were evaluated. As shown in figure 18, HCD-fed GK rats (GKHCD and GKHCDVh) exhibited increased epididymal fat mass (fig. 18B) and adipocyte volume (fig. 18C) compared to GK rats fed a standard diet (GKSD, $p<0.01$). Bromocriptine significantly decreased epididymal fat mass (fig. 18B) ($p<0.05$ vs GKHCD and $p<0.01$ vs GKHCDVh) and adipocyte size (fig. 18C) ($p<0.05$ vs GKDS, $p<0.0001$ vs GKHCD and GKHCDVh). Representative haematoxylin-eosin images are shown in figure 18A and it is clear from the images the effect of bromocriptine in decreasing

adipocyte size. Decreased adiposity in GKHCDBr rats was accompanied by lower plasma leptin levels (fig. 18D, $p<0.001$ vs GKHCD and $p<0.0001$ vs GKHCDVh), while adiponectinemia remained unchanged between groups (fig. 18E).

Concerning insulin sensitivity and metabolic pathways, bromocriptine treatment increased GLUT4 (fig 18F, $p<0.001$ vs WSD, GKSD, GKHCD and GKHCDVh), insulin receptor (InsR) (fig. 18G, $p<0.001$ vs GKSD and $p<0.0001$ vs GKHCD) and PPARy (fig. 18I, $p <0.001$ vs GKSD, $p<0.001$ both vs GKHCD and GKHCDVh), while no differences were observed in total AMPK levels between groups (fig. 18H). The activation of the InsR and AMPK was determined in post-absorptive and post-prandial states. In the postabsorptive stage (6h fasting), no differences were found between groups regarding InsR activation (IR-Tyr1361, fig. 18J). Nevertheless, increased levels of AMPK-Thr172 (fig. 18K, $p<0.05$ vs GKSD) and a trend to increase ACL-Ser563 after bromocriptine treatment. Such results suggest increased catabolic activity, and particularly lipid oxidation, which was confirmed by the increase in β-hydroxybutyrate levels in pEWAT ($p<0.01$ vs WSD, $p<0.001$ vs GKSD, $p<0.01$ vs GKHCD and $p<0.05$ vs GKHCDVh) by bromocriptine treatment (fig. 18M), while no differences were found in the serum (data not shown). Increased catabolic activity is thought to reduce lipotoxicity and insulin resistance. Accordingly, in the pos-prandial state, bromocriptine increased IR-Tyr1361 levels (fig. 18N, $p<0.05$ vs GKHCD), while AMPK-Thr172 levels remained similar to the other groups (fig. 18O).

pEWAT

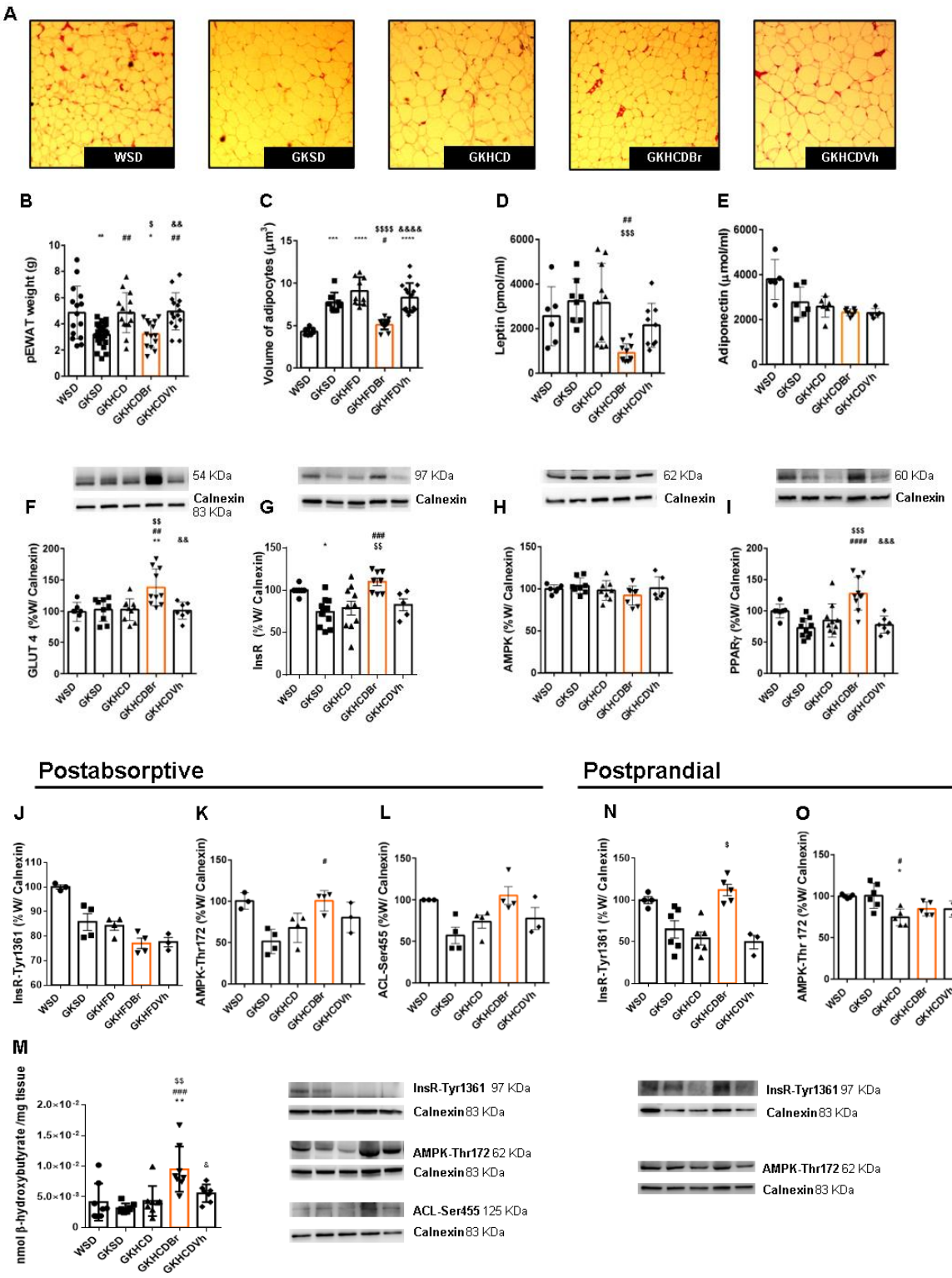

A
WSD
GKSD
GKHCD
GKHCDBr
GKHCDVh
B
pEWAT weight (g)
C
Volume of adipocytes (µm³)
GKHFD
GKHFDBr
GKHFDVh
D
Leptin (pmol/ml)
E
Adiponectin (µmol/ml)
F
54 KDa
Calnexin
83 KDa
GLUT 4 (%W/ Calnexin)
G
97 KDa
InsR (%W/ Calnexin)
H
62 KDa
AMPK (%W/ Calnexin)
I
60 KDa
PPARγ (%W/ Calnexin)
Postabsorptive
Postprandial
J
InsR-Tyr1361 (%W/ Calnexin)
K
AMPK-Thr172 (%W/ Calnexin)
L
ACL-Ser455 (%W/ Calnexin)
N
InsR-Tyr1361 (%W/ Calnexin)
O
AMPK-Thr 172 (%W/ Calnexin)
M
nmol β-hydroxybutyrate /mg tissue
InsR-Tyr1361 97 KDa
Calnexin 83 KDa
AMPK-Thr172 62 KDa
ACL-Ser455 125 KDa

Figure 18 – Improvement of insulin sensitivity and remodeling of adipose tissue catabolic pathways by bromocriptine.

Haematoxylin eosin staining (A, 100X) of peri-epididymal adipose tissue (pEWAT). Bromocriptine treatment decreased pEWAT weight (B), adipocytes volume (C) and plasma leptin levels (D), while no changes were observed for plasma adiponectin levels (E). Bromocriptine treatment increased glucose 4 transporter (GLUT4) (F), insulin receptor (InsR) (G) and Peroxisome proliferator-activated receptor gamma (PPARγ, H), while AMP-activated protein kinase (AMPK, I) remained similar between groups. In the bottom, no changes were observed in postabsorptive activation of InsR (InsR-Tyr1361, J), but Bromocriptine increased AMPK-Thr172 (K), ACL-Ser455 (L) and β-hydroxybutyrate levels (M). In the post-prandial stage, the InsR was more activated (N), while no differences were found AMPK activation (O) in Bromocriptine-treated rats. Bars are means ± SD. One-way ANOVA test with Tukey multiple comparisons was used to assess differences between groups. Kruskal Wallis test was used to analyse InsR-Tyr1361 and AMPK-Thr172. * Different from WSD; # different from GKSD; $ different from GKHCD; & different from GKHCDBr. Level of significance: * p<0,05; **p<0,01; ***p<0,001.

4.2.4 Bromocriptine reduces liver triglycerides content without activating liver AMPK pathway.

Figure A shows liver hematoxylin-eosin staining for the different studied groups. A high amount of lipid droplets consistent with hepatic steatosis phenotype was observed in animals fed with a HCD (fig. 19A, GKHCD and GKHCDVh), consistent with the observed increase in liver weight (fig. 19B, p<0.001 vs WSD and GKSD) and triglycerides content (fig 19C, p<0.001 vs WSD and GKSD). Bromocriptine notably reduced the presence of lipid droplets in the hematoxylin-eosin staining and reversed hepatic steatosis (fig. 19A), which was accompanied by decreased liver weight (fig. 19B, p<0.001 vs GKHCD) and hepatic triglycerides (fig. 19C, p<0.01 vs GKHCD and GKHCDVh).

In order to understand the role of bromocriptine in the regulation of hepatic metabolic function, InsR and AMPK activation, as well as GLUT2 levels were determined. Animals treated with bromocriptine showed increased total InsR levels (fig. 19E, p<0.05 vs GKSD p<0.01 vs

GKHCD) but no changes in AMPK (fig. 19F). Intriguingly, lower levels of GLUT2 were also observed after bromocriptine treatment (fig. 19D, $p<0.001$ vs WSD, $p<0.01$ vs GKSD and $p<0.01$ vs both GKHCD and GKHCDVh), as well as lower InsR-Tyr1361 (fig. 19I, $p<0.05$ vs GKHCD) and tendentially lower AMPK-Thr172 levels (fig. 19J, $p=0.07$ vs GKSD) in the post-prandial state. No differences were observed in InsR-Tyr1361 and AMPK-Thr172 levels in the postabsorptive state (fig. 19G and 19H).

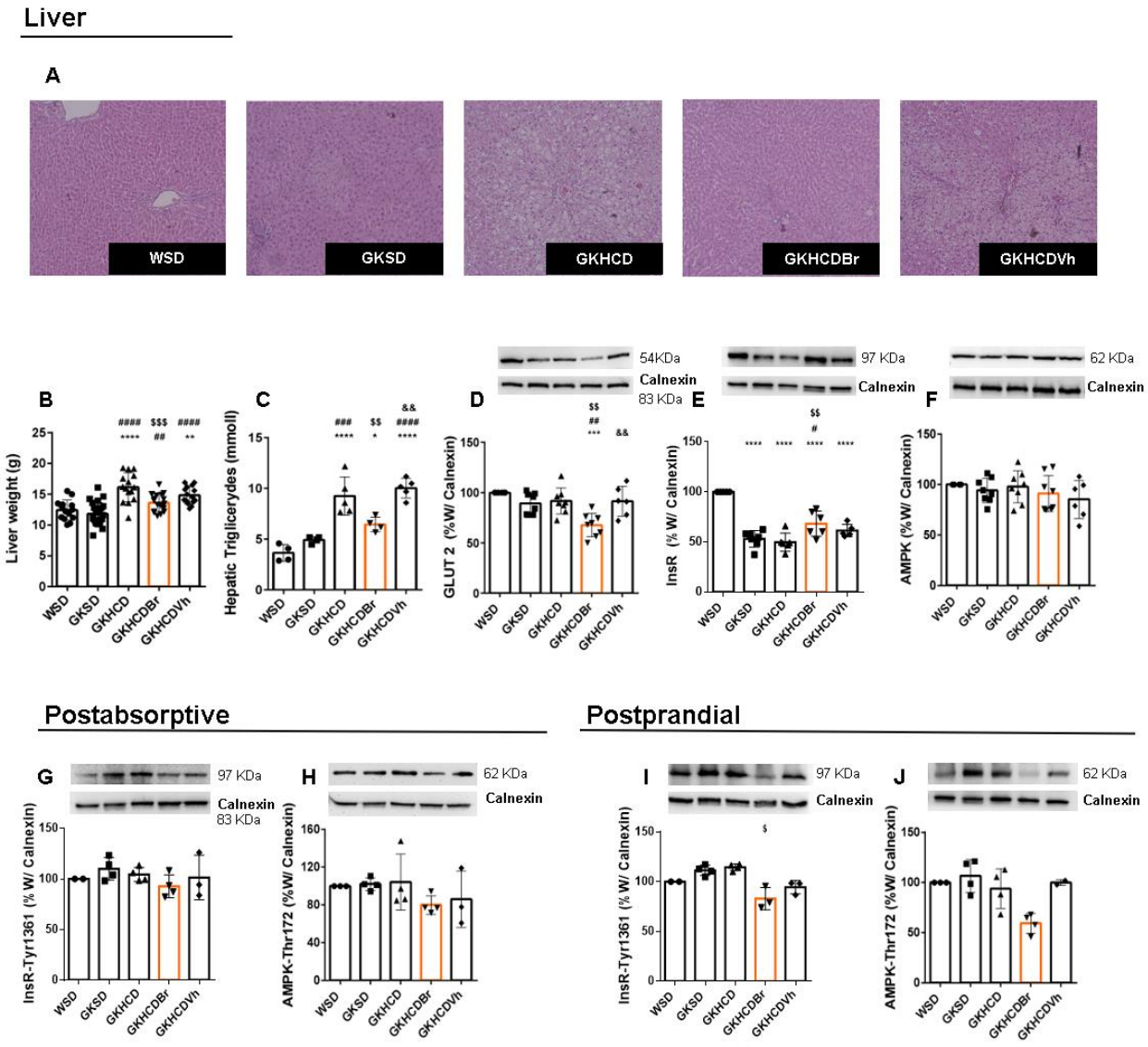


Figure 19 – Bromocriptine reduces liver triglycerides content without activating liver AMPK pathway.

Liver haematoxylin-eosin staining (A, 100X) showing a reduction of hepatic steatosis after bromocriptine treatment, in accordance with lower liver weight (B) and hepatic triglycerides (C). Bromocriptine treatment decreased GLUT2 (D), but increased InsR (E), while AMPK (F) levels did not alter between groups. No changes were observed in InsR-Tyr1361 (G) nor AMPK-Thr172 (H) in the postabsorptive stage. Postprandially lower levels of InsR-Tyr1361 (I) and no changes in AMPK-Thr172 (J) were observed. Bars are means ± SD. One-way ANOVA was used for liver weight, hepatic triglycerides, GLUT2, InsR and AMPK; Kruskal Wallis test was used for InsR-Tyr1361 and AMPK-Thr172. *Different from WSD; # different from GKSD; $ different from GKHCD; & different from GKHCDBr. Level of significance: *p <0.05; **p <0.01; ***p <0.001.

4.2.5 Bromocriptine increased fasting insulin signalling in brown adipose tissue but has no effects in the skeletal muscle.

Although bromocriptine did not change BAT dopaminergic system, increased GLUT4 levels were observed after the treatment (fig. 20A, $p<0.001$ vs WSD, $p<0.01$ vs GKSD, $p<0.001$ vs GKHCD and $p<0.05$ vs GKHCDVh), while both total levels of IR and AMPK remained similar between groups (fig. 20B and C). Furthermore, bromocriptine also increased postabsorptive levels of InsR-Tyr1361 (fig. 20D $p<0.05$ vs GKHCD), but not AMPK-Thr172 (fig. 20E). Animals sacrificed at post-prandial stage presented similar levels of InsR-Tyr1361 (fig. 20F) and AMPK-Thr172 (fig. 20G) between groups. In the skeletal muscle, GLUT4, InsR and AMPK levels did not change between groups (fig 20H, I and J). Moreover, either postabsorptive or post-prandial InsR-Tyr1361and AMPK-Thr172 levels also remained similar between groups (fig. 20K-N).

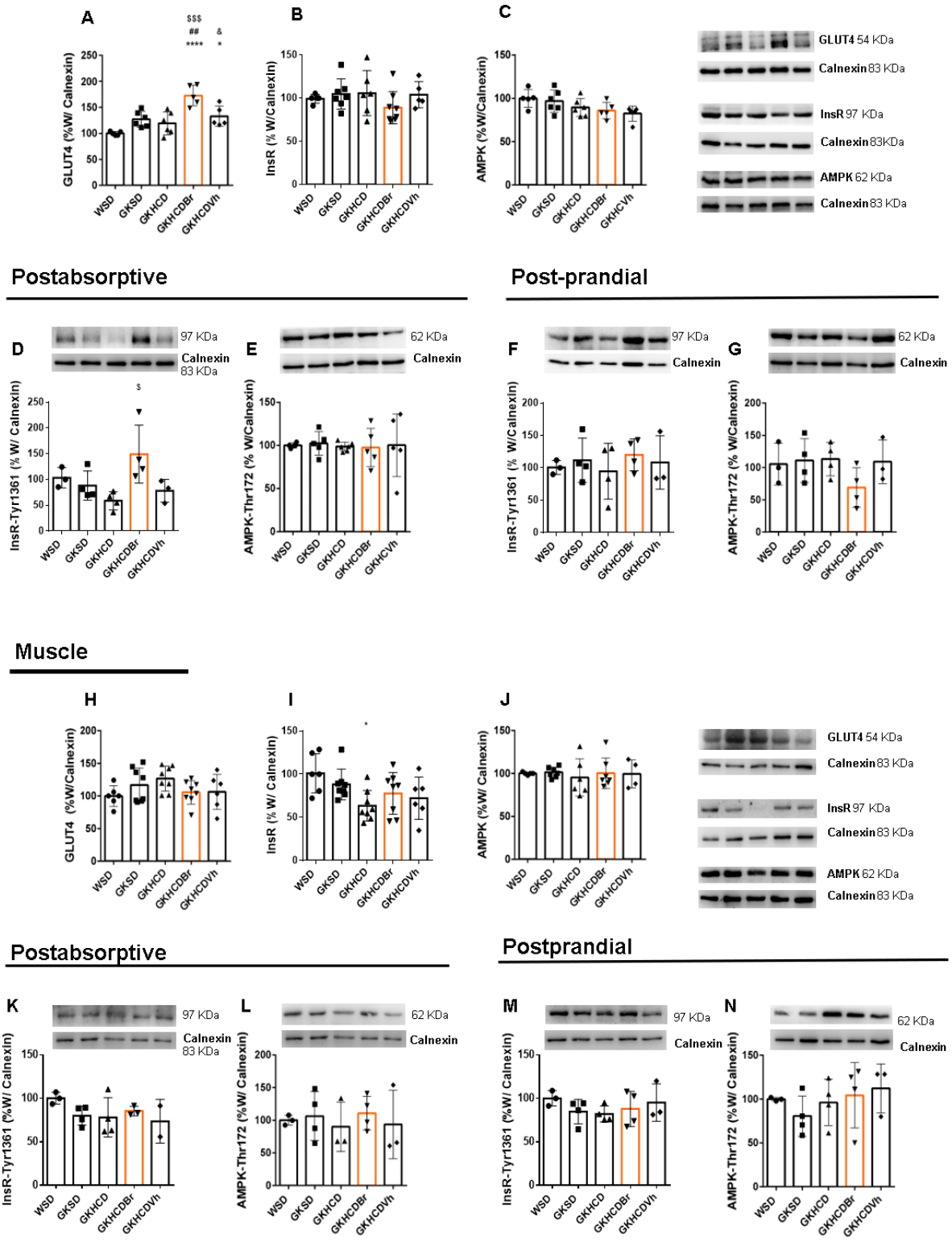

BAT
A
B
C
GLUT4 (%W/ Calnexin)
InsR (% W/Calnexin)
AMPK (%W/Calnexin)
WSD
GKSD
GKHCD
GKHCDBr
GKHCDVh
GLUT4 54 KDa
Calnexin 83 KDa
InsR 97 KDa
Calnexin 83KDa
AMPK 62 KDa
Calnexin 83 KDa
Postabsorptive
Post-prandial
D
E
F
G
97 KDa
62 KDa
Calnexin
83 KDa
InsR-Tyr1361 (% W/ Calnexin)
AMPK-Thr172 (% W/Calnexin)
Muscle
H
I
J
Postabsorptive
Postprandial
K
L
M
N

Figure 20 – Bromocriptine increased fasting insulin signaling in brown adipose tissue but presented no effect in the skeletal muscle.

Bromocriptine treatment increased levels of GLUT4 (A) in BAT, while neither InsR (B) nor AMPK (C) levels changed. In the postabsorptive stage increased InsR-Tyr172 (D) after bromocriptine treatment, but no differences were observed for postabsorptive AMPK-Thr172 (E), nor postprandial InsR-Tyr172 (F) and AMPK-Thr172 (G). In the skeletal muscle, no significant changes were observed for GLUT4 (H), InsR (I) total AMPK (J), nor InsR and AMPK activation in the postabsorptive (K, L) or postprandial (M and N) stages. Bars are means ± SD. One-way ANOVA test with Tukey multiple comparisons was used to analyse GLUT4, InsR and AMPK total proteins levels; Kruskal Wallis test was used for IR-Try1361 and AMPK-Thr172 analyses. * Different from WSD; # different from GKSD; $ different from GKHCD; & different from GKHCDBr. Level of significance: 1 symbol, $p<0.05$; 2 symbols, $p<0.01$; 3 symbols, $p<0.001$.

4.2.6 Improved adipose tissue insulin sensitivity and metabolic function after bromocriptine treatment is associated with better metabolic profile

In accordance with previous reports from our laboratory (Rodrigues et al., 2017) GK rats naturally present lower body weight than Wistar rats (fig. 21A and 21A1; $p<0.001$). HCD-fed GK rats showed higher weight gain in the last month (fig. 21B $p<0.05$ vs GKSD), which was associated with increased caloric intake during the same period of time (fig. 21C $p<0.001$, WSD vs GKHCD and GKSD vs GKHCD; $p<0.01$ WSD vs and $p<0.001$ GKSD vs both GKHCD and GKHCDVh). On the other hand, bromocriptine treatment has shown to prevent weight gain (fig. 21A2 and 21B, $p<0.001$ vs GKHCD and $p<0.05$ vs GKHCDVh) and decrease the caloric intake (fig. 21C, $p<0.0001$ vs GKHCD and GKHCDVh).

The increased body weight and caloric intake in HCD-fed rats was followed by increased fasting plasma triglycerides (fig. 21D, $p<0.0001$, WSD vs GKHCD and $p<0.001$ WSD vs GKHCDVh; $p<0.0001$, GKSD vs both GKHCD and GKHCDVh) and total cholesterol levels (fig. 21E $p<0.0001$ WSD vs GKHCD and $p<0.0001$ GKSD vs GKHCD). Interestingly, bromocriptine

treatment improved plasma lipid profile by decreasing both plasma triglycerides (fig. 21D $p<0.001$ vs GKHCD and $p<0.05$ vs GKHCDVh) and cholesterol levels (fig. 21E $p<0.001$ vs GKHCD), but no significant changes were observed for plasma free fatty acids (fig. 21F). Such results are in accordance with the lower fat accumulation observed in the pEWAT and liver as well as with increased pEWAT lipid oxidation, as depicted in figure 18 and 19.

GK rats are described as a model of glucose intolerance and mild fasting hyperglycaemia (Rodrigues et al., 2017), which was now confirmed by increased glycemia after 6h fasting and AUC during the IPITT as presented at figures 21G and 21H respectively (fig. 21G $p<0.0001$ WSD vs GKSD; fig. 21H $p<0.05$ WSD vs GKSD). The HCD further aggravated such phenotype, by increasing fasting glycemia (fig. 21G $p<0.0001$ WSD vs GKHCD and $p<0.001$ vs GKHCDVh), AUC during the IPITT (fig. 21H $p<0.0001$ WSD vs GKHCD and $p<0.001$ vs GKHCDVh) and glycemia at 1 hour during the IPITT (fig. 21I $p<0.0001$ WSD vs GKHCD and $p<0.05$ vs GKHCDVh). Conversely, bromocriptine treatment improved glycemic control by decreasing 6h fasting glycemia (fig. 21G) in relation to GKHCD group ($p<0.01$) and reducing the difference to Wistar rats ($p<0.05$). Moreover, bromocriptine significantly reduced AUC (fig. 21H, $p<0.001$ vs GKHCD) and glycemia at 1h during IPITT (fig. 21I, $p<0.0001$ vs GKHCD). Plasma insulin levels were decreased in GK rats (fig.21J, $p<0.0001$ vs WSD), as consequence of the described β-cell impairment in this animal model (Almon et al., 2014; Movassat et al., 2008), and remained similar in all the GK groups tested (fig. 21J), as well as glucagon plasma levels (fig. 21K).

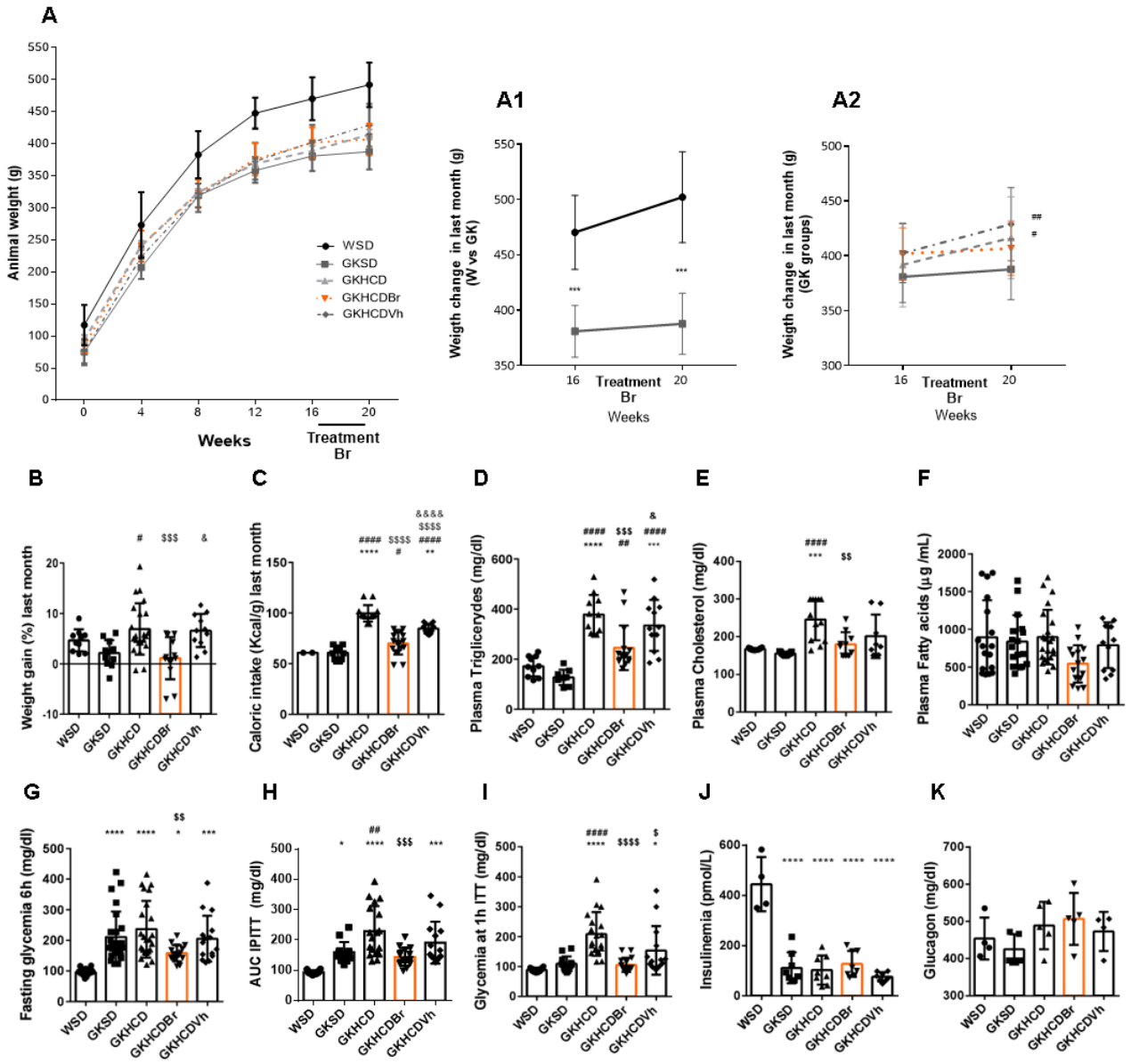


Figure 21 – Bromocriptine improves metabolic profile in HCD-fed GK rats.

In A is represented the weight gain curves during the all period of the study, and specifically in the last month. Bromocriptine prevented HCD-induced weight gain in the last month (A2 and B). Bromocriptine also reduced caloric intake (C) plasma triglycerides (D) and cholesterol levels (E), without modifying plasma free fatty acids (F). Glucose homeostasis also improved with bromocriptine treatment, namely fasting glycaemia (G), AUC during the ITT (H) and glycemia at 1h during the IPITT (I), despite no differences were observed in plasma insulin (J) and glucagon levels (K). Values are means ± SD.

Differences between groups were assessed through the One-way ANOVA test with Tukey multiple comparisons. * Different from WSD; # different from GKSD; $ different from GKHCD; & different from GKHCDBr. Level of significance: 1 symbol, p<0,05; 2 symbols, p<0,01; 3 symbols, p<0,001.

4.2.7 Discussion

Although the therapeutic effects of the D2R dopaminergic agonist, bromocriptine, in T2D have been attributed to central actions, peripheral dopamine is acknowledged to directly modulate glucose uptake in insulin-sensitive tissues and lipid metabolism in white adipose tissue. Herein we hypothesized that patients with T2D may have an impairment of adipose tissue dopaminergic system, which may be therapeutic target for bromocriptine in T2D patients. We showed that IR patients exhibit a downregulation of *DRD4* and *DRD1* expression in the adipose tissue, which is correlated with markers of adipocyte metabolic function. Additionally, we found in an animal model of T2D and HCD-induced obesity, that bromocriptine remodeled the dopaminergic system of white adipose tissue and liver, through increased D1R and TH levels. Such mechanisms were associated with higher catabolic function, namely lipid oxidation, and insulin sensitivity in adipose tissue, as well as lower hepatic steatosis. Importantly, no effects were observed in skeletal muscle and BAT, suggesting a predominant action of bromocriptine in the white adipose tissue.

Borcherding et al., have shown the presence of dopamine receptors in human adipocytes, specifically in the subcutaneous adipose tissue of healthy volunteer (Borcherding et al., 2011). Similar to our results, they showed that the D1R was the most expressed receptor subtype in white adipose tissue, while dopamine receptors type 2 (D2R and D4R) were the less expressed (Borcherding et al., 2011). In our study, no expression of D3R and D5R was observed, which may be attributed to their absence or very reduced expression or to technical hints, as the

selected primers. Nevertheless, similar observations were made by Borcherding et al., which did not observe, as well, expression of these two receptors in the subcutaneous adipose tissue (Borcherding et al., 2011). Importantly, similarly to our findings, the same authors found a higher expression of D1R in adipocytes than stromovascular fraction and a 10-fold difference between D1R and D2R expression. In the present study, we showed for the first time, a deregulation of dopamine receptors expression in the visceral adipose tissue from patients with IR and T2D. It is known that dopamine is involved in the regulation of GSIS, decreasing insulin secretion through D2R-dependent mechanisms (Movasst 2008, Farino 2019). It has also been proposed that dopamine-induced decreased GSIS could be a protective mechanism to avoid β-cell exhaustion (de Leeuw van Weenen et al., 2010; Roden & Shulman, 2019). Accordingly, it has been reported that obese humans present reduced dopamine levels and/or function, which may be related to the compensatory hyperinsulinemia usually observed in such patients (Roden and Shulman 2019, Wang 2001). Herein, we show that IR is a condition also characterized by an imbalance of the dopaminergic system in the adipose tissue, which was correlated with the downregulation of genes implicated in lipid oxidation, *UCP1* and *PPARA*, as well as with *INSR* gene. Given that D1R is a Gs-linked receptor, it is possible that such results reflect lipid oxidation reduction, lipotoxicity and IR (Vichi 2016, Wang 2001, Nash 2017). An interesting observation is that lower D1R levels were also found in the peri-epididymal adipose tissue of T2D rats, confirming the results obtained in human samples, highlighting a translatability of mechanisms between species.

The D2R dopaminergic agonist bromocriptine is the only dopaminergic modulator approved for the treatment of T2D, being also commonly used for Parkinson and prolactinomas treatment. It was also shown to have insulin sensitizing and beta-cell protecting effects (Roe 2015, DeFronso

2011, Beaulieu 2019). To the best of our knowledge, our study is the first to show that chronic bromocriptine treatment leads to an increase in D1R protein levels in both pEWAT and liver, what may be explained as a possible feedback or balance between the two dopamine receptor families. Importantly, TH protein level also increased with bromocriptine treatment suggesting increased local production of dopamine.

It is possible that the increased TH levels may reflect increased neuronal dopamine production within the adipose tissue (Pirzgalska et al., 2017), but questions remain if this is caused by the increase of neuronal projections to the adipose tissue or direct actions of bromocriptine in the tissue. Nevertheless, increased TH levels suggest increased dopamine synthesis in the adipose tissue, possibly released by TH-positive neurons terminals or by the adipocytes themself, which signals through the D1R to regulate adipocyte metabolism. In fact, the machinery for production of dopamine has been described to be present in adipocytes (Vargovic et al., 2011).

The physiological role of higher dopamine synthesis is not well understood. However, the predominant expression of D1R, which is coupled to Gs protein-cAMP signalling pathway and is further increased by bromocriptine treatment, suggests that D1R can be related to the modulation of catabolic pathways in the pEWAT. Indeed, we have observed increased fasting levels of AMPK-Thr172 and ACL-Ser455 in pEWAT, suggesting higher fasting catabolic activity, towards lipid oxidation. This is in accordance with previous results from our team, showing that the inhibition of D2R by domperidone, when adipose tissue explants were stimulated with dopamine plus insulin, resulted in acute and marked activation of AMPK, ACL and HSL, suggesting a stimulatory effect of D1R on lipid catabolism. Bromocriptine also increased the levels of the InsR and GLUT4 in the pEWAT, as well as the activation of the InsR after the ingestion of a mixed meal. In adipose tissue explants, activation of D2R was shown to acutely

potentiate insulin-mediated glucose uptake, which may, nevertheless, denote a role for D2R in the modulation of adipose tissue glucose uptake following bromocriptine treatment.

Increased peripheral metabolism in fat depots result not only in a better adipose tissue function, but also in the protection of liver from lipotoxicity-induced insulin-resistance. Interestingly, herein we found that although the InsR levels were increased in the liver, its postprandial activation was reduced, together with lower AMPK activation. Our group has previously showed in liver explants, that D2R activation by dopamine or bromocriptine induce an acute insulin-independent glucose uptake. Here, the observed upregulation of D1R after chronic bromocriptine treatment may result in the opposite effects. Although the liver is expected to significantly contribute to glucose uptake upon a meal ingestion (Adeva-Andany et al., 2016), in this study we actually observed lower hepatic GLUT2 levels. Altogether, our results suggest a metabolic remodeling towards a higher metabolic activity in the periphery, and particularly in the white adipose tissue, rather than in the liver, which is consistent with the dramatic decrease of liver fat and adipose tissue weight/adipocyte volume. Importantly, given that no changes were observed in insulin receptor and AMPK activation in the liver, the direct effects of dopamine are questionable. Thus, it is possible that D1R upregulation may be just implicated in lipid mobilization rather than oxidation in the liver. In the adipose tissue, reduced lipotoxicity is associated with postprandial insulin sensitivity and glucose uptake, which is in line with the increment of InsR and GLUT4 levels. The observed decreased adipose tissue weight is associated with lower leptin levels, which is in accordance with the work by Borcherding et al. showing that modulation of D1R decreased leptin levels both in subcutaneous adipose explants and isolated adipocytes (Borcherding et al., 2011). Moreover, Kok et al., observed decreased

plasma leptin levels after bromocriptine treatment in a cohort of obese women (Kok, et al., 2006a).

In the present work, it was also possible to observe a metabolic shift in the pEWAT of bromocriptine-treated rats, characterized by higher lipid oxidation rather than glucose utilization in the post-absorptive period and vice-versa in the post-prandial state. This is physiologically relevant and shows that bromocriptine restores the balance of energy supply according to the energetic status and body energy requirement, which may be very important for the prevention of lipotoxicity and glucotoxicity, two hallmarks of insulin resistance and metabolic dysregulation.

Both the BAT and skeletal muscle are considered tissues with higher metabolic rates, since the majority of disposable post-prandial glucose is taken up by the skeletal muscle (DeFronzo & Tripathy, 2009) while BAT converts nutrients energy into chemical energy in the form of heat (Kajimura 2010, Berry 2013). Both GLUT4 and fasting InsR-Tyr1361 levels were increased in the BAT of bromocriptine-treated animals. We previously showed in BAT explants, that dopamine and bromocriptine exert no direct effects on glucose uptake and InsR and AMPK activation. Thus, our findings obtained herein in the whole-body animal model are likely to result from the overall improvement of the metabolic status and reduction of lipotoxicity, or from other indirect actions of bromocriptine. On the other hand, activation of D1R in skeletal muscle explants was shown to, directly and independently of insulin, lead to glucose uptake (Tavares et al., 2020, 56th EASD Annual Meeting of the European Association for the Study of Diabetes). However, herein no alterations on dopamine receptors and metabolic pathways were found in the skeletal muscle on the bromocriptine treated animals. In accordance with the results found herein that show a lack of effect of bromocriptine in skeletal muscle, a clinical trial in obese type 2

diabetic patients reported that bromocriptine did not improve insulin sensitivity in the skeletal muscle within the physiologic range of hyperinsulinemia (DeFronso 2011, Berry 2013).

As expected, and in accordance with the increased GLUT4 and InsR activation in adipose tissues, bromocriptine treatment resulted in enhanced peripheral insulin sensitivity and improved fasting glycaemia. Particularly, in the study from Pijl et al. (2010), T2D individuals treated with bromocriptine for 16 weeks showed lower fasting glycaemia and improved glucose tolerance tests (Pijl et al., 2000). Also, bromocriptine treatment in spontaneous hypertensive rats reduced retroperitoneal body fat, C-reactive protein, plasma insulin and glucose levels, and HOMA-IR (Ezrokhi 2014. Some studies have shown that body weight was unaltered in T2D individuals after bromocriptine administration (Beaulieu 2019, Piji 2000, Kamath 1997) which disagrees with our findings. One factor contributing to the lower body weight in our animal model could be the decrease in caloric intake. In fact, it has been shown that bromocriptine treatment restores hypothalamic dopamine levels in rodents, a mechanism shown to be reduced in hibernating animals and involved in insulin resistance during such period (DeFronso 2011, Kamath 1997). However, we could not exclude the possibility of a lower caloric intake in bromocriptine-treated animals due to an improvement in peripheral metabolism or other unknown peripheral actions. In agreement with this, we also found an overall improvement in triglyceride and FFA levels, by diminishing their levels in the liver and their mobilization from adipose tissue to circulation, effects that were also observed in clinical trials (Kok, et al., 2006b)).

CHAPTER 5 – Closing remarks and future perspectives

5.1 Conclusion

DA is not exclusively produced in the CNS, with several peripheral organs being responsible for its production (carotid body, adrenal glands, GI tract, pancreas, adipose tissue among others) as well as its signalling machinery proteins where in some of them dopamine exert its effects in an autocrine and paracrine manner (Armando et al., 2015; Borcherding et al., 2011; Bucolo et al., 2019; Chaudhry et al., 2016; Maffei et al., 2015; Vargovic et al., 2011). The present work demonstrated that this signalling machinery is present in insulin-sensitive organs and may be impaired in the adipose tissue of insulin resistant patients and animal models of T2D.

Thus, by directly injecting dopamine (100nmol) in heathy lean Wistar rats, we found that peripheral DA modulates glucose uptake, in part through direct actions in insulin-sensitive tissues. Moreover, by selectively treated tissue explants (liver, soleus muscle, mWAT, eWAT and BAT) with DA or bromocriptine, in the presence or the absence of insulin and dopamine receptor antagonists (domperidone and haloperidol), we concluded that D1R and D2R are differentially involved in glucose uptake and in the regulation of insulin signalling pathway and AMPK activation in insulin-sensitive tissues. Particularly, dopamine regulates glucose uptake in soleus muscle via D1R while D2R regulates glucose uptake in the liver, and increase insulin-mediated glucose uptake in mWAT, without direct effects on BAT. Nevertheless, the direct effects of DA only partially explain *in vivo* tissue glucose uptake observed after its exogenous peripheral administration. Given that dopamine is not able to cross the blood-brain-barrier, other peripheral indirect mechanisms of glucose homeostasis regulation by DA may be involved and should be addressed in the future.

Regarding insulin signalling, only in the presence of domperidone (selective D2R antagonist) IR-Tyr972 in the liver and both mWAT and eWAT was increased. Interestingly, domperidone-

induced IR-Tyr972 increase is an independent mechanism of glucose uptake, suggesting that insulin receptor phosphorylation and insulin pathway may be involved in other metabolic functions in response to DA.

In contrast, both DA and bromocriptine directly regulates AMPK phosphorylation via D2R in the soleus muscle while D2R inhibition with domperidone in mWAT and eWAT, increased AMPK, HSL and ACL phosphorylation, activating lipolytic and lipid oxidation pathways and inhibiting lipid synthesis by inhibiting ACC. Thus, DA impacts on lipid metabolism regulation in WAT showing its importance as possible therapeutical approach for obesity and obesity-related metabolic disorders as cardiovascular disease, metabolic syndrome and T2D.

In this work we also demonstrate an imbalance of dopamine receptors in VAT from obese patients with IR, even before glycemic dysmetabolism. Lower *DRD1* expression correlated with reduced expression of key markers of AT lipid oxidation function (*UCP1* and *PPARA*) and the insulin receptor gene (*INSR)*.

Despite of several medications helps to manage T2D, there is an increasing need for more effective treatments than those currently available. For instance, not all T2D patients can tolerate metformin (Brown et al., 2010; Cook et al., 2005), the first line medication for T2D treatment. As well metformin is not efficacious in all T2D patients (Florez, 2017). Additionally, thiazolidinediones (TZD), a class of insulin-sensitizing medications, improve systemic glucose homeostasis, but present harmful side effects such as: weight gain (Nichols & Gomez-Caminero, 2007), heart problems (Lipscombe et al., 2007), and bladder cancer (Turner et al., 2014). Therefore, the importance of finding alternative treatment that attaining their benefits without the associated side effect risks is urgent (Chamarthi & Cincotta, 2017; F. L. Dunn et al., 2011; Townsend et al., 2017). Bromocriptine has shown to exhibit low side effects, preventing

hypoglycemia and heart diseases complication, at the same time presented good adherence and treatment effectiveness (Lamos et al., 2016; Lopez Vicchi et al., 2016; Roe et al., 2015; Schwartz & Zangeneh, 2016).

In an animal model of dysmetabolism (T2D and diet-induced obesity), the modulation of dopaminergic system through bromocriptine restored both D1R and TH in pEWAT and liver. Such changes were associated with higher activation of catabolic pathways and lipid oxidation in AT during the postabsorptive phase and increased insulin receptor activation in the postprandial phase. Altogether, our data suggest that the modulation of peripheral dopaminergic system induces a higher catabolic activity in pEWAT, leading to the mobilization of hepatic lipidic content to be taken up and oxidized in AT, contributing to reduced hepatic and AT lipotoxicity, as well as improved insulin sensitivity and systemic metabolic function. Although additional information on the mechanisms involved on dopaminergic regulation of glucose and lipid metabolism should be better elucidated in the future, we hypothesize that D1R activation could exert a role in enhancing metabolic activity. Thus, the disclosure of the mechanisms involved in bromocriptine therapeutic effects provide new insights regarding the mechanism of action of bromocriptine in T2D and highlight the relevance of the modulation of peripheral dopaminergic system for the treatment of metabolic diseases.

5.2 Future perspectives

In the present work we used the available D2R agonist drug to modulates dopaminergic signalling, in order to study its effects on insulin sensitive tissue, namely in the improvement of AT metabolic function. However, bromocriptine can cross the blood brain barrier, being impossible to fully disclose its direct effects in peripheral tissues excluding the central input. It is also true that in our methodology, instead of intraventricular bromocriptine injections (as

mentioned in some works), we chose intraperitonially injection, to ensure a more directed drug delivery to the abdominal wall of the animals comprising mostly the liver and pEWAT tissues. Moreover, we use a dose of 10mg/kg administered daily according to what has been described in the literature. Although we know that clinically the Bromocriptine-QR dosage used in T2D patient is a much lower comparing to the one used for Parkinson's treatment sometimes is not so linear to extrapolate this treatment dosages for animal use, despite of choosing the one mostly described and used in animal studies.

Thus, it was impossible to fully disclose the direct effects of bromocriptine in peripheral tissues excluding the CNS input. Conscious of this limitation, it would be interesting in the future, to follow a similar approach presented in the methodology (T2D diet-obese induced, with respective healthy Wistar and GK control groups) but adding a group treated with both bromocriptine and domperidone in order to discriminate the direct mechanisms of action of bromocriptine in insulin sensitive tissues. Nevertheless, this work has provided evidences to promote investigation by pharmaceutical companies to develop new therapeutic approaches, based on the modulation of dopaminergic signalling, allowing the management of T2D and obesity in Europe, with the approval of the European Medicines Agency (EMA).

Furthermore, as above-mentioned, the glucose uptake results from tissue explants treated with dopamine or bromocriptine did not resemble the results of tissue glucose uptake with exogenous administration of dopamine. Thus, such evidence suggest that dopamine could modulate other signalling pathways or hormones to indirectly modulate peripheral glucose uptake by insulin sensitive tissues. These mechanisms were not addressed in this study although it would be interesting to address this issue in the future.

Moreover, we have learnt from the literature that direct effect of both dopamine and bromocriptine thought D2R activation of pancreatic β-cells modulates insulin secretion, which could constitute a negative feedback to glucose and incretins action (stimulation of insulin secretion) preserving β-cells function and indirectly contributing to peripheral glucose homeostasis (Chaudhry et al., 2016; de Leeuw van Weenen et al., 2010; Farino et al., 2019; Garcia-Tornadú et al., 2010; Maffei et al., 2015). Taking this into consideration, we hypothesise that dopamine could interact with the incretin system, namely with glucagon-like peptide-1 (GLP1), enhancing peripheral insulin sensitivity. It is well documented that sleeve gastrectomy improves metabolic outcome by improving glucose homeostasis and peripheral insulin sensitive previous to the promotion of weight loss (Eickhoff et al., 2015). The remodelling of GI tract by decreasing 80% of the stomach volume suggested an increase of incretins release (GLP1 and GIP (glucose-dependent insulinotropic polypeptide)) or at least an early action effect in the improvement of peripheral metabolism (Chambers et al., 2014; Eickhoff et al., 2015; Larraufie et al., 2019; Svane et al., 2019). Analogues of GLP1 such as liraglutide has been used for T2D treatment as it has shown to improve glucose homeostasis and insulin sensitivity as well it stimulates β-cell proliferation, differentiation and inhibits apoptosis (Bessesen & Van Gaal, 2018; Caporarello et al., 2017; Rask et al., 2001). Moreover, recent finding from our lab has shown that GLP1 improves insulin sensitivity in WAT as well as angiogenesis improving adipose tissue function by enhancing its vascular supply (Eickhoff et al., 2019; Rodrigues, 2020). Thus, to test this hypothesis of a possible crosstalk between dopamine and GLP1 we aim in the future to evaluate dopamine receptors expression in an T2D diet induced obesity animal model submitted to sleeve gastrectomy and on the other hand evaluate GLP1 receptor expression in animals treated with bromocriptine and its respective control groups.

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APENDIX

1. PhD work achievements: publications, awards, oral and posters communications

1.1 Publications:

The results of the first part of the work (first aim) were currently submitted and under revision on Frontiers in Pharmacology Journal.

- **"Dopamine, a key modulator of glucose uptake and lipid metabolism in insulin sensitive tissues in rats"** authored by Drs. Gabriela Tavares, Bernardete F. Melo, Fátima O. Martins, Paulo Matafome and Silvia V. Conde.

The results concerning the last two specific aims are currently published at Molecular Metabolism Journal. DOI: 10.1016/j.molmet.2021.101241

- **"Dopamine D2 receptor agonist, bromocriptine, remodels adipose tissue dopaminergic signalling and upregulates catabolic pathways, improving metabolic profile in type 2 diabetes"** authored by Drs. Gabriela Tavares, Daniela Marques, Cátia Barra, Daniela Rosendo-Silva, Ana Costa, Tiago Rodrigues, Patricia Gasparini, Bernardete F. Melo, Joana F. Sacramento, Raquel Seiça, Silvia V. Conde and Paulo Matafome.

1.2 Award:

- Best poster communication at 55th Annual Scientific Meeting of European Society for Clinical Investigation (ESCI), 9 to 11 of June.

  "Peripheral dopamine directly acts on insulin sensitive tissue to regulate insulin signalling and metabolic function".

1.3 Oral communications:

Gabriela Tavares, Daniela Rosendo Silva, Flavia Simões, Hans Eickhoff, Daniela Marques, Raquel Seiça, Sílvia V. Conde & Paulo Matafome
"Circulating dopamine is regulated by gut nutritional signals and acts in the adipose tissue to sensitize for GLP-1 action". Oral communication at 57th Annual Scientific Meeting of the European Association for the Study of Diabetes (EASD), Virtual meeting, 27 of September to 1st of October 2021.

Gabriela Tavares, Daniela Rosendo Silva, Flavia Simões, Hans Eickhoff, Daniela Marques, Raquel Seiça, Sílvia V. Conde & Paulo Matafome
"Circulating dopamine is regulated by gut nutritional signals and acts in the adipose tissue to sensitize for GLP-1 action". Oral communication at 55th Annual Scientific Meeting of European Society for Clinical Investigation (ESCI), virtual meeting, 9 to 11 of June 2021

Gabriela Tavares, Daniela Marques, Cátia Barra, Daniela Rosendo-Silva, Ana Costa, Tiago Rodrigues, Bernardete F. Melo, Fátima O. Martins, Raquel Seiça, Sílvia V. Conde & Paulo Matafome
"Peripheral dopamine directly acts on insulin sensitive tissue to regulate insulin signalling and metabolic function". Oral communication at 55th Annual Scientific Meeting of European Society for Clinical Investigation (ESCI), virtual meeting, 9 to 11 of June 2021.

Gabriela Tavares, Bernardete F. Melo, Fátima O. Martins, Daniela Marques, Cátia Barra, Daniela Rosendo-Silva, Sílvia V. Conde& Paulo Matafome
"A dopamina periférica atua diretamente nos tecidos sensíveis à insulina para regular a sinalização da insulina e a função metabólica". Oral communication at 17º Congresso Português de Diabetes, virtual meeting, 11 to 17 of March 2021

Gabriela Tavares, Bernardete F. Melo, Fátima O Martins, Paulo Matafome & Sílvia V. Conde
"A dopamina modula a captação de glucose nos tecidos sensíveis à insulina via recetores D1 e D2 e através da ativação de diferentes vias de sinalização".
Oral communication at 16º Congresso Português de Diabetes, Vilamoura, 6 to 8 of March, 2020.

Gabriela Tavares, Bernardete F. Melo, Fátima O Martins, Paulo Matafome & Sílvia V. Conde
"Tissue-specific modulation of glucose uptake and lipid metabolism by dopaminergic signalling". Oral communication at 50ª Reunião da Sociedade Portuguesa de Farmacologia, Coimbra, 5 to 7 of February, 2020.

1.4 Poster Presentations:

Gabriela Tavares, Bernardete F. Melo, Fátima O Martins, Paulo Matafome & Sílvia V. Conde
"Dopamine acts through distinct mechanisms in liver, adipose tissue and skeletal muscle regulating glucose uptake and insulin receptor and AMPK phosphorylation".
Poster communication at 56th Annual Scientific Meeting of the European Association for the Study of Diabetes (EASD), Virtual meeting, 21 to 26 of September 2020.

Gabriela Tavares, Daniela Marques, Ana Costa, Cátia Barra, Tiago Rodrigues, Joana Sacramento, Bernardete F. Melo, Raquel Seiça, Sílvia V. Conde & P. Matafome.
"Modulation of dopaminergic signaling with Bromocriptine improves glucose and lipid metabolism in an obese type 2 diabetic animal model".
Poster presentation at 55th Annual Scientific Meeting of the European Association for the Study of Diabetes (EASD), Barcelona, 16 to 20 of September 2019.

Gabriela Tavares, Daniela Marques, Ana Costa, Cátia Barra, Tiago Rodrigues, Joana Sacramento, Bernardete F. Melo, Raquel Seiça, Sílvia V. Conde & P. Matafome.
"Modulation of dopaminergic signalling with Bromocriptine improves glucose and lipid metabolism in white and brown adipose tissue in a type 2 diabetic obese animal model".
Poster presentation at 53rd Annual Scientific Meeting of the European Society for Clinical Investigation (ESCI), Coimbra, 22 to 24 of May 2019.

Gabriela Tavares, Daniela Marques, Ana Costa, Cátia Barra, Tiago Rodrigues, Joana Sacramento, Bernardete F. Melo, Raquel Seiça, Sílvia V. Conde & P. Matafome.
"**A modulação da sinalização dopaminérgica no tecido adipose branco e castanho pela Bromocriptina melhora o metabolismo da glucose e dos lípidos no modelo animal diabetes tipo 2 e obesidade".**
Poster presentation at 15º Congresso Português de Diabetes, Vilamoura, 8 to 10 of March, 2019

Original Article

MOLECULAR METABOLISM

Dopamine D2 receptor agonist, bromocriptine, remodels adipose tissue dopaminergic signalling and upregulates catabolic pathways, improving metabolic profile in type 2 diabetes

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ABSTRACT

Background and objectives: The therapeutic effects of the dopamine D2 receptor (D2R) agonist, bromocriptine, in type 2 diabetes (T2D) have been attributed to central nervous system actions. However, peripheral dopamine directly modulates glucose uptake in insulin-sensitive tissues and lipid metabolism in adipose tissue (AT). We hypothesized that the dopaminergic system may be impaired in the adipose tissue of patients with T2D and that the therapeutic actions of bromocriptine could involve the modulation of metabolism in this tissue.

Methods: The expression of dopamine receptors was evaluated in visceral AT samples from patients with obesity and stratified in several groups: insulin sensitive (IS); insulin resistance (IR) normoglycaemic; insulin resistant prediabetic; insulin resistant diabetic, according to Ox-HOMA2IR, fasting glycaemia and HbA1c levels. T2D Goto-Kakizaki rats (GK) were fed a high-caloric diet (HCD) for five months and treated with bromocriptine (10 mg/kg/day, i.p.) in the last month. The levels of dopaminergic system mediators and markers of insulin sensitivity and glucose and lipid metabolism were assessed in the peri-epididymal adipose tissue (pEWAT) and brown (BAT) adipose tissues, liver, and skeletal muscle.

Results: Patients with IR presented a decreasing trend of *DRD1* expression in the visceral adipose tissue, being correlated with the expression of *UCP1*, *PPARA*, and insulin receptor (*INSR*) independently of insulin resistance and body mass index. Although no differences were observed in *DRD2, DRD4* expression was significantly decreased in patients with prediabetes and T2D. In HCD-fed diabetic rats, bromocriptine increased D1R and tyrosine hydroxylase (TH) levels in pEWAT and the liver. Besides reducing adiposity, bromocriptine restored GLUT4 and PPARγ levels in pEWAT, as well as postprandial InsR activation and postabsorptive activation of lipid oxidation pathways. A reduction of liver fat, GLUT2 levels and postprandial InsR and AMPK activation in the liver was observed. Increased insulin sensitivity and GLUT4 levels in BAT and an improvement of the overall metabolic status were observed.

Conclusions: Bromocriptine treatment remodels adipose tissue and the liver dopaminergic system, with increased D1R and TH levels, resulting in higher insulin sensitivity and catabolic function. Such effects may be involved in bromocriptine therapeutic effects, given the impaired expression of dopamine receptors in the visceral adipose tissue of IR patients, as well as the correlation of D1R expression with InsR and metabolic mediators.

Keywords Type 2 diabetes; Obesity; Dopamine; Bromocriptine; D2 dopamine receptor; Adipose tissue; Liver

1. INTRODUCTION

Obesity, body mass index (BMI)>30 kg/m^2, is a major cause of morbidity and mortality, associated with an increased risk of metabolic syndrome and type 2 diabetes (T2D) [1,2]. Moreover, insulin resistance (IR) is a core feature of the metabolic syndrome, leading to the development of T2D, dramatically changing glucose and fatty acids metabolism [3]. However, the mechanisms of adipose tissue and liver insulin resistance are still unknown and under investigation.

The D2 receptor (D2R) agonist, bromocriptine, was approved by the Food and Drug Administration (FDA) for the treatment of T2D in USA, given its effects in improving glucose tolerance and insulin sensitivity [4].

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Abbreviations

AMPK	AMP-activated protein kinase
AMPK-Thr172	Phosphorylated AMP-activated protein kinase on 172 threonine residue
BAT	Brown adipose tissue
DARPP 32	Dopamine- and cAMP-regulated neuronal phosphoprotein
DRD1	Gene encoding for human dopamine receptor 1
DRD2	Gene encoding for human dopamine receptor 2
DRD4	Gene encoding for human dopamine receptor 4
D1R	Dopamine receptor 1
D2R	Dopamine receptor 2
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
HCD	High caloric diet
INSR	Gene encoding for human insulin receptor
InsR	Insulin receptor
InsR-Tyr1361	Phosphorylated insulin receptor on 1361 tyrosine residue
IR	Insulin resistance
IS	Insulin sensitive
T2D	Type 2 Diabetes
TH	Tyrosine hydroxylase
pEWAT	peri-epididymal adipose tissue
PPARA	Gene encoding for human peroxisome proliferator-activated receptor alfa
PPARγ	Peroxisome proliferator-activated receptor gamma
UCP1	Gene encoding for human uncoupling protein 1
VAT	Visceral adipose tissue

Bromocriptine-QR appears to be a unique insulin sensitizing therapy with a good safety profile, without risk of weight gain or hypoglycaemia and with a potential to reduce adverse cardiovascular risk [4,5]. Moreover, it also presents important pharmacological advantages, such as lower side effects, safety, tolerability, and good adherence to treatment [5–7]. Bromocriptine acts on pancreatic beta cells to downregulate glucose-stimulated insulin secretion (GSIS), avoiding long-term beta cell exhaustion [8]. On the other hand, it also has positive effects on insulin sensitivity, which have been attributed to the regulation of the hypothalamic sympathetic output and prolactin secretion [6,9]. Nevertheless, the existence of functional dopamine receptors in adipose tissue suggests that this drug may exert direct regulatory functions in this tissue [10]. Although catecholamines have well-known lipolytic effects in the adipose tissue, recent evidences support the theory that bromocriptine also acts on adipose tissue to modulate insulin action and glucose and fatty acid metabolism [11]. Accordingly, we have recently observed that peripheral dopamine stimulates glucose uptake *in vivo* in insulin-sensitive tissues, such as the adipose tissue, liver and skeletal muscle, and bromocriptine directly acts in the same tissues to regulate, not only glucose uptake, but also insulin sensitivity and metabolic function [12]. Despite these direct and new effects of bromocriptine that we described, the effects of chronic bromocriptine stimulation in such tissues have never been addressed before, as well as the role of local dopaminergic signalling. Therefore, revealing bromocriptine peripheral actions on insulin-sensitive tissues may enable to identify new therapeutic targets and strategies in T2D.

Given the current knowledge of direct dopamine effects in adipose tissue, in this study, our first objective was to evaluate the expression of dopamine receptors in the visceral adipose tissue of patients with metabolic syndrome and correlate it with markers of adipocyte metabolism. Additionally, our second aim was to investigate the effects of chronic bromocriptine treatment in an animal model of T2D fed a hypercaloric diet (HCD) in remodelling adipose tissue dopaminergic signalling, insulin sensitivity, and metabolic function.

2. RESEARCH DESIGN AND METHODS

2.1. Human study

A cohort of obese patients aged 25–65 years old (diabetic and non-diabetic) was selected at the obesity surgery appointment at the Hospital Geral de Coimbra (Covões) - Centro Hospitalar Universitário de Coimbra. All subjects signed an informed consent and the study was approved by the institutional ethics committee (Ethics Committee of the Coimbra University Hospital Centre), according to the principles outlined in the Declaration of Helsinki. Exclusion criteria were as follows: active inflammatory and chronic diseases (neurodegenerative diseases or active tumours), previous restrictive (sleeve gastrectomy) or mal-absorptive (gastric bypass or duodenal switch) surgeries and T2D medication other than metformin (GLP-1RA, DPPIV inhibitors or insulin). On the day before surgery, height and body weight were recorded and fasting blood samples were collected for biochemical analysis. Visceral white adipose tissue samples were collected during surgery and kept in liquid nitrogen to be then stored at - 80 °C.

2.2. Patient selection and characterization

Ninety-two obese patients (77 women and 15 men) were divided in groups according to glycaemic profile: fasting glucose levels, HbA1c, and Ox-HOMA2IR. Subject characterization resulted in four different groups: 1 – insulin sensitive group (IS) ($n = 17$), composed by individuals that were both IS and normoglycaemic (NG) (Ox-HOMA2IR < 1); 2 – insulin resistant (IR) and NG group ($n = 29$), with insulin resistant patients (Ox-HOMA2IR>1) that were normoglycaemic (fasting glucose$<$100 mg/dL and HbA1c$<$5.7%); 3 – prediabetic group ($n = 28$), that allocated IR patients with fasting glucose levels from 100 to 125 mg/dL or HbA1c between 5.7 and 6.4%; 4 – T2D group ($n = 18$), constituted by IR subjects diagnosed with T2D (fasting blood glucose$>$125 mg/dL or HbA1c$>$6.4%) (Supplementary Table 1) [13].

2.3. RNA extraction

Total RNA was extracted from VAT human biopsies (100 mg) using an RNeasy Lipid Tissue Mini Kit (Qiagen, Germany). RNA samples were analysed by NanoDrop One/One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 260 nm to evaluate its concentration. RNA integrity was also analysed through capillary electrophoresis with an Agilent RNA 6000 Nano Kit and the results obtained with the Agilent 2100 Bioanalyser (Agilent Technologies, CA, USA).

2.4. Quantitative real-time polymerase chain reaction using the high throughput platform biomark$_{tm}$ HD system

RNA sample was diluted into the same concentration (25 ng/μL). Reverse transcriptase enzyme [qScript cDNA super mix (Quanta BioSciences)] was added to each RNA sample to obtain cDNA. To amplify cDNA samples, they were mixed with a mix of pooled primers (500 nM final concentration each), with a PreAmp Master Mix enzyme (Fluidigm), following 12 cycles of thermal cycling according to the enzyme manufacturer. Samples were then treated with Exonuclease I (New

England Biolabs) to remove unincorporated primers and were diluted 5 × in TE buffer (10 mM Tris—HCl, 1 mM EDTA). For each sample a Pre-Mix was prepared (SsoFast Eva Green Supermix, BioRad), with DNA binding dye sample reagent at 20 × (Fluidigm). Thereafter, samples were pipetted into the respective inlet of a Fluidigm® 96.96 Gene expression IFC. For each gene assay, a mix with 2 × Assay loading reagent (Fluidigm), forward and reverse primers (50 μM stock) diluted TE buffer was individually prepared. The primers sequences were: *DRD1* forward- ACACAATTAACTCCGTTTCC and reverse- GTAGTGTCCCTGTTTGATTG; *DRD2* forward- TCCACTAAAGGGCAACTG and reverse- GGAAACTCCCATTAGACTTC; *DRD4* forward- CATCTACACTGTCTTCAACG and reverse- ATTAACGTACAAAAGCGCC; *UCP-1* forward- ACAGCACCTAGTTTAGGAAG and reverse- CTGTACGCATTATAAGTCCC; *PPARA* forward- CCTAAAAAGCCTAAGGAAACC and reverse- GATCTCCACAGCAAATGATAG; and *INSR* forward- GATCCAATCTCAGTGTCTAAC and reverse- CCTTTGAGGCAATAATCCAG, obtained by Sigma Aldrich, (USA) and reconstituted in water to a final concentration of 100 μM. Then each gene assay was pipetted into their respective assay inlets on the chip and loaded with the Load mix (136 ×) script of the HX controller (HD Biomark), using the BioMark HDTM, accordingly to the cycling parameters recommended by Fluidigm® for 96.96 Gene expression Integrated Fluidic Circuit. Then data were collected with Data Collection Software and analysed using Fluidigm® Real Time PCR Analysis v2.1 software. All data were normalized for the reference gene *ATCB*.

2.5. Animal study

Wistar rats from our breeding colonies (Faculty of Medicine, University of Coimbra) were kept under standard conditions [13,14]. The experimental protocol was approved by the local Institutional Animal Care and Use Committee (ORBEA 04—2015), 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). At 1 month of age, type 2 diabetic GK rats were divided into two groups; the first group was maintained with the same standard diet until 6 months of age (A03; 5% triglycerides and 45% carbohydrates, SAFE, France) (GK group, n = 33), while the other group was fed a high-caloric diet (HCD) in the same period (customized A03 HCD with 20% fat plus 20% sucrose, SAFE, France) to induce weight gain. The HCD-fed group was randomly divided into three groups: the first group without further treatment apart from the diet (GKHCD, n = 20), the second group with bromocriptine treatment in the last month (GKHCDBr, n = 17), and the third group with vehicle administration during the same period (GKHCDVh, n = 15). Male Wistar rats fed with a standard diet for 6 months were used as control (n = 22). Bromocriptine gently supplied by Generis®, (Amadora, Portugal) was diluted 1:4 DMSO/H_2O and administered daily via intraperitoneal (i.p) injection (10 mg/kg/day) during the last month. In the vehicle group, the same volume (100 μL) of the vehicle 1:4 DMSO/H_2O was administered i.p. during the same period.

2.6. Insulin tolerance teste

Two days prior to the sacrifice, an intraperitoneal insulin tolerance test (IPITT) was performed. Fasted (6 h) animals were injected with insulin (0.25 U/kg, Lilly, Portugal) and glucose was measured before and after 15, 30, 60, and 120 min using a Glucometer (Bayer, Germany). The area under the curve was then calculated.

2.7. Blood sampling and tissue collection

On the day of the sacrifice, fasting (6 h) glycaemia, triglyceridemia, and cholesterol levels were measured in the blood sample obtained from the tail vein by using a Glucometer (Bayer, Germany) and the Accutrend system (Roche, Germany) with the respective reactive test stipes. Before sacrifice, the animals were divided into 2 subgroups, those sacrificed after 6 h fasting — "postabsorptive" (WSD = 15, GKSD = 21, GKHCD = 13, GKHCDBr = 11, GKHCDVh = 9) and those sacrificed 1 h after a mixed meal ingestion — "post-prandial" (WSD = 7, GKSD = 12, GKHCD = 7, GKHCDBr = 6, GKHCDVh = 6). Then, the animals were anesthetized with ketamine chloride (75 mg/kg, Nimatek, Dechra, UK) and chlorpromazine chloride (2.65 mg/kg, Lab. Vitória, Portugal).

In the set of animal sacrificed after 6 h fasting, plasma, and serum samples were collected by cardiac puncture as previously described [13,15] for further evaluation of insulin, glucagon, leptin, adiponectin, and free fatty acid levels. The animals were sacrificed by cervical displacement, and peri-epididymal adipose tissue (pEWAT), brown adipose tissue (BAT), muscle, and the liver were collected, weighted and stored in 4% formalin solution or at −80 °C. In the set of animals sacrificed after a mixed meal, 3 mL of Nutricia, Fortimel (Nestle, Switzerland) was administered through gavage and animals were sacrificed 1 h later for tissue collection.

2.8. Western blotting

Tissues were homogenized as previously described [13,14] and samples were loaded in 8% polyacrylamide gels, separated by SDS-PAGE and transferred to a PVDF membrane (Advansta, USA). Membranes were incubated with the specific primary antibodies overnight at 4 °C (listed below), and then incubated 2 h at room temperature with secondary antibodies. The secondary antibodies were anti-mouse (GE Healthcare, UK), anti-rabbit and anti-goat (Bio-Rad, USA). Membranes were revealed using ECL substrate in a Versadoc system (Bio-Rad, USA) and analysed with Image Quant® (Molecular Dynamics, USA).

2.9. Reagents, ELISA kits and antibodies

Salts and organic solvents used in solution preparations were purchased from Thermo Fisher Scientific (Leicestershire, UK), Sigma Chemicals (United States of America - USA) or Merck Darmstad (Germany), with the highest grade of purity commercially available. Antibodies used were AMPK (#2532), AMPK-Thr172 (#2535 GLUT 4, PPARγ (#2213S and #2443S, Cell Signaling, USA), InsRβ (sc-57342, Santa Cruz Biotechnology, USA), GLUT2 (ab54460) and InsR-Tyr1361 (ab60946, Abcam, UK). Antibodies D1R, D2R, DARPP32, DARPP32-Thr34 and tyrosine hydroxylase (TH) were also used (ab81296, ab85367, ab40801 and ab51114 respectively, Abcam, UK; T1299, Sigma Aldrich, USA). Calnexin was used as loading control (AB0037, Sicgen, Portugal). Plasma insulin levels were assessed through the Rat Insulin ELISA Kit, (Mercodia, Sweden), plasma glucagon using the ELISA Kit (Wako, Germany), and plasma levels of leptin and adiponectin were measured using the Mouse/Rat Leptin Quantikine ELISA Kit and Rat Total Adiponectin/Acrp30 Immunoassay (R&D system, USA). Free fatty acids were assessed using the FFA Assay Kit (ZenBio, NC, USA). Furthermore, 100 mg of liver samples were homogenised in the lysis buffer (1 mL of 5% NP-40/ddH_2O solution) and hepatic triglycerides content was quantified by the colorimetric Triglyceride Quantification Assay Kit (Abcam 65336, UK). Moreover, 30 mg of pEWAT were homogenised in PBS 0.02 M, pH 7.2 (BAM R61, FDA) to quantify β-hydroxybutyrate by a colorimetric assay (Biovison K632-100, USA).

2.10. Statistical analysis

Non-parametric tests were performed on the clinical human data (sample size < 30/group), and results were presented as median and interquartile range. Kruskal–Wallis and Mann–Whitney tests were used to compare gene expression between groups according to its division. Spearman correlation test was performed to assess correlation between variables. In the animal study, results were presented as mean $\pm$ standard deviation. One-way ANOVA test with Tukey's multiple comparisons was used to performed systemic parameters analysis and total proteins quantification. For phosphorylated proteins, Kruskal–Wallis test (all pairwise multiple comparisons) was applied to determine statistical differences between the groups. Differences were considered significant at $p < 0.05$. All computation analyses were performed using Graphpad Prism (6.0 version, USA).

3. RESULTS

3.1. Expression of dopamine receptors is impaired in the visceral adipose tissue of patients with insulin resistance

Patients included in the diabetic group (fasting blood glucose>125 mg/dL or HbA1c>6.4%), were previously shown to have a failure of beta-cell function, and lower levels of high density lipoprotein cholesterol and adiponectin, markers of adipose tissue dysfunction [13]. Detailed clinical characterization of the groups is described in Supplementary

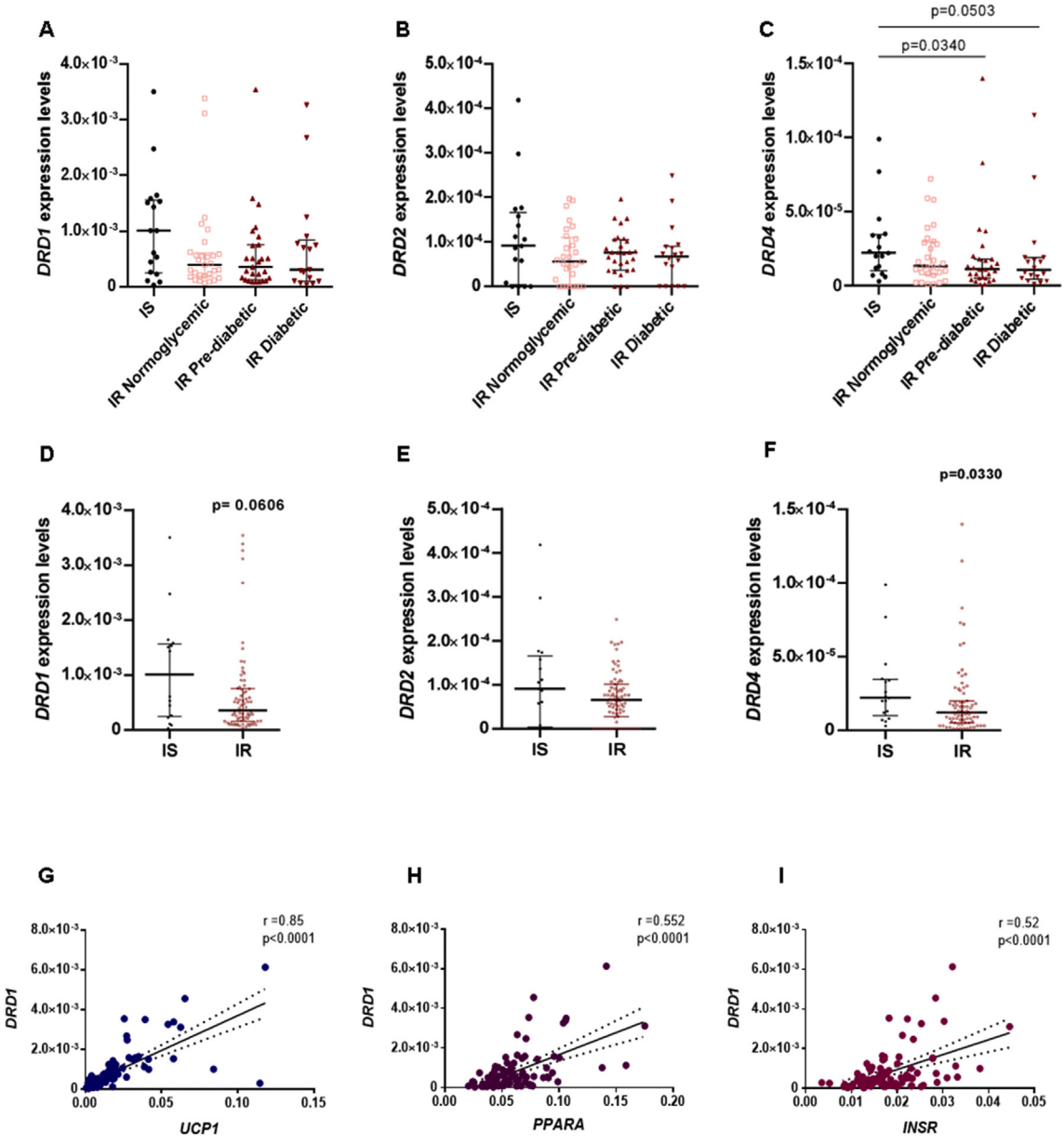

Figure 1: Expression of dopamine receptor in visceral adipose tissue (VAT) of patients with metabolic syndrome. Upper line shows VAT expression of DRD1 (A), DRD2 (B) and DRD4 (C) in each group of patients: insulin-sensitive (IS), and insulin resistant (IR) normoglycemic, prediabetic, and type 2 diabetic patients. All IR patients were grouped and compared with IS patients for DRD1 (D), DRD2 (E) and DRD4 (F). Bottom line shows the correlations between DRD1 expression and UCP-1(G) and PPARA (H) and the insulin receptor INSR (I). Values are median $\pm$ interquartile range. Kruskal–Wallis and Mann–Whitney tests were used to analyse gene expression data according to the patient's group. Spearman's correlation test was used to performed correlation analysis.

Table 1. Here, we investigated the gene expression of dopamine receptors in the adipose tissue of these patients and correlated their expression with markers of adipocyte metabolic function. Expression of *DRD1*, *DRD2* and *DRD4* was evaluated in the four groups of patients, showing decreased *DRD4* expression in patients with prediabetes and T2D when compared with IS patients ($p < 0.05$, Figure 1C). No significant differences were observed for *DRD1* and *DRD2* expression (Figure 1A–C), and no expression was detected for *DRD3* and *DRD5*. Importantly, *DRD1* expression was 10-fold higher than that of the other dopamine receptors (Figure 1A). In order to evaluate the relation between insulin resistance (IR) and dopamine receptor expression, all IR patients (groups 2, 3, and 4) were compared to IS patients, as depicted in Figure 1 (D, E, F). Decreased *DRD4* expression in patients with IR was also observed when comparing to IS patients (Figure 1F, $p < 0.05$). Despite no differences were observed for *DRD2* expression, a trend to decreased *DRD1* expression ($p = 0.06$) was observed in IR patients when compared to IS individuals. In order to understand the possible involvement of dopamine receptors in regulating adipocyte metabolism, the correlation between *DRD1* expression with *INSR* and genes involved in lipid metabolism was determined. Figure 1 shows a positive correlation between *DRD1* expression and *UCP-1* ($p < 0.001$, $\rho = 0.85$) (G), *PPARA* ($p < 0.001$, $\rho = 0.55$) (H) and *INSR* expression ($p < 0.001$, $\rho = 0.52$) (I). Importantly, the expression of *UCP-1*, *PPARA* and *INSR* was also decreased in diabetic patients, suggesting that the impairment of adipose tissue dopaminergic signalling is correlated with impaired insulin sensitivity and metabolic function (data not shown). Multiple regression analysis has shown that the correlations of D1DR with UCP-1, PPAR and INSR are independent of IR and BMI (Supplementary Fig. 1).

3.2. Bromocriptine treatment increases D1R expression in pEWAT and the liver in an animal model of T2D and obesity

Although previous evidences indicate the central actions of bromocriptine, recent findings suggest a direct action of dopamine and bromocriptine in peripheral insulin-sensitive tissues [12]. In order to understand the role of bromocriptine treatment in regulating dopaminergic signalling in insulin-sensitive tissues, the protein levels of D1R, D2R, the downstream effector DARPP32 and tyrosine hydroxylase (TH) were determined in pEWAT, the liver, BAT and soleus muscle. Figure 2A shows decreased D1R levels in pEWAT of GK rats feeding a standard or a HCD (GKHCD) ($p < 0.05$), similarly to what is observed in human adipose tissue in Figure 1. Bromocriptine treatment significantly improved D1R levels ($p < 0.01$ vs GKSD and vs GKHCD) to levels similar to that in Wistar control animals. TH levels were also increased in treated animals, suggesting higher local dopamine production (Figure 2C, $p < 0.05$ vs GK and GKHCD). Similarly, in the liver, D1R (Figure 2D, $p < 0.05$ vs GK and GKHCD) and TH levels (2F, $p < 0.05$ vs GK, GKHCD and GKHCDVh) were increased after bromocriptine treatment. On the other hand, D2R and DARPP32 levels were not altered among groups in both tissues (Figure 2B,E, representative western blot images in the right panel). No significant changes in dopaminergic receptors and TH levels were observed in BAT and skeletal muscle (representative western blot images at Figure 2 G and H).

3.3. Bromocriptine increases insulin sensitivity and drives metabolic pathways towards lipid oxidation in pEWAT

In order to understand the impact of dopaminergic system remodelling induced by bromocriptine in pEWAT, markers for the endocrine and metabolic function of adipose tissue were evaluated. As shown in Figure 3, HCD-fed GK rats (GKHCD and GKHCDVh) exhibited increased epididymal fat mass (Figure 3B) and adipocyte volume (Figure 3C) compared to GK rats fed a standard diet (GKSD, $p < 0.01$). Bromocriptine significantly decreased epididymal fat mass (Figure 3B) ($p < 0.05$ vs GKHCD and $p < 0.01$ vs GKHCDVh) and adipocyte size (Figure 3C) ($p < 0.05$ vs GKDS, $p < 0.0001$ vs GKHCD and GKHCDVh). Representative haematoxylin-eosin images are shown in Figure 3A and it is clear from the images the effect of bromocriptine in decreasing adipocyte size. Decreased adiposity in GKHCDBr rats was accompanied by lower plasma leptin levels (Figure 3D, $p < 0.001$ vs GKHCD and $p < 0.0001$ vs GKHCDVh), while adiponectinemia remained unchanged between groups (Figure 3E) (see Figure 4).

Concerning insulin sensitivity and metabolic pathways, bromocriptine treatment increased GLUT4 (Figure 3F, $p < 0.001$ vs WSD, GKSD, GKHCD and GKHCDVh), insulin receptor (InsR) (Figure 3G, $p < 0.001$ vs GKSD and $p < 0.0001$ vs GKHCD) and PPARy (Figure 3I, $p < 0.001$ vs GKSD, $p < 0.001$ both vs GKHCD and GKHCDVh), while no differences were observed in total AMPK levels between groups (Figure 3H). The activation of the InsR and AMPK was determined in postabsorptive and post-prandial states. In the postabsorptive stage (6 h fasting), no differences were found between groups regarding InsR activation (IR-Tyr1361, Figure 3J). Nevertheless, increased levels of AMPK-Thr172 (Figure 3K, $p < 0.05$ vs GKSD) and a trend to increase ATP citrate lyase (ACL-Ser455, Figure 3L, $p = 0.056$ vs GKSD) were found in pEWAT after bromocriptine treatment. Such results suggest increased catabolic activity, and particularly lipid oxidation, which was confirmed by the increase in β-hydroxybutyrate levels in pEWAT ($p < 0.01$ vs WSD, $p < 0.001$ vs GKSD, $p < 0.01$ vs GKHCD and $p < 0.05$ vs GKHCDVh) by bromocriptine treatment (Figure 3M), while no differences were found in the serum (data not shown). Increased catabolic activity is thought to reduce lipotoxicity and IR. Accordingly, in the post-prandial state, bromocriptine increased IR-Tyr1361 levels (Figure 3N, $p < 0.05$ vs GKHCD), while AMPK-Thr172 levels remained similar to the other groups (Figure 3O).

3.4. Bromocriptine reduces liver triglyceride content without activating liver AMPK pathway

Figure 4A shows liver haematoxylin-eosin staining for the different studied groups. A high amount of lipid droplets consistent with hepatic steatosis phenotype was observed in animals fed with a HCD (Figure 4A, GKHCD and GKHCDVh), consistent with the observed increase in liver weight (Figure 4B, $p < 0.001$ vs WSD and GKSD) and triglyceride content (Figure 4C, $p < 0.001$ vs WSD and GKSD). Bromocriptine notably reduced the presence of lipid droplets in the haematoxylin-eosin staining and reversed hepatic steatosis (Figure 4A), which was accompanied by decreased liver weight (Figure 4B, $p < 0.001$ vs GKHCD) and hepatic triglycerides (Figure 4C, $p < 0.01$ vs GKHCD and GKHCDVh).

In order to understand the role of bromocriptine in the regulation of hepatic metabolic function, InsR and AMPK activation, as well as GLUT2 levels were determined. Animals treated with bromocriptine showed increased total InsR levels (Figure 4E, $p < 0.05$ vs GKSD $p < 0.01$ vs GKHCD) but no changes in AMPK (Figure 4F). Intriguingly, lower levels of GLUT2 were also observed after bromocriptine treatment (Figure 4D, $p < 0.001$ vs WSD, $p < 0.01$ vs GKSD and $p < 0.01$ vs both GKHCD and GKHCDVh), as well as lower InsR-Tyr1361 (Figure 4I, $p < 0.05$ vs GKHCD) and tendentially lower AMPK-Thr172 levels (Figure 4J, $p = 0.07$ vs GKSD) in the post-prandial state. No differences were observed in InsR-Tyr1361 and AMPK-Thr172 levels in the postabsorptive state (Figure 4G,H).

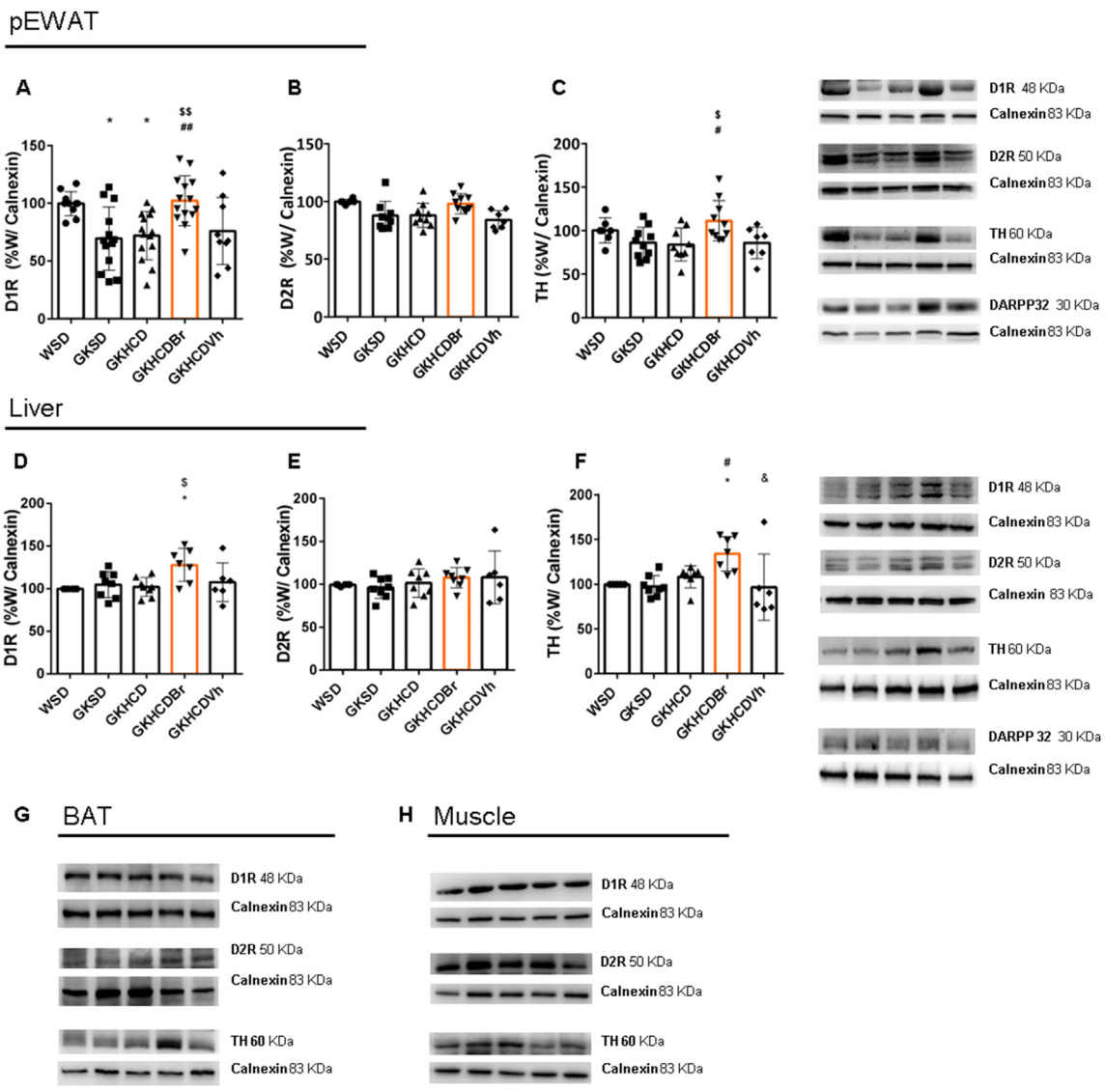

Figure 2: Alterations of dopaminergic signalling in peri-epididymal adipose tissue (pEWAT) and liver after bromocriptine treatment in an animal model of type 2 diabetes and obesity. Increased dopamine D1 receptor (D1R) (A) and tyrosine hydroxylase (TH) (C) in the pEWAT, while no differences were observed for dopamine D2 receptor (D2R) (B). Similar results were observed for D1R (D), D2R (E) and TH (F) in the liver. Representative western blot images are shown in the right panel. The bottom line shows representative western blot images from dopaminergic signalling in the brown adipose tissue (BAT) (G) and the skeletal muscle (H), where no differences were found between groups. Bars represent means ± standard deviation. Differences between groups were assessed by one-way ANOVA with Tukey's multiple comparisons. * Different from WSD; # different from GKSD; $ different from GKHCD; & different from GKHCDBr. *$p < 0,05$; **$p < 0,01$; ***$p < 0.001$.

3.5. Bromocriptine increased fasting insulin signalling in brown adipose tissue but has no effects in the skeletal muscle

Although bromocriptine did not change the BAT dopaminergic system, increased GLUT4 levels were observed after the treatment (Figure 5A, $p < 0.001$ vs WSD, $p < 0.01$ vs GKSD, $p < 0.001$ vs GKHCD and $p < 0.05$ vs GKHCDVh), while both total levels of IR and AMPK remained similar between groups (Figure 5B,C). Furthermore, bromocriptine also increased postabsorptive levels of InsR-Tyr1361 (Figure 5D $p < 0.05$ vs GKHCD), but not AMPK-Thr172 (Figure 5E). Animals sacrificed at the post-prandial stage presented similar levels of InsR-Tyr1361 (Figure 5F) and AMPK-Thr172 (Figure 5G) between groups. In the skeletal muscle, GLUT4, InsR, and AMPK levels did not change between groups (Figure 5H–J). Moreover, either postabsorptive or post-prandial InsR-Tyr1361 and AMPK-Thr172 levels also remained similar between groups (Figure 5K–N).

3.6. Improved adipose tissue insulin sensitivity and metabolic function after bromocriptine treatment is associated with better metabolic profile

In accordance with previous reports from our laboratory [16], GK rats naturally present lower body weight than Wistar rats (Figure 6A and 6A1; $p < 0.001$). HCD-fed GK rats showed higher weight gain in the last month (Figure 6B $p < 0.05$ vs GKSD), which was associated with increased caloric intake during the same period of time (Figure 6C $p < 0.001$, WSD vs GKHCD and GKSD vs GKHCD; $p < 0.01$ WSD vs and $p < 0.001$ GKSD vs both GKHCD and GKHCDVh). On the

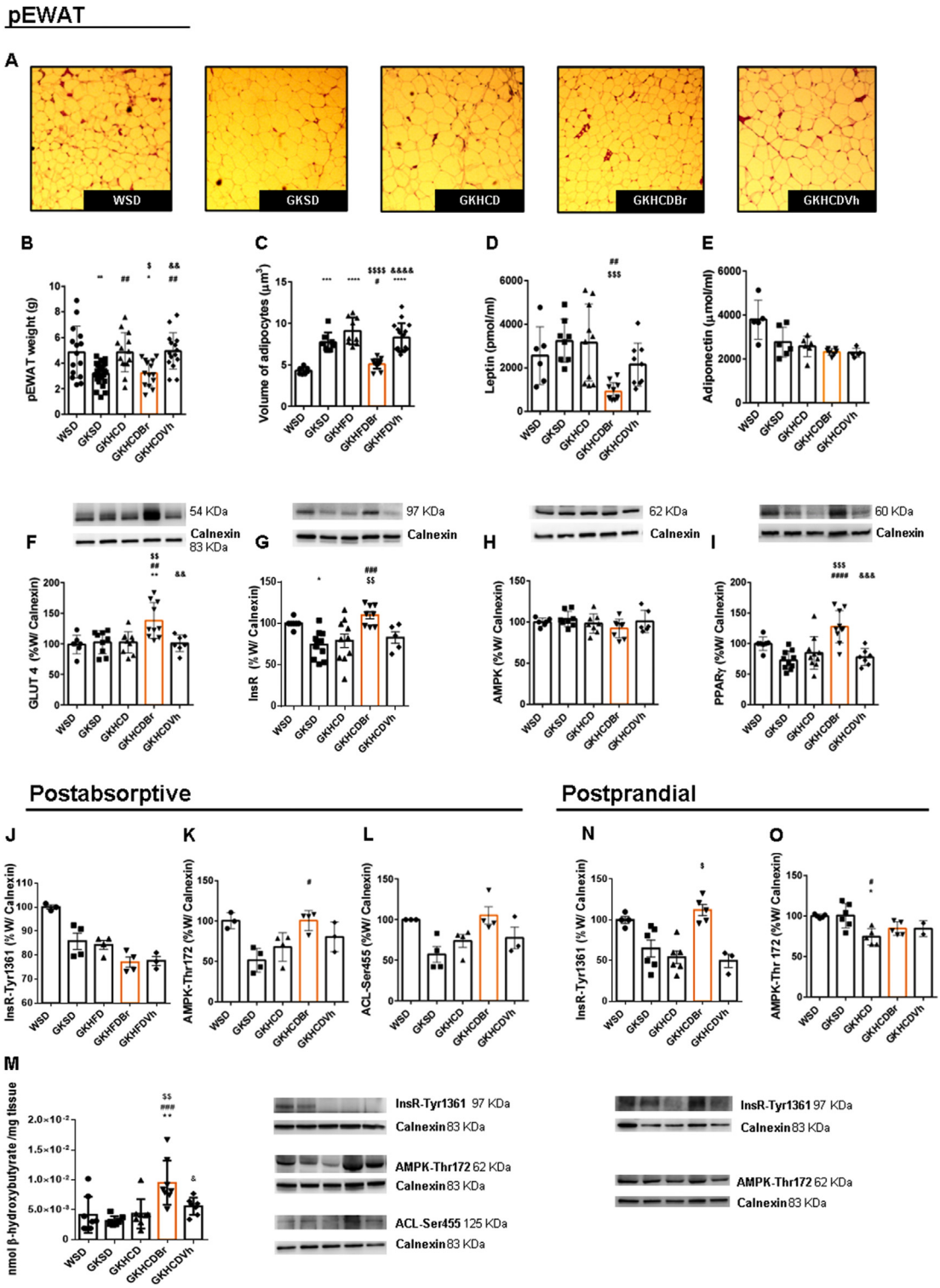

Figure 3: Improvement of insulin sensitivity and remodelling of adipose tissue catabolic pathways by Bromocriptine. Haematoxylin-eosin staining (A, 100 ×) of peri-epididymal adipose tissue (pEWAT). Bromocriptine treatment decreased pEWAT weight (B), adipocytes volume (C) and plasma leptin levels (D), while no changes were observed for plasma adiponectin levels (E). Bromocriptine treatment increased glucose 4 transporter (GLUT4) (F), insulin receptor (InsR) (G) and Peroxisome proliferator-activated receptor gamma (PPARγ, H), while AMP-activated protein kinase (AMPK, I) remained similar between groups. In the bottom panel, no changes were observed in postabsorptive activation of InsR (InsR-Tyr1361, J), but Bromocriptine increased AMPK-Thr172 (K), ACL-Ser455 (L) and β-hydroxybutyrate levels (M). In the post-prandial stage, the InsR was more activated (, N), while no differences were found AMPK activation (O) in Bromocriptine-treated rats. Bars show means ± standard deviation. One-way ANOVA with Tukey's multiple comparisons was used to assess differences between groups. Kruskal–Wallis test was used to analyse InsR-Tyr1361 and AMPK-Thr172. * Different from WSD; # different from GKSD; $ different from GKHCD; & different from GKHCDBr. Level of significance: *p < 0,05; **p < 0,01; ***p < 0.001.

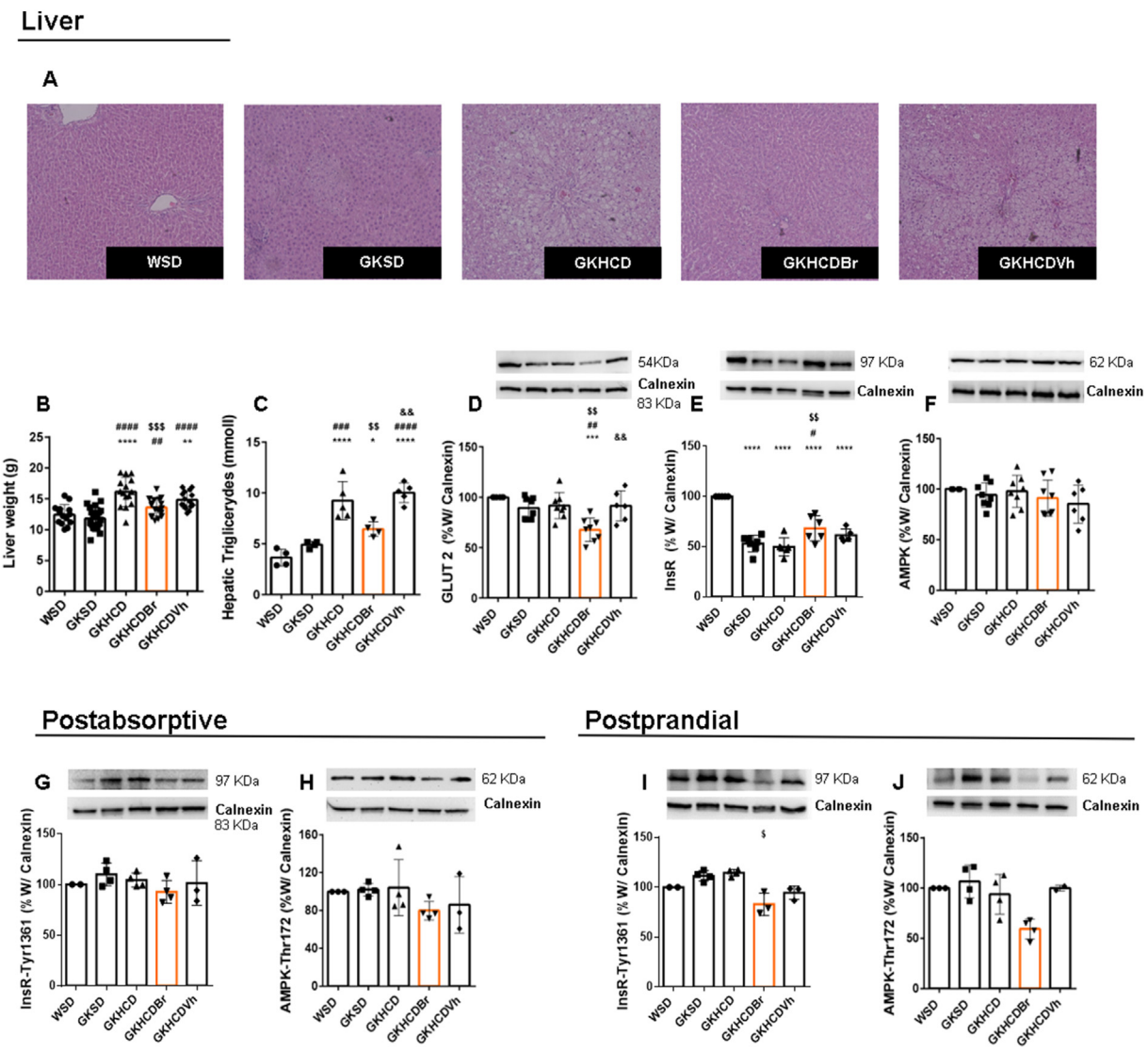

Figure 4: Bromocriptine reduces liver triglycerides content without activating liver AMPK pathway. Liver haematoxylin-eosin staining (A, 100 ×) showing a reduction of hepatic steatosis after bromocriptine treatment, in accordance with lower liver weight (B) and hepatic triglycerides (C). Bromocriptine treatment decreased GLUT2 (D), but increased InsR (E), while AMPK (F) levels did not alter between groups. No changes were observed in InsR-Tyr1361 (G) nor AMPK-Thr172 (H) in the postabsorptive stage. Postprandially lower levels of InsR-Tyr1361 (I) and no changes in AMPK-Thr172 (J) were observed. Bars represent means ± standard deviation. One-way ANOVA was used for liver weight, hepatic triglycerides, GLUT2, InsR and AMPK; Kruskal–Wallis test was used for InsR-Tyr1361 and AMPK-Thr172. *Different from WSD; # different from GKSD; $ different from GKHCD; & different from GKHCDBr. Level of significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

other hand, bromocriptine treatment has been shown to prevent weight gain (Figure 6A2 and 6B, $p < 0.001$ vs GKHCD and $p < 0.05$ vs GKHCDVh) and decrease the caloric intake (Figure 6C, $p < 0.0001$ vs GKHCD and GKHCDVh).

The increased body weight and caloric intake in HCD-fed rats was followed by increased fasting plasma triglycerides (Figure 6D, $p < 0.0001$, WSD vs GKHCD and $p < 0.001$ WSD vs GKHCDVh; $p < 0.0001$, GKSD vs both GKHCD and GKHCDVh) and total cholesterol levels (Figure 6E $p < 0.0001$ WSD vs GKHCD and $p < 0.0001$ GKSD vs GKHCD). Interestingly, bromocriptine treatment improved plasma lipid profile by decreasing both plasma triglycerides (Figure 6D $p < 0.001$ vs GKHCD and $p < 0.05$ vs GKHCDVh) and cholesterol levels (Figure 6E $p < 0.001$ vs GKHCD), but no significant changes were observed for plasma free fatty acids (Figure 6F). Such results are in accordance with the lower fat accumulation observed in the liver and pEWAT as well as with increased pEWAT lipid oxidation, as depicted in Figures 3 and 4.

GK rats are described as a model of glucose intolerance and mild fasting hyperglycaemia [16], which was now confirmed by increased glycaemia after 6 h fasting and AUC during the IPITT as presented at Figure 6G,H respectively (Figure 6G $p < 0.0001$ WSD vs GKSD; Figure 6H $p < 0.05$ WSD vs GKSD). The HCD further aggravated such phenotype, by increasing fasting glycaemia (Figure 6G $p < 0.0001$ WSD vs GKHCD and $p < 0.001$ vs GKHCDVh), AUC during the IPITT (Figure 6H $p < 0.0001$ WSD vs GKHCD and $p < 0.001$ vs GKHCDVh) and glycaemia at 1 h during the IPITT (Figure 6I $p < 0.0001$ WSD vs GKHCD and $p < 0.05$ vs GKHCDVh). Conversely, bromocriptine treatment improved glycaemic control by decreasing 6 h fasting glycaemia (Figure 6G) in relation to GKHCD group ($p < 0.01$) and reducing the difference to Wistar rats

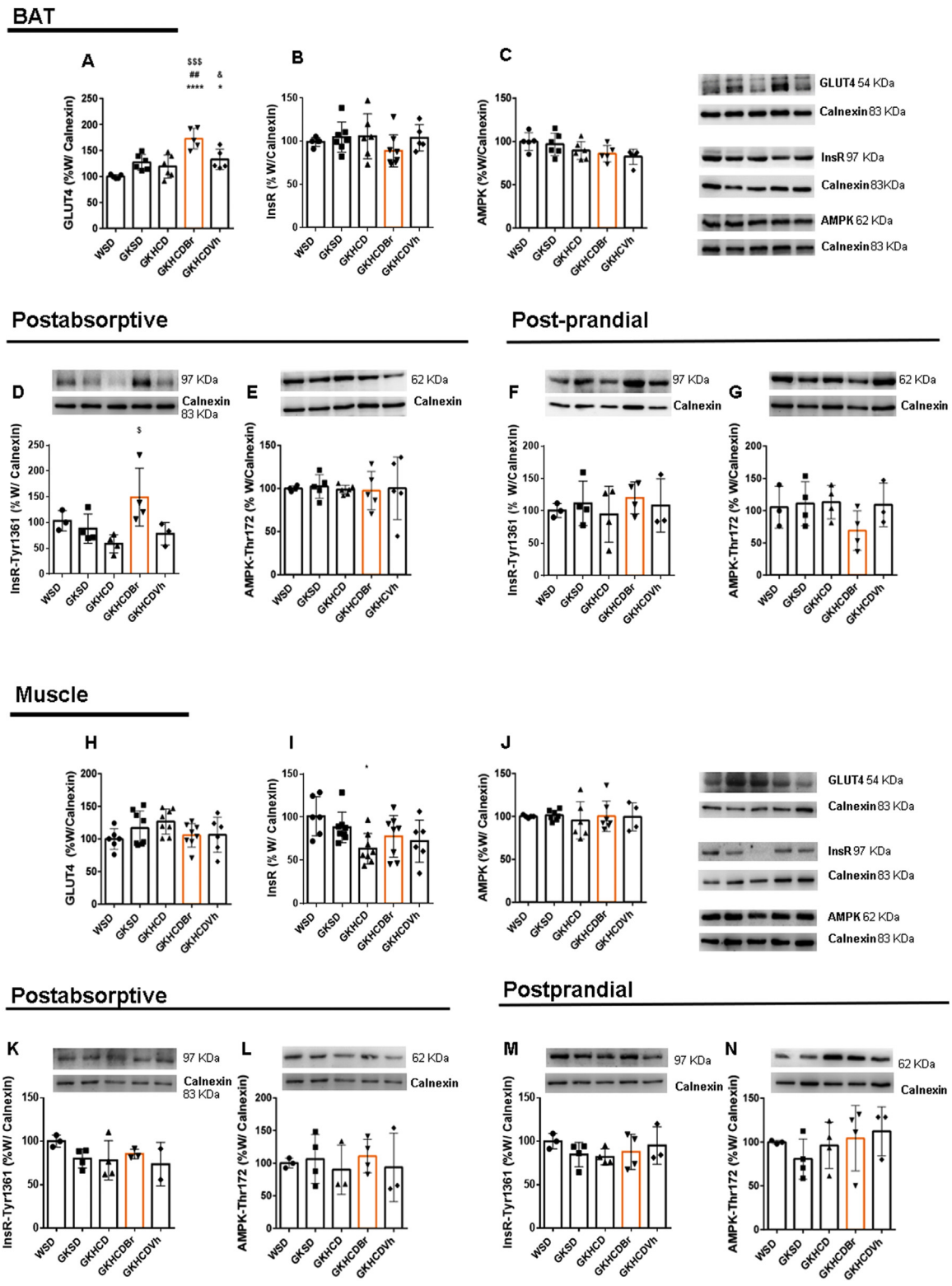

Figure 5: Bromocriptine increased fasting insulin signalling in brown adipose tissue but has no effects in the skeletal muscle. Bromocriptine treatment increased levels of GLUT4 (A) in BAT, while neither InsR (B) nor AMPK (C) levels changed. In the postabsorptive stage increased InsR-Tyr172 (D) after bromocriptine treatment, but no differences were observed for postabsorptive AMPK-Thr172 (E), nor postprandial InsR-Tyr172 (F) and AMPK-Thr172 (G). In the skeletal muscle, no significant changes were observed for GLUT4 (H), InsR (I) total AMPK (J), nor InsR and AMPK activation in the postabsorptive (K, L) or postprandial (M and N) stages. Bars represent means ± standard deviation. One-way ANOVA test with Tukey's multiple comparisons was used to analyse GLUT4, InsR and AMPK total proteins levels; Kruskal—Wallis test was used for IR-Try1361 and AMPK-Thr172 analyses. * Different from WSD; # different from GKSD; $ different from GKHCD; & different from GKHCDBr. Level of significance: 1 symbol, $p < 0.05$; 2 symbols, $p < 0.01$; 3 symbols, $p < 0.001$.

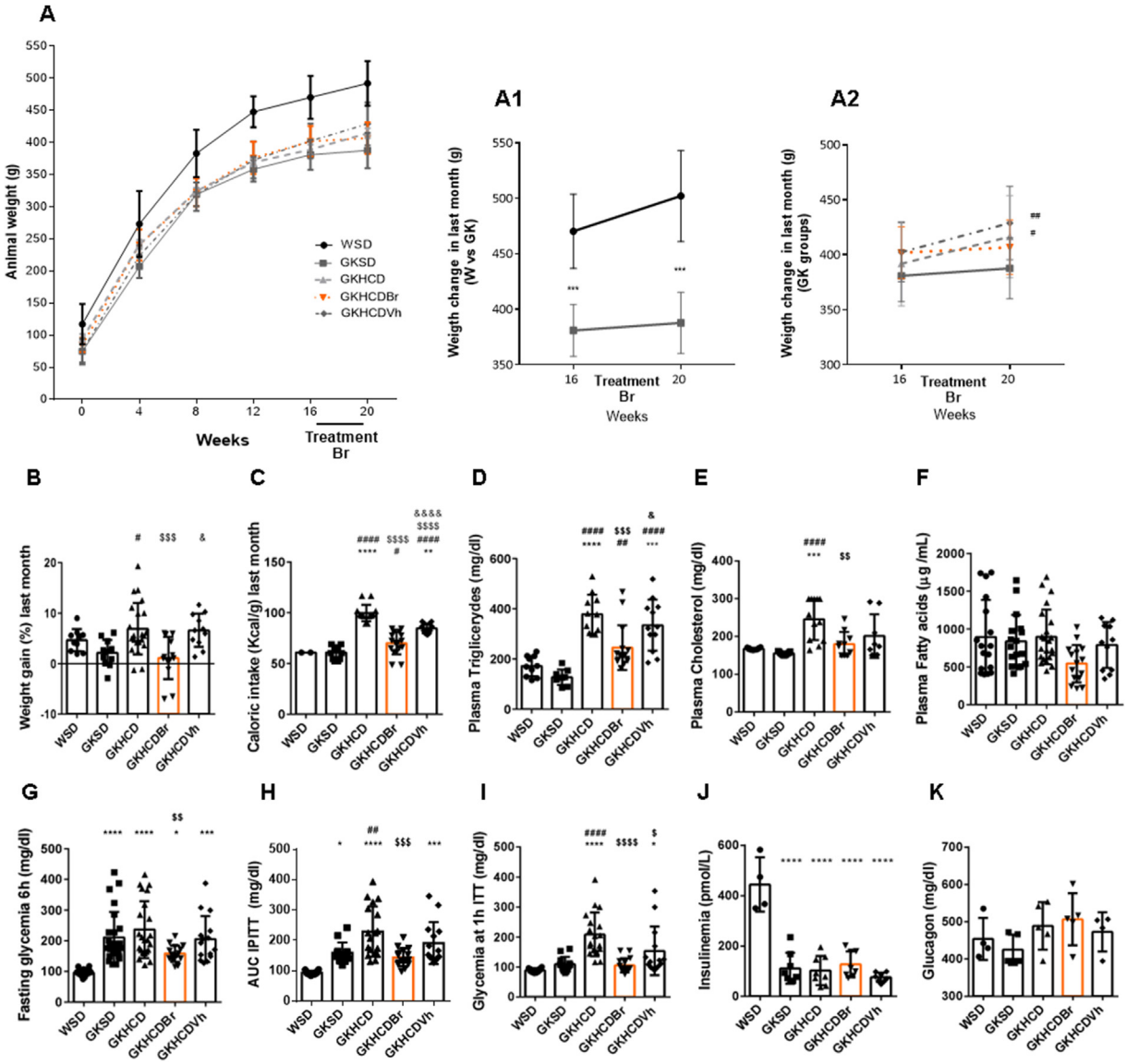

Figure 6: Bromocriptine improves metabolic profile in rats. (A) Weight gain curves during the entire period of the study, and specifically in the last month (A1). Bromocriptine prevented HCD-induced weight gain in the last month (A2 and B). Bromocriptine also reduced caloric intake (C) plasma triglycerides (D) and cholesterol levels (E), without modifying plasma free fatty acids (F). Glucose homeostasis also improved with bromocriptine treatment, namely fasting glycaemia (G), AUC during the ITT (H) and glycemia at 1 h during the IPITT (I), despite no differences were observed in plasma insulin (J) and glucagon levels (K). Values are means ± standard deviation. Differences between groups were assessed by one-way ANOVA test with Tukey's multiple comparisons. * Different from WSD; # different from GKSD; $ different from GKHCD; & different from GKHCDBr. Level of significance: 1 symbol, $p < 0.05$; 2 symbols, $p < 0.01$; 3 symbols, $p < 0.001$.

($p < 0.05$). Moreover, bromocriptine significantly reduced AUC (Figure 6H, $p < 0.001$ vs GKHCD) and glycaemia at 1 h during IPITT (Figure 6I, $p < 0.0001$ vs GKHCD). Plasma insulin levels were decreased in GK rats (Figure 6J, $p < 0.0001$ vs WSD), as consequence of the described β-cell impairment in this animal model [17,18], and remained similar in all the GK groups tested (Figure 6J), as well as glucagon plasma levels (Figure 6K).

4. DISCUSSION

Although the therapeutic effects of the D2R dopaminergic agonist bromocriptine in T2D have been attributed to central actions, peripheral dopamine is acknowledged to directly modulate glucose uptake in insulin-sensitive tissues and lipid metabolism in white adipose tissue. Herein, we hypothesized that patients with T2D may have an impairment of adipose tissue dopaminergic system, which may be a therapeutic target for bromocriptine in T2D patients. We showed that IR patients exhibit a downregulation of *DRD4* and *DRD1* expression in the adipose tissue, which is correlated with markers of adipocyte metabolic function. Additionally, we found in an animal model of T2D and HCD-induced obesity, that bromocriptine remodelled the dopaminergic system of white adipose tissue and the liver, through increased D1R and TH levels. Such mechanisms were associated with higher catabolic function, namely lipid oxidation, and insulin sensitivity in adipose tissue, as well as

lower hepatic steatosis. Importantly, no effects were observed in skeletal muscle and BAT, suggesting a predominant action of bromocriptine in the white adipose tissue.

Borcherding et al., have shown the presence of dopamine receptors in human adipocytes, specifically in the subcutaneous adipose tissue of healthy volunteers [10]. Similar to our results, they showed that the D1R was the most expressed receptor subtype in white adipose tissue, while dopamine receptors type 2 (D2R and D4R) were the less expressed [10]. In the present study, no expression of D3R and D5R was observed, which may be attributed to their absence or very reduced expression or to technical hints, as the selected primers. Nevertheless, similar observations were made by Borcherding et al., who also did not find expression of these two receptors in the subcutaneous adipose tissue [10]. Importantly, similarly to our findings, the same authors found a higher expression of D1R in adipocytes than stromovascular fraction and a 10-fold difference between D1R and D2R expression. In the present study, we showed for the first time, a dysregulation of dopamine receptors expression in the visceral adipose tissue from patients with IR and T2D. It is known that dopamine is involved in the regulation of GSIS, decreasing insulin secretion through D2R-dependent mechanisms [19,20]. It has also been proposed that dopamine-induced decrease of GSIS could be a protective mechanism to avoid β-cell exhaustion [8,21]. Accordingly, it has been reported that obese humans present reduced dopamine levels and/or function, which may be related to the compensatory hyperinsulinemia usually observed in such patients [22]. Herein, we show that IR is a condition also characterized by an imbalance of the dopaminergic system in the adipose tissue, which was correlated with the downregulation of genes implicated in lipid oxidation, *UCP1* and *PPARA*, as well as with *INSR* gene. Given that D1R is a Gs-linked receptor, it is possible that such results reflect lipid oxidation reduction, which leads to lipotoxicity and IR [9,23]. An interesting observation is that lower D1R levels were also found in the pEWAT of T2D rats, confirming the results obtained in human samples and highlighting a translatability of mechanisms between species.

The D2R dopaminergic agonist bromocriptine is the only dopaminergic modulator approved for the treatment of T2D, and it is also commonly used for Parkinson disease and prolactinomas treatment. It was also shown to have insulin sensitizing and beta-cell protecting effects [4,8,24]. To the best of our knowledge, our study is the first to show that chronic bromocriptine treatment leads to an increase in D1R protein levels in both pEWAT and the liver, which may be explained as a possible feedback or balance between the two dopamine receptor families. Importantly, TH protein level also increased with bromocriptine treatment. It is possible that the increased TH levels may reflect increased neuronal dopamine production within the adipose tissue [25], but questions remain whether this is caused by the increase of neuronal projections to the adipose tissue or direct actions of bromocriptine in the tissue. Nevertheless, increased TH levels suggest increased dopamine synthesis in the adipose tissue, possibly released by TH-positive neuron terminals or by the adipocytes themselves, which signals through the D1R to regulate adipocyte metabolism. In fact, the machinery for production of dopamine has been described to be present in adipocytes [10].

The physiological role of higher dopamine synthesis is not well understood. However, the predominant expression of D1R, which is coupled to the Gs protein-cAMP signalling pathway and is further increased by bromocriptine treatment, suggests that D1R can be related to the modulation of catabolic pathways in the pEWAT. Indeed, we have observed increased fasting levels of AMPK-Thr172 and ACL-Ser455 in pEWAT, suggesting higher fasting catabolic activity, towards lipid oxidation. This is in accordance with previous results from our team, showing that the inhibition of D2R by domperidone, when adipose tissue explants were stimulated with dopamine plus insulin, resulted in acute and marked activation of AMPK, ACL and HSL, suggesting a stimulatory effect of D1R on lipid catabolism [12]. Bromocriptine also increased the levels of the InsR and GLUT4 in the pEWAT, as well as the activation of the InsR after the ingestion of a mixed meal. In adipose tissue explants, activation of D2R was shown to acutely potentiate insulin-mediated glucose uptake, which may, nevertheless, denote a role for D2R in the modulation of adipose tissue glucose uptake following bromocriptine treatment [12].

Increased peripheral metabolism in fat depots result not only in a better adipose tissue function, but also in the protection of the liver from lipotoxicity-induced insulin-resistance. Interestingly, herein we found that although the InsR levels were increased in the liver, its postprandial activation was reduced, together with lower AMPK activation. Our group has previously showed that D2R activation by dopamine or bromocriptine induce an acute insulin-independent glucose uptake in liver explants [12]. Here, the observed upregulation of D1R after chronic bromocriptine treatment may result in the opposite effects. Although the liver is expected to significantly contribute to glucose uptake upon a meal ingestion [26], in this study we actually observed lower hepatic GLUT2 levels. Altogether, our results suggest a metabolic remodelling towards a higher metabolic activity in the periphery, and particularly in the white adipose tissue, rather than in the liver, which is consistent with the dramatic decrease of liver fat and adipose tissue weight/adipocyte volume. The marked reduction of steatosis is in line with the observations made by Davis et al. (2006), who also reported lower liver steatosis after Bromocriptine treatment to obese Zucker rats [27]. Importantly, given that no changes were observed in insulin receptor and AMPK activation in the liver, the direct effects of dopamine are questionable. Thus, it is possible that D1R upregulation may be just implicated in lipid mobilization rather than oxidation in the liver. In the adipose tissue, reduced lipotoxicity is associated with postprandial insulin sensitivity and glucose uptake, which is in line with the increment of InsR and GLUT4 levels.

The observed decreased adipose tissue weight is associated with lower leptin levels, which is in accordance with the work by Borcherding et al. showing that modulation of D1R decreased leptin levels both in subcutaneous adipose explants and isolated adipocytes [10]. Moreover, Kok et al., observed decreased plasma leptin levels after bromocriptine treatment in a cohort of obese women [28].

In the present work, it was also possible to observe a metabolic shift in the pEWAT of bromocriptine-treated rats, characterized by higher lipid oxidation rather than glucose utilization in the postabsorptive period and vice-versa in the post-prandial state. This is physiologically relevant and shows that bromocriptine restores the balance of energy supply according to the energetic status and body energy requirement, which may be very important for the prevention of lipotoxicity and glucotoxicity, two hallmarks of insulin resistance and metabolic dysregulation.

Both the BAT and skeletal muscle are considered tissues with higher metabolic rates, since the majority of disposable post-prandial glucose is taken up by the skeletal muscle [29] while BAT converts nutrients energy into chemical energy in the form of heat [30,31]. Both GLUT4 and fasting InsR-Tyr1361 levels were increased in the BAT of bromocriptine-treated animals. We previously showed in BAT explants, that dopamine and bromocriptine exert no direct effects on glucose uptake and InsR and AMPK activation. Thus, our findings obtained herein in the whole-body animal model are likely to result from the overall improvement of the metabolic status and reduction of lipotoxicity, or from other indirect actions of bromocriptine. On the other hand, the activation of D1R in skeletal muscle explants was shown to, directly and independently of insulin, lead to glucose uptake [12]. However, herein no alterations on

dopamine receptors and metabolic pathways were found in the skeletal muscle on the bromocriptine-treated animals. In accordance with the results found herein that show a lack of effect of bromocriptine in skeletal muscle, a clinical trial in obese T2D patients reported that bromocriptine did not improve insulin sensitivity in the skeletal muscle within the physiologic range of hyperinsulinemia [6,29].

As expected, and in accordance with the increased GLUT4 and InsR activation in adipose tissues, bromocriptine treatment resulted in enhanced peripheral insulin sensitivity and improved fasting glycaemia. Particularly, in the study from Pijl et al. (2010), T2D individuals treated with bromocriptine for 16 weeks showed lower fasting glycaemia and improved glucose tolerance tests [32]. Also, bromocriptine treatment in spontaneous hypertensive rats reduced retroperitoneal body fat, C-reactive protein, plasma insulin and glucose levels, and HOMA-IR [33]. Some studies have shown that body weight was unaltered in T2D individuals after bromocriptine administration [28,34,35], which disagrees with our findings. One factor contributing to the lower body weight in our animal model could be the decrease in caloric intake. In fact, it has been shown that bromocriptine treatment restores hypothalamic dopamine levels in rodents, a mechanism shown to be reduced in hibernating animals and involved in insulin resistance during such periods [6,36]. Likewise, Davis et al. (2009) described higher hypothalamic D2R levels after Bromocriptine treatment to obese Zucker rats and associated such increase with reduced hyperphagia and fat mass [37]. However, we could not exclude the possibility of a lower caloric intake in bromocriptine-treated animals due to an improvement in peripheral metabolism or other unknown peripheral actions. In agreement with this, we also found an overall improvement in triglyceride and FFA levels, by diminishing their levels in the liver and their mobilization from adipose tissue to circulation, effects that were also observed in clinical trials [38].

5. CONCLUSION

In summary, our results demonstrate the imbalance of dopamine receptors in visceral adipose tissue from patients with IR, even before glycaemic dysmetabolism. Lower *DRD1* expression correlated with reduced expression of key markers of adipose tissue metabolic function and the *INSR*. Moreover, in an animal model of dysmetabolism, modulation of dopaminergic system through bromocriptine, restored both D1R and TH in white adipose tissue and the liver. Such changes were associated with higher activation of catabolic pathways and lipid oxidation in the adipose tissue during the postabsorptive state and insulin receptor activation in the postprandial state. Altogether, our data suggest that the modulation of peripheral dopaminergic system induces a higher catabolic activity in the white adipose tissue, leading to mobilization of liver lipids, reduced hepatic and adipose tissue lipotoxicity, as well as improved insulin sensitivity and overall metabolic status. Although additional information on the mechanisms involved on dopaminergic regulation of glucose and lipid metabolism should be generated in the future, we can hypothesize that D1R activation could play a role in enhancing metabolic activity. The disclosure of the mechanisms involved in bromocriptine therapeutic effects provide new insights regarding the mechanism of action of bromocriptine in T2D, and highlight the relevance of the modulation of peripheral dopaminergic system for the treatment of metabolic diseases.

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CONFLICT OF INTEREST

The authors have no conflict of interest relevant to this study to declare.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101241.

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