MODULATION OF ADENOSINERGIC SYSTEM AS A THERAPEUTIC TARGET FOR DYSMETABOLISM

JOSÉ FERNANDO GERALDES MALHEIRO PONCE DE LEÃO

A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Biomedical Research (Specialization Area: Ageing and Chronic Diseases) at Faculdade de Ciências Médicas | NOVA Medical School of NOVA University Lisbon

September, 2023

nms.unl.pt

MODULATION OF ADENOSINERGIC SYSTEM AS A THERAPEUTIC TARGET FOR DYSMETABOLISM

José Fernando Geraldes Malheiro Ponce de Leão
Supervisors: Sílvia Vilares Conde, Associate Professor, Principal Investigator at NOVA Medical School

A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Biomedical Research (Specialization Area: Ageing and Chronic Diseases)

September, 2023

Part of the results in this thesis originated:

The following presentations at National Congresses:

As oral presentations

- **2023** Ponce de Leão J, Martins FO, Rosendo-Silva D, Melo BF, Farreca SF, Capucho AM, Sacramento JF, Matafome P, **Conde SV.** O dismetabolismo em humanos e ratos está associado a alterações na sinalização de adenosina no tecido adiposo branco e castanho. 19º Congresso de Diabetes. Vilamoura, Portugal
- **2023** Ponce de Leão J, Martins FO, Rosendo-Silva D, Melo BF, Farreca SF, Capucho AM, Sacramento JF, Matafome P, **Conde SV.** Dysmetabolism in humans and rats is associated with changes in adenosine signaling in white and brown adipose tissue. LIII Reunião Anual da Sociedade Portuguesa de Farmacologia, XLI Reunião da Farmacologia Clínica e XX Reunião de Toxicologia, Coimbra, Portugal.

As poster presentations

- **2022** Ponce de Leão J, Martins FO, Rosendo-Silva D, Melo BF, Farreca SF, Capucho AM, Sacramento JF, Matafome P, **Conde SV**. Chronic caffeine intake ameliorates white adipose tissue adenosine signaling and its metabolism alterations in dysmetabolic states. 2º Congresso Internacional de Fisiologia SPFis - 2022. Coimbra, Portugal

Acknowledgements

Em primeiro lugar, quero agradecer à minha orientadora **Professora Doutora Sílvia Conde** por um infindável apoio, por tudo que me ensinou a nível académico e por me acolher no âmbito dos seus projetos sempre com entusiasmo. Um muito obrigado por todos os votos de confiança.

Ao **Marcos** e ao **António Júnior** por todas as conversas científicas, pela troca de ideias e discussões de otimização de métodos, por me apoiarem quando a continuar quando em várias situações era mais fácil desistir. Não existe gratidão maior ou respeito em poderem ser além de colegas bons amigos.

Aos meus colegas de bancada **Adriana** e **Gonçalo,** quero reconhecer todo o vosso tempo e esforço no trabalho colaborativo científico. Pelas brincadeiras e gargalhadas mesmo em situações sérias, ou por darem com todos os outros em doidos, desejo os melhores votos para o vosso PhD

Às Doutoras **Fátima Martins** e **Joana Sacramento**, por toda a ajuda e apoio quotidiano no laboratório, por interromperem os seus trabalhos para ajudar e ensinar enquanto não se é autónomo, pelos bons e maus feitios, e mesmo "puxões de orelhas" quando foram precisos.

Ao **Professor Doutor Paulo Matafome e Daniela Rosedo,** pela ajuda na obetenção de melhores resultados de western e me receberem na sua instituição em Coimbra.

Aos meus **amigos** de toda a vida, sem precisar nomear ninguém, vamos continuar a apoiar-nos e ajudar quando possível pelo resto da vida.

Por último, à minha **família** que sustentou as minhas ambições na ciência, me educou e motivou a ser independente e resiliente. Obrigado por todo o carinho incondicional e pelas oportunidades.

Abstract

Adenosine is involved in the regulation of white (WAT) and brown (BAT) adipose tissues and implicated in obesity and related diseases, however the specific contribution is still unknow. Moreover, the therapeutics for obesity and type 2 diabetes are scarce and not completely effective. Therefore, the present work aimed to investigate if dysmetabolic states are associated with alterations in adenosine receptors and adenosine metabolism in WAT and BAT and the mechanisms behind. Moreover, we explored the impact of chronic caffeine, an antagonist of adenosine receptors, treatment on these mechanisms.

Three groups of Wistar rats were used: a control group that fed 25 weeks of standard diet, a HFHSu group that fed for 25 weeks a 60%-lipid rich diet plus 35% sucrose) in drinking water and a HFHSuCAF group submitted to HFHSu diet plus caffeine intake (1g/kg) in the last 11 weeks of diet. Rats were evaluated for weight gain, insulin sensitivity and glucose tolerance. WAT and BAT depots were collected and weighted. Ado was quantified in WAT and BAT by HPLC and Ado receptor levels and proteins involved in Ado metabolism analysed.

We found that HFHSu animals exhibit insulin resistance and glucose intolerance, effects that were rescued and ameliorated by chronic caffeine treatment, respectively. HFHSu diet promoted an increase in weight gain and WAT depots without changing BAT amount, which was associated with decreased levels of adenosine content. These effects were rescued by chronic caffeine treatment. HFHSu diet decreased A_{2A} and A_{2B} levels in WAT in contrast with BAT where HFHSu diet promoted an increase in A_{2A} levels and a decrease in A_{2B} levels. All these effects were rescued by chronic caffeine administration. In both tissues no changes in A_1 levels were observed. In WAT, HFHSu diet did not change ENT1 or CD73 levels but caffeine intake in HFHSu rats increased CD73 levels in comparison with HFHSu rats, and in BAT the diet produced an increase in ENT1 and CD73 levels.

We can conclude that alterations in Ado receptor levels and Ado metabolism in adipose tissues are associated with metabolic dysfunction and that the modulation of Ado receptors with chronic caffeine intake can be useful to improve dysmetabolic states.

Resumo

A adenosina está envolvida na regulação dos tecidos adiposos branco (WAT) e castanho (BAT) e está implicada na obesidade e doenças relacionadas, tal como a diabetes do tipo 2. No entanto, a sua contribuição específica para estas patologias é ainda desconhecida. Para além disso, as terapias para obesidade e diabetes tipo 2 são escassas, não sendo totalmente eficazes. Deste modo, o presente trabalho teve como objetivo investigar se estados dismetabólicos estão associados a alterações nos recetores de adenosina e no metabolismo da adenosina no WAT e no BAT, bem como os mecanismos por trás dessas alterações. Além disso, explorámos o impacto do tratamento crónico com cafeína, um antagonista dos recetores de adenosina, nessas alterações.

Foram utilizados três grupos de ratos Wistar: um grupo controlo que recebeu uma dieta padrão por 25 semanas, um grupo HFHSu que recebeu uma dieta rica em lipídios (60%) e 35% de sacarose na água de beber por 25 semanas, e um grupo HFHSuCAF submetido à dieta HFHSu com ingestão de cafeína (1g/kg) nas últimas 11 semanas da dieta. Os ratos foram avaliados quanto ao ganho de peso, sensibilidade à insulina e tolerância à glucose. No final do período das dietas os depósitos de WAT e BAT foram recolhidos e pesados. A adenosina (Ado) foi quantificada no WAT e BAT por HPLC, e os níveis de receptores de Ado e proteínas envolvidas no metabolismo da adenosina foram analisadas.

Descobrimos que os animais do grupo HFHSu apresentaram resistência à insulina e uma diminuição da tolerância à glucose, efeitos foram revertidos e melhorados, respetivamente com a ingestão crónica de cafeína. A dieta HFHSu promoveu um aumento no ganho de peso e nos depósitos de WAT, sem alterar a quantidade de BAT, que se encontra associado a níveis diminuídos de adenosina nestes tecidos. Esses efeitos foram revertidos com o tratamento crónico com cafeína. A dieta HFHSu diminuiu os níveis de recetores A2A e A2B no WAT, ao contrário do BAT, onde a dieta HFHSu aumentou os níveis de recetores A2A e diminuiu os níveis de A2B. Todos esses efeitos foram revertidos pela administração crónica de cafeína. Em ambos os tecidos, não foram observadas alterações nos níveis dos recetores A1. No WAT, a dieta HFHSu não alterou os níveis do transportador ENT1 ou do enzima CD73, mas a ingestão de cafeína nos ratos HFHSu aumentou os níveis de CD73 em comparação com os ratos HFHSu. No BAT, a dieta produziu um aumento nos níveis de ENT1 e CD73.

Pode-se concluir que as alterações nos níveis dos recetores de Ado e no metabolismo da adenosina nos tecidos adiposos estão associadas à disfunção metabólicas e que a modulação dos recetores de Ado com a ingestão crónica de cafeína pode ser útil para melhorar os estados dismetabólicos.

Index

List of figures

List of tables

List of Acronyms

ADA – American Diabetes Association

Ado-r – adenosine receptors

AKT – protein kinase B

aPKC – atypical protein kinase C

AS160 – Akt substrate of 160 kDa

AT – adipose tissue

BAT – brown adipose tissue

BMI - body mass index

cAMP – cyclic adenosine monophosphate

CD39 – cluster of differentiation 39

CD73 – cluster of differentiation 73

CSN – central nervous system

DM – Diabetes Mellitus

FFA – free fatty acid

Foxo1 – Forkhead box protein O1

GDP - gross domestic product

GLUT4 – glucose transporter type 4

GPCRs – G protein-coupled receptors

Gsk3b – glycogen synthase kinase b

HSL – hormone-sensitive lipase

IDF – International Diabetes Federation

IFG – impairment of fasting glycemia

IGT – impaired glucose tolerance

I-R – insulin receptor

IR – Insulin Resistance

IRS – insulin receptor substrates

MAPK – mitogen-activated protein kinase

NCD - non-communicable diseases

NEFA – non-esterified fatty acid

PDE3B – phosphodiesterase 3B

PDE3B – phosphodiesterase 3B

PDK1 – phosphoinositide-dependent protein kinase-1

PDK2 – phosphoinositide-dependent protein kinase-2

PI3K – phosphatidylinositide-3-kinase

PIP3 – phosphatidylinositol (3,4,5)-triphosphate

SREBP1 – phosphorylating sterol regulatory element-binding protein 1

T2DM - Type 2 Diabetes Mellitus

WAT – white adipose tissue

WHO - World Health Organization

I. State of the art

1.1 Metabolic Disorders

The XXI century life context rose from the rapid development of urbanization, technology modernization and the acceleration of socio-economic growth and prosperity. Although improving the standard of living and life expectancy it catastrophically pushed individuals to a sedentary (almost one-third of the world's population is inactive) and more stressful lifestyle, marked by lack of physical activity and harmful dietary patterns across the globe (Arocha Rodulfo, 2019).

More than 70% of early deaths worldwide result from noncommunicable diseases (NCDS) including cardiovascular diseases, Type 2 Diabetes Mellitus (T2DM) and cancer. As a high impact risk factor, obesity is estimated to decrease life expectancy up to 20 years depending on comorbidities and severity. Altogether being the leading causes of premature disability and mortality (Blüher, 2019).

Over the past couple of decades metabolic disorders have exponentially increased the burden in health and health systems being the most prevalent and deeply interconnected obesity and T2DM (Anon, 2021*a*, Anon, 2023; Ruze *et al.*, 2023).

A metabolic disorder is characterized by abnormal chemical reactions disrupting body's metabolism. This often manifests as a problem in energy regulation either from difficulties in processing and distributing macronutrients or low efficiency in cell production of energy. They can be acute, late-onset acute, progressive, or even permanent and vary from genetic to environmental/behavioural causes (Zakir *et al.*, 2022).

For the past years researchers have pulled a collective effort to investigate deeper correlations between the pathogeneses of these two most common metabolic disorders (Piché *et al.*, 2020). These efforts urge as the increasing boom of obesity is spread across all age groups and is pointed as a main driver of the expansion of the T2DM population. Hence effective and decisive actions are necessary to prevent and treat obesity and other obesity-related comorbidities while we are facing a global health emergency (Kumanyika & Dietz, 2020).

1.2 Obesity Epidemics

Obesity has been steadily increasing in prevalence for the past 50 years reaching officially pandemic levels and recognized as a multifactorial, relapsing, and chronic condition. According to the World Health Organization, the global prevalence of obesity has tripled since the mid-1970s. In 2016, more than 1.9 billion adults were overweight and of these over 650 million were obese.(Kumanyika & Dietz, 2020, Anon, 2021*b*)

It is a major health challenge as it aggravates the risk and onset of T2DM, steatotic liver disease, hypertension, myocardial infarction, stroke, dementia and other neurodegenerative disorders, osteoarthritis, obstructive sleep apnea and numerous cancers. Either alone or with related comorbidities compromises every organ system.(Hu, 2008)

It is influenced by biological and genetical predisposition and features, access to education and healthcare, environmental context, and personal lifestyle as well as mental status, sociocultural or economic factors. (Smith & Smith, 2016)

Besides impacting both physical and mental health obesity also promotes an increase in economic burdens as it is associated to unemployment, social disadvantages and reduced socio-economic productivity which can hardly ever be easily restored by weight loss (Smith & Smith, 2016; Kumanyika & Dietz, 2020, Anon, 2023).

From an evolutionary perspective, humans have survived periods of undernutrition contributing to centuries of natural selection resulting in a survival genotype that enhances overeating, low energy expenditure and physical inactivity. To endure famine genetic variants that promoted the ability to eat faster, mobilize calories more efficiently and to expand energy stores in adipose tissue were key adaptations that promoted the generational passage and survival of the fittest.(Arocha Rodulfo, 2019)

The pilar in the pathogenesis of obesity is a tip in the scale of metabolism and energy, where long-term consumption of too many calories weigh more than the few calories expended.

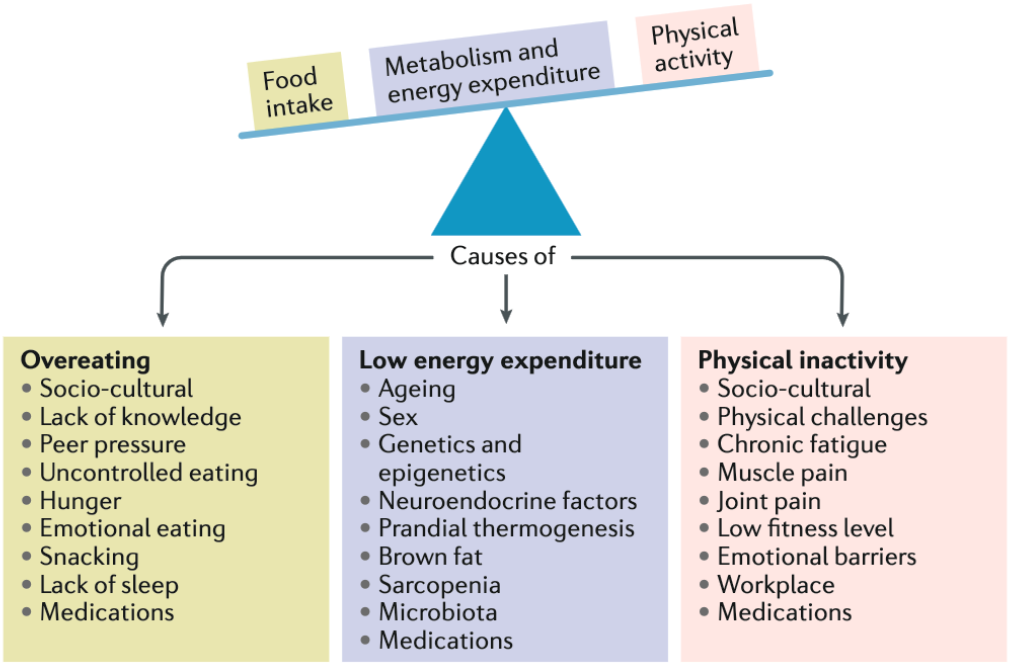

Figure 1. Metabolic balance and energy expenditure

Factors that can influence the chronic positive energy balance, thus subsequently causing obesity. Weight gain can result from a combination of increased energy intake, low physical activity, and reduced energy expenditure. Adapted from (Blüher, 2019)

Commonly obesity is described and stratified into classes by the body mass index (BMI), at least on a population level (table 1). BMI is obtained by dividing weight in kilograms by height in meters squared. However, this parameter does not comprehend individual disease complexity lacking the assessment of more comprehensive and systemic parameters. (Chooi *et al.*, 2019, Anon, 2022)

Obesity prevalence is higher amongst women than in men accounting for age and reproductive status (menopause etc.), though if we include global prevalence of overweight then it is higher in men. (Anon, 2023)

BMI classifications parameters can be adjusted according to ethnicity and sex (Smith & Smith, 2016).

Table 1. Overweight and obesity definitions

Body mass index (kg/m^2)	Class	Body mass index (kg/m^2)	Class
25–29.9	Overweight	25–29.9	Overweight
30–34.9	Class I obesity	≥30	Obese
35–39.9	Class II obesity	35–39.9	Severe obesity
≥40	Class III obesity	40–49.9	Morbid obesity
		≥50	Super obesity

Adapted from: World Health Organization "Body mass index (BMI)", 2019

According to The World Obesity Atlas 2022 and 2023 global estimated levels of overweight and obesity suggest an increase from 38% of the world's population in 2020 to over 50% by 2035 (over 4 billion). By 2030 the percentage of adults with obesity Class I, II and III, will be 10%, 4%, and 2%, respectively.(Anon, 2022, Anon, 2023) it also predicts that the global economic impact of overweight and obesity will reach $4.32 trillion annually by 2035 if prevention and treatment measures do not improve. At almost 3% of global GDP, this is comparable with the impact of COVID-19 in 2020.(Anon, 2022, Anon, 2023)

In Europe obesity-related healthcare economic burden ranged from 0.09% to 0.61% of each country's gross domestic product (GDP) costing around to 10.4 billion euros. (Müller-Riemenschneider *et al.*, 2008). In Portugal, the 1st National Health Examination Survey (INSEF 2015) points gender bias to overweight prevalence in men and obesity in women, also that a high prevalence of overweight and obesity are found in older individuals, married, with lower education levels and non-smoking women (Gaio et al., 2018).

Research efforts both in humans and animals have enlightened the understanding of how obese individuals crave for food; how appetite and satiety centered in the hypothalamus are regulated by adipose tissue, gut or liver hormones; and how dysfunction of adipose tissue increases health burden (ANAND & BROBECK, 1951; Murray

et al., 2014; Di Cesare *et al.*, 2016; Heymsfield & Wadden, 2017). Moreover, in a pooled analyses BMI is detrimental for the risk in NCDs across globe geographical areas (Di Cesare *et al.*, 2016)

So far, we are failing to address treatment and prevention strategies and reaching the established prevalence goals.(World Obesity Federation, 2022). Race, Ethnicity, and Culture need to be further considered. (Caprio *et al.*, 2008) As well as the heterogenicity of "obesities" no longer represented by BMI as the author(Piché *et al.*, 2020), reveals. Lifestyle and behavioural approaches have reduced effectiveness because genetic background often influences metabolic, hormonal, and neuronal pathways that protect against weight loss and promote weight regain. Hence, several new strategies combined with more conservative treatments are necessary. (Tsai & Bessesen, 2019)

Obesity is believed to be a promoter of T2DM as they share similar genetic susceptibilities and the accumulation of fat being simultaneously an aggravating cause and consequence of T2DM.(Malone & Hansen, 2019; Piché *et al.*, 2020; Sørensen *et al.*, 2022; Ruze *et al.*, 2023)

As mentioned, excessive accumulation of certain nutrients and metabolites sabotage the metabolic balance provoking expansion of the adipose tissues.

Adipose tissue is a complex structured array of cell types that release numerous cytokines, chemokines, and hormones. Adipocytes or fat cells make up roughly 33% of the adipose tissue and remaining components include endothelial cells, macrophages, fibroblasts, stromal cells, immune cells, and pre-adipocytes (Chait & den Hartigh, 2020).

Traditionally it has been categorized into two main types: white adipose tissue (WAT) and brown adipose tissue (BAT).

In mammals WAT is the most predominant adipose tissue crucial as an energy reservoir, but also as an active endocrine organ, secreting a wide array of bioactive molecules known as adipokines (Trayhurn & Beattie, 2001). These adipokines affect various physiological processes, including appetite regulation, insulin sensitivity, inflammation, and lipid metabolism (Fasshauer & Blüher, 2015; Chait & den Hartigh, 2020).

In contrast, BAT is primarily responsible for maintaining body temperature through a process called “non-shivering thermogenesis” and is most prevalent in mammals during postnatal development and hibernation.

White and brown adipocytes exhibit distinct sizes, shapes, and intracellular organelle composition. White adipocytes typically adopt a spherical shape and contain a single large lipid droplet, that displaces organelles toward the cell’s periphery. Conversely, brown adipocytes are ellipsoidal in shape and contain multiple dispersed lipid droplets along with mitochondria (Richard *et al.*, 2000).

More recently, a new subtype of adipocytes has been described, it displays characteristics of both white and brown adipocytes and therefore named beige. These beige adipocytes develop within WAT deposits, originating from a distinct subset of preadipocytes or through the transdifferentiation of existing adipocytes. Various stimuli, including cold exposure, dietary factors, and exercise, can trigger the emergence of beige adipocytes(Seale *et al.*, 2008; Wu *et al.*, 2012; Shao *et al.*, 2019; Chait & den Hartigh, 2020).

Adipose tissue is not a uniform entity but rather exists in distinct depots throughout the body, primarily classified as subcutaneous and visceral (Tchernof & Després, 2013).Subcutaneous adipose tissue is situated beneath the skin and acts as a thermal insulator, while visceral adipose tissue is found within the abdominal cavity, enveloping vital organs such as the liver, pancreas, and intestines(Ibrahim, 2010). These different depots exhibit unique characteristics in terms of adipokine secretion, metabolic activity, and susceptibility to metabolic dysregulation.

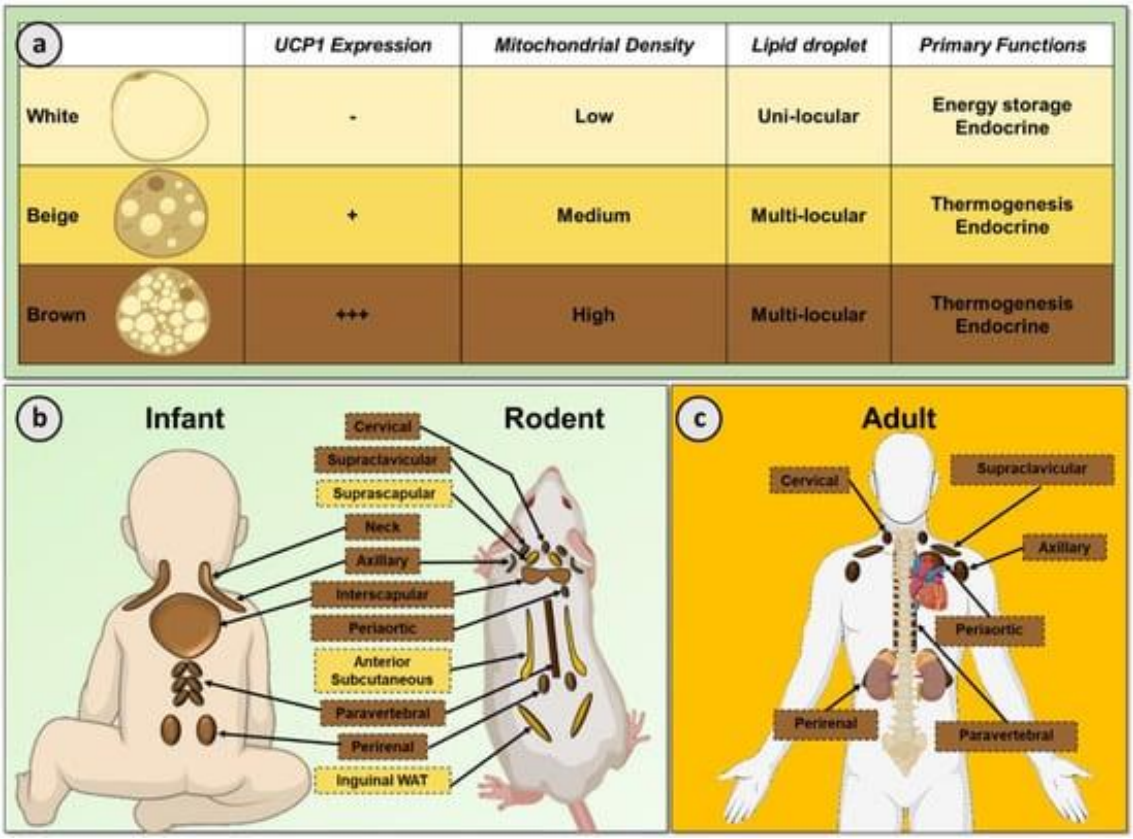

Figure 2. Adipose tissue function and location.

A) Types of adipocytes, functions, and features; B) Dorsal homology of BAT in infant humans and rodents and dorsal WAT depots; C) Ventral distribution of BAT in adult humans

Adapted from: (Suchacki & Stimson, 2021) (https://www.mdpi.com/2072-6643/13/6/1748)

1.3 Type 2 Diabetes Mellitus

T2DM is part of the four different types of diabetes mellitus (DM) according to the American Diabetes Association (ADA) them being: Type 1 Diabetes, Type 2 Diabetes, Gestational Diabetes, and specific types of diabetes due to other causes.(ADA, 2023) The World Health Organization (WHO) estimates that over 95% of DM cases are T2DM.(WHO, 2023)

The principal drivers of the T2DM epidemic are hypercaloric diets, the global increase of obesity, sedentary lifestyles, and the aging population.(Chatterjee *et al.*, 2017) further aetiology presented at (Zheng *et al.*, 2018)

In addition, latest international diabetes federation (IDF) 2021 report estimates that 537 million people aged between 20-79 years suffer from diabetes, representing 10.5%

of world's population with high correlation to mortality numbers (~6.7million) due to DM. By 2030 and 2045 statistics predict even worse scenarios of the numbers rising to 643 million people (11.3%) and 783 million (12.2%) respectively, this phenomenon is probable to occur in middle-income countries due to their ageing populations. One last potentially catastrophic factor is that almost 1 in 2 adults are unaware they have diabetes.(Anon, 2021*a*) In opposition to obesity, data in the report shows that T2D is most prevalent in men. (Kautzky-Willer *et al.*, 2023)

As mentioned above T2D is one of the most prevalent metabolic disorders. It is characterized primarily by a combination of two main factors: defects in insulin secretion by pancreatic β-cells and the development of insulin resistance (IR) resulting in insulin-sensitive tissues inefficiency when responding to insulin. (Roden & Shulman, 2019)

Regarding the pathophysiology of the disease beta cell dysfunction and insulin resistance induce hyperglycemia and therefore increase insulin demand. In these states glucose sensing is impaired, and secretion of insulin stimuli reduced, leading to glucose concentrations above the physiological range (fig.3). (Weyer *et al.*, 1999; Stumvoll *et al.*, 2005)

Both pathological states influence each other and are thought to synergistically exacerbate diabetes by dysregulating glucose homeostasis. (Cerf, 2013) Moreover, patients with T2DM are often obese or present a higher body fat percentage, distributed predominantly in the abdominal region. In this condition, visceral adipose tissue deposition promotes IR through various inflammatory mechanisms, including increased free fatty acid (FFA) release and adipokine deregulation. (Cerf, 2013; Gastaldelli *et al.*, 2017; Galicia-Garcia *et al.*, 2020)

Therefore, in T2D pathogenesis the role of adipocytes involves glucose transport, phosphorylation and oxidation, glycogen and its synthesis, glycolysis, and insulin signaling. (DeFronzo, 2004)

Insulin release and action must meet the metabolic demand. Hence, the molecular mechanisms involved in the synthesis and release of insulin, as well as the insulin response in tissues must be tightly regulated. Furthermore, glucose homeostasis is highly influenced by IR mechanisms and will be presented in detail.

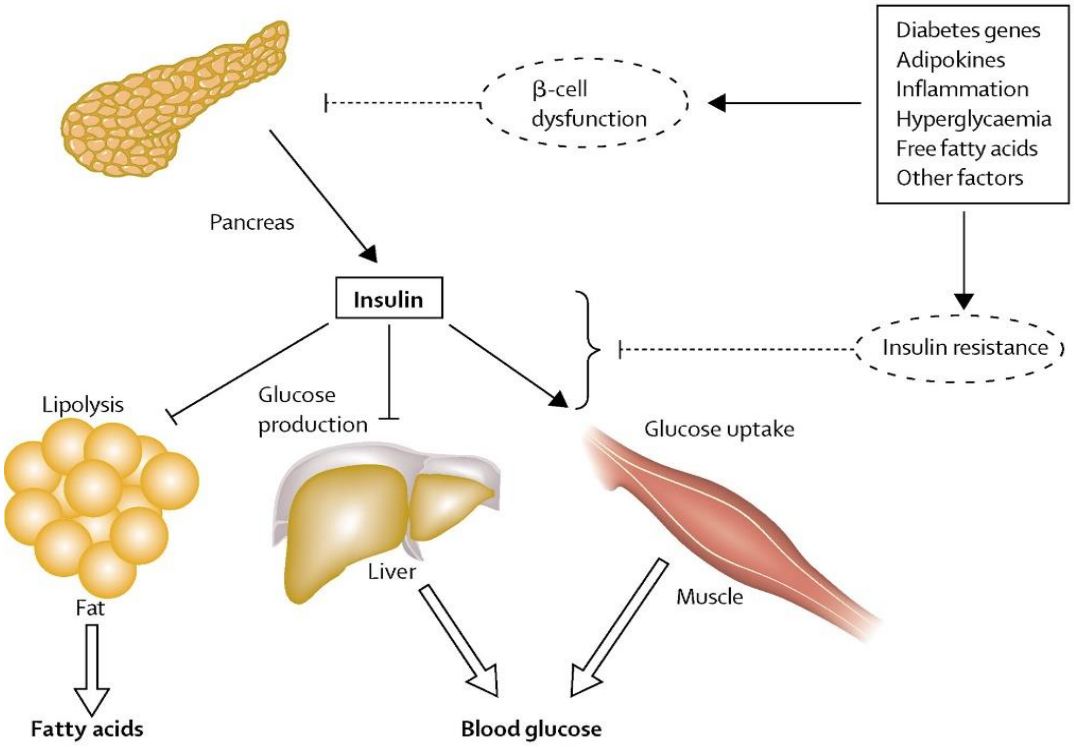

Figure 3. Insulin action on insulin sensitive tissues.

Insulin secreted by the pancreas normally reduces glucose output by the liver, enhances glucose uptake by skeletal muscle, and suppresses fatty acid release from fat tissue. Insulin resistance pathways affect the action of insulin in each of the major target tissues, leading to increased circulating fatty acids and the hyperglycemia of diabetes. Several factors like genetic background, inflammation, hyperglycemia and increase in adipokines and free fatty acids promote IR in insulin sensitive tissues and β-cell disfunction reducing insulin action and production levels Adapted from: (Stumvoll et al.*, 2005).*

1.4 Insulin and Glucouse Homeostasis

1.4.1 Insulin signalling

Insulin is a vital anabolic hormone in the body, with a crucial role in regulating various physiological processes, such as metabolism, cell growth, and differentiation (Kahn & White, 1988; Guo, 2014). The effects of insulin are diverse and vary depending on the specific insulin-sensitive tissue being targeted. In terms of peripheral metabolism regulation, insulin primarily acts on three major tissues: skeletal muscle, adipose tissue (AT), and the liver (Petersen & Shulman, 2018)

Insulin regulatory effects are multifaceted and tissue specific to maintain glucose homeostasis. In terms of peripheral metabolism regulation, insulin promotes glucose uptake in skeletal muscle and adipose tissue, inhibits hepatic glucose production, and facilitates the storage of substrates, including lipogenesis, glycogen, and protein

synthesis, while concurrently inhibiting lipolysis, glycogenolysis, and protein degradation. (Petersen & Shulman, 2018).Beyond peripheral tissues, insulin acts within the central nervous system (CNS), where it contributes to appetite control by acting upon specific CNS centers responsible for appetite suppression (Timper & Brüning, 2017).

Insulin is a peptide hormone composed of 51 amino acids, organized into two polypeptide chains: A (21 amino acids) and B (30 amino acids), interconnected by disulfide bridges and activates cells signaling cascade through its receptor I-R. Insulin receptor is a glycoprotein belonging to the tyrosine kinase receptor family and consists of an α extracellular subunit and a β transmembrane subunit.(Guo, 2014)

Pancreatic β-cells, within the pancreatic islets, sense elevated plasma glucose levels are responsible for the production and release of insulin to control fat and carbohydrate metabolism, convert these into storable macromolecules like glycogen, proteins, and lipids.

The precise control of plasma glucose levels hinges on a delicate equilibrium between glucose absorption from the intestines post-prandial, hepatic glucose production, and uptake and its consumption as energy fuel by tissues.(Kahn & White, 1988; Lizcano & Alessi, 2002; Tokarz *et al.*, 2018)

1.4.2 Insulin cascade

Upon insulin bound to the I-r α subunit, it initiates receptor dimerization, forming an α2β2 complex. This dimerization leads to the autophosphorylation of the β subunit at specific tyrosine residues (Tyr1158, 1162, and 1163), activating a phosphorylation cascade(Guo, 2014)

Activation of the IR signaling pathway results in the recruitment and phosphorylation of various proteins, including insulin receptor substrates (IRS 1-4). These proteins initiate intracellular pathways, such as the phosphatidylinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades (Guo, 2014)

Insulin-induced activation of Ras → MAPK that regulates cell growth and mitogenesis, while PI3K activation generates phosphatidylinositol (3,4,5)-triphosphate (PIP3). PIP3

triggers phosphoinositide-dependent protein kinase-1 and -2 (PDK1 and PDK2), which, in turn, regulate insulin's effects on metabolism and cell survival. PDK1 and PDK2 also play a crucial role in the activation of Protein Kinase B (AKT/PKB) (Saltiel & Kahn, 2001; Guo, 2014)

AKT is central to insulin signaling, phosphorylation of downstream proteins, such as glycogen synthase kinase b (Gsk3b), leading to the inhibition of glycogen synthesis (Hermida *et al.*, 2017; Huang *et al.*, 2018). AKT also phosphorylates mediators like AKT substrate 160 kDa (AS160), activating Rab10GTPase, which facilitates glucose transporter type 4 (GLUT4) translocation to the plasma membrane for glucose uptake (Koepsell, 2020). AKT activates Phosphodiesterase 3B (PDE3B), an enzyme that degrades cyclic adenosine monophosphate (cAMP). Additionally, AKT inhibits cAMP response element-binding protein (CREB)-regulated transcription coactivator 2 (CRTC2), important for reducing hepatic gluconeogenesis. AKT also stimulates liver lipogenesis by phosphorylating sterol regulatory element-binding protein 1 (SREBP1) and inhibits Forkhead box protein O1 (Foxo1) by phosphorylation, suppressing liver glucose production (Guo, 2014; Hermida *et al.*, 2017)

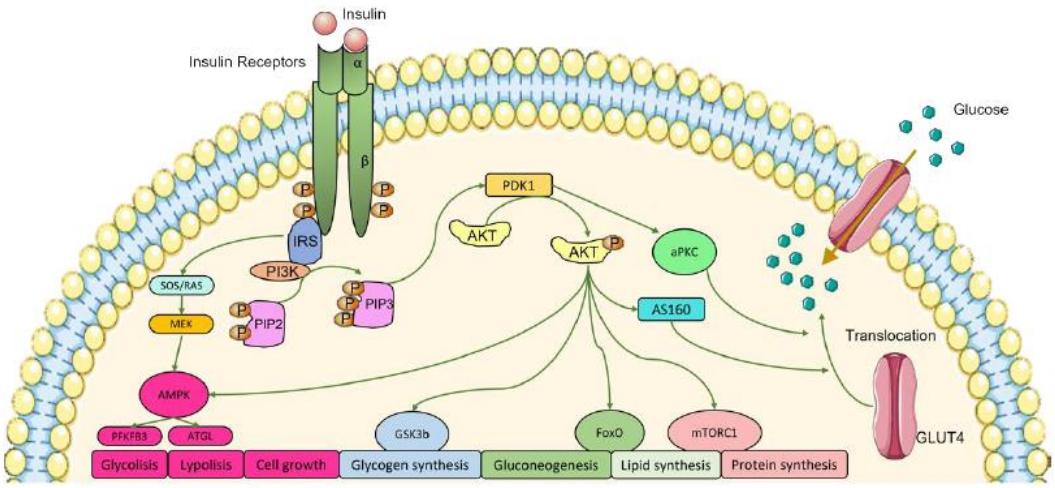

Figure 4. Schematic representation of insulin signaling pathways.

IRS, insulin receptor substrate; PI3K, phosphatidylinositide-3-kinase; PIP2, phosphatidylinositol (4,5)-biphosphate; PIP3, phosphatidylinositol (3,4,5)- triphosphate; PDK1, phosphoinositide dependent protein kinase-1; AKT, protein kinase B; AS160, Akt substrate of 160 kDa; aPKC, atypical protein kinase C; SOS/RAS, son of evenless; MEK, mitogen-activated protein kinase; AMPK, mitogen-activated protein kinase; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; ATGL, adipose triglyceride lipase; GSK3b, glycogen synthase kinase b; FoxO, forkhead box protein O1; mTORC1, mammalian target of rapamycin complex 1; GLUT4, glucose transporter type 4. Adapted with consent from (Capucho et al.*, 2022)*

1.4.3 Insulin action and glucose homeostasis

Following a meal, plasma glucose levels rise due to intestine nutrient absorption. Herein insulin and glucagon act as the masters' hormone regulators of blood glucose levels. The liver is responsible for 85% of endogenous glucose production, with the remaining 15% produced by the kidneys. Both glycolysis and glycogenesis contribute equally to the basal rate of hepatic glucose production.

While insulin release suspends hepatic glucose production it also produces an antilipolytic effect, inhibiting lipolysis and reducing circulating free fatty acid (FFA) levels. To counter-balance insulin action, secretion of glucagon by pancreatic α-cells activates glycogenolysis to increase circulating glucose availability [(Saltiel & Kahn, 2001; Yu *et al.*, 2002; DeFronzo, 2004)

1.4.4 White Adipocyte Insulin signaling: Effectors and effects

The white adipocyte is exquisitely sensitive to insulin in vivo. Insulin potency in managing plasmatic non-esterified fatty acid (NEFA) levels is crucial for maintaining euglycemia. In WAT, insulin is also key in lipolysis suppression (Reaven, 1988) and stimulation of glucose transport, although WAT only accounts for a small fraction of whole-body glucose disposal.

In rats insulin's rapid antilipolytic action is facilitated by the brief half-life of plasma NEFA, resulting in a substantial reduction NEFA levels within 5 minutes of elevated insulin levels [(Griffin *et al.*, 1999) The principal mechanism through which insulin curtails lipolysis revolves around the dampening or reversal of adrenergic signaling mediated via cyclic AMP (cAMP) and protein kinase A (PKA) (Duncan *et al.*, 2007; Jaworski *et al.*, 2007) PKA, in turn, phosphorylates pivotal lipolysis-associated proteins, including hormone-sensitive lipase (HSL) and perilipin (PLIN) (VAUGHAN *et al.*, 1964; Jaworski *et al.*, 2007).

In addition, insulin acts largely through phosphodiesterase 3B (PDE3B) to suppress lipolysis. PDE3B degrades cAMP to attenuate pro-lipolytic PKA signaling toward HSL and PLIN (Smith *et al.*, 1991; Rahn *et al.*, 1994; Young *et al.*, 2006; Jaworski *et al.*, 2007). This

way insulin both attenuates adrenergic kinase activity through PDE3B activation and actively dephosphorylates lipolytic regulatory proteins through protein phosphatase activation.

1.4.5 Insulin resistance and metabolic disorders

Insulin resistance, a state where insulin loses its effectiveness in insulin-sensitive tissues, is a complex phenomenon, while the precise mechanisms of insulin resistance remain largely unknown, several factors have been implicated: including hyperinsulinemia often associated with T2DM; obesity; and inflammation, among others (IDF Diabetes Atlas, 10th Edn., 2021; Huang et al., 2018; Ye, 2013)

In relation to adipose tissue dysfunction, hypertrophy caused in adipocytes arises from excessive absorption of nutrients. This hypertrophy occurs, above all, in visceral adipose tissue, as this is the deposit most susceptible to the flow of nutrients (Matafome & Seiça, 2017). In fact, some authors have currently sought to associate hypertrophy with the development of hypoxic regions, being a determining factor in the influence and maintenance of the secretory and metabolic functions of adipose tissue. Triglycerides are continually accumulated in adipocytes during the fat accumulation process, resulting in increased expression of enzymes involved in the esterification of fatty acids. When the capacity of adipocytes to accumulate fat from the diet is exhausted, fatty acids from adipocytes are released into the systemic circulation, which end up accumulating in other tissues such as the liver and skeletal muscle (Matafome & Seiça, 2017).

Clinical conditions such as hepatic and muscular steatosis, present in individuals with type 2 diabetes, lead to morphological changes and insulin resistance in tissues accumulated with fatty acids (Guilherme *et al.*, 2008; Lonardo *et al.*, 2015; Matafome & Seiça, 2017). Since the successive availability of fatty acids increases lipid oxidation and this is a limited process, this will result in the accumulation of intermediate metabolites (i.e., diacylglycerols and ceramides), inhibiting glucose uptake and insulin signaling (Bugianesi *et al.*, 2005; Golay & Ybarra, 2005). When these mechanisms are activated excessively and chronically, they generate insulin resistance, contributing to the development of T2D ((Guilherme *et al.*, 2008; Matafome & Seiça, 2017)

A bidirectional relationship exists between insulin resistance and hyperinsulinemia. When insulin action is impaired in insulin-sensitive tissues, pancreatic β-cells respond by increasing insulin production to normalize blood glucose levels. However, prolonged overstimulation of these β-cells diminishes their capacity to produce high insulin levels, leading to impaired insulin secretion. When insulin secretion falls below the threshold required for maintaining normal blood glucose levels, individuals become insulin dependent.(Ye, 2013, Anon, 2021*a*)

In fact, animal studies have shown that administering high doses of insulin can induce insulin resistance, and in humans, elevated plasma insulin levels persist even 40 hours after insulin infusion, leading to decreased glucose uptake and indicating impaired insulin signaling (Fukagawa *et al.*, 1985; Shanik *et al.*, 2008)

These findings support the notion that hyperinsulinemia, as seen in many insulin-resistant states, contributes to the development of insulin resistance.

Moreover, this effects in increasing impairment of fasting glycemia (IFG), and impaired glucose tolerance (IGT) in addition to the onset of IR and β-cells failure can be summarized as depicted in the following figure:

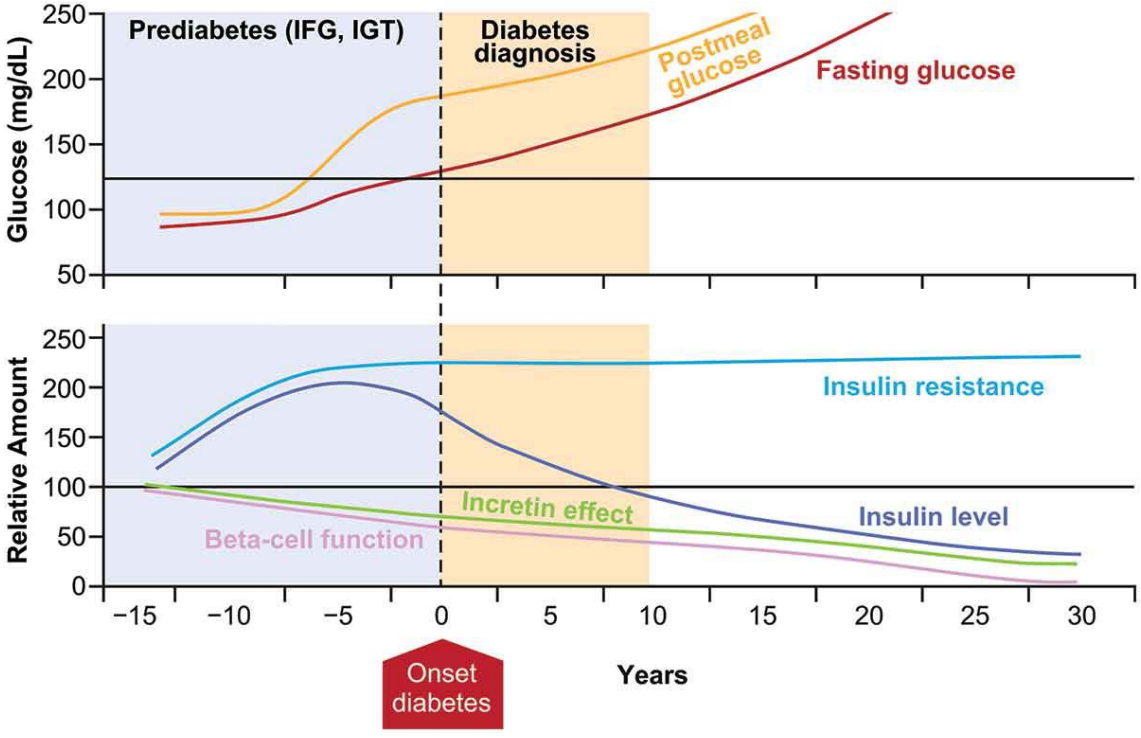

Figure 5. Graphic representation of the natural history of type 2 diabetes.

Most pathophysiological changes occur prior to any diabetes diagnosis. IFG, impaired fasting glycemia; IGT, impaired glucose tolerance are conditions that characterize the state of prediabetes where blood glucose levels are increased but not high enough to determine a diabetes diagnosis. In addition, with glucose levels deregulation, coping hyperinsulinemia promotes IR in insulin sensitive tissues. Concomitantly with β-cell dysfunction insulin levels decrease also promoting increase in circulating glucose. Adapted from (Wysham & Shubrook, 2020)

1.5 Purinergic system as a target for dysmetabolism treatment

Via IR metabolic balance is sabotaged by the hypertrophy of the AT and excessive accumulation of detrimental nutrients and metabolites. These effects exacerbate the dysregulation of physiological metabolism through inflammation, disruption of microbiome-gut-brain axis and impaired autophagy. (Rohm *et al.*, 2022)

Given the intricate links between obesogenic and diabetogenic pathogenesis, current therapeutic strategies are focused as previously mentioned in lifestyle interventions, pharmacotherapies, and bariatric surgeries. Although strategies still lack in significant improvements on metabolic disorders burden. (Lingvay *et al.*, 2022)

Meanwhile, new strategies are currently being evaluated to improve treatment effectiveness. According to (Liu *et al.*, 2019), increasing evidence has revealed that thermogenic regulators have enhanced therapeutic effects on pathological adipose tissue. G protein-coupled receptors (GPCRs) are under the scope as a possible target of this regulators, namely the adenosine receptors (Ado-r) (fig.6). (de Oliveira *et al.*, 2020*a*). Adenosine and analogues were found to inhibit damaging processes in adipocytes from rodents (Fain *et al.*, 1972; Schimmel & McCarthy, 1984). While other study suggested adenosine enhanced the thermogenic effect in brown and white adipocytes either from human or murine also found that the effect of adenosine was stronger in brown than white adipocytes. (Gnad *et al.*, 2014*a*)

In the adipose tissue ATP can be released together with norepinephrine upon stimulation of sympathetic nerves (Burnstock, 2007), but it can also be originated directly from adipocytes (Gnad *et al.*, 2014*a*). However, it is still unknown (1) the contribution of each of these components to the purinergic communication among adipose tissue cells, and (2) the role of adenosine originated from the extracellular catabolism of released ATP in the proliferation, differentiation, and activity of adipocytes from different adipose tissue origins.

Findings indicated the role of adenosinergic signalling and metabolism is still controversial but may prove to be beneficial. Hence, more studies are needed.

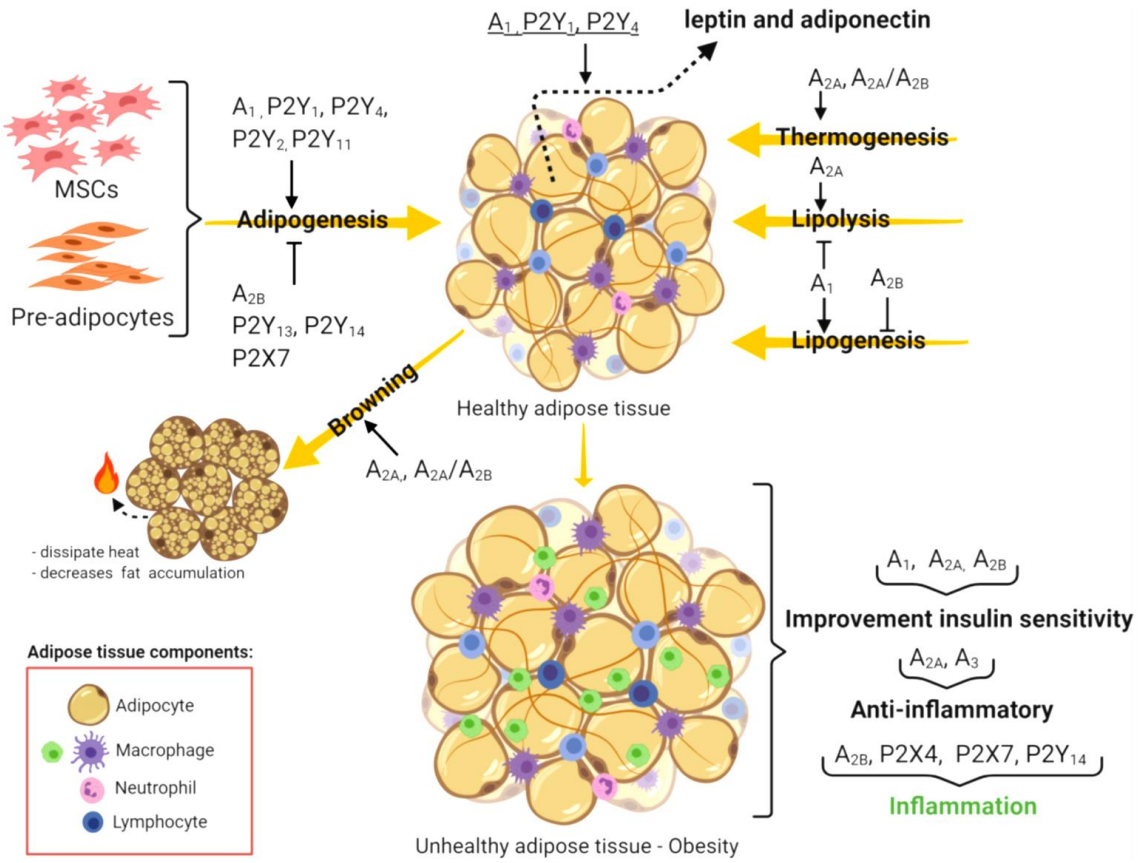

Figure 6. Effect of purinergic receptors in multipotent stem cell (MSC), pre-adipocytes and adipocytes.

Key contribution of the adenosine receptors either inducing or inhibiting adipogenesis, lipolysis, lipogenesis, and browning, but also thermogenesis and insulin sensitivity. Adenosine receptors are, in addition, important regulator in tissue inflammatory environment. Adapted from (de Oliveira et al.*, 2020a)*

1.5.1 Adenosine Receptors (Ado-R)

The adenosine system consists of catabolic enzymes such as ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and CD73, transporters responsible for molecules translocation across cell membranes by equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs). In addition, it bounds to four known subtypes of adenosine receptors (Ado-R) belonging to the superfamily of GPCRs – A1 and A3 Gi inhibitory and A2A and A2B Gs stimulatory each has a unique pharmacological profile, tissue distribution and effector coupling (Jacobson & Gao, 2006*a*; Antonioli *et al.*, 2015; Borea *et al.*, 2018).

1.6 Caffeine

Caffeine is a naturally occurring alkaloid found in varying quantities within the seeds, leaves, and fruits of over 60 different plant species. However, roasted coffee beans and tea leaves from Camellia sinensis are widely acknowledged as the primary sources of dietary caffeine worldwide (Harland, 2000; Heckman *et al.*, 2010). Furthermore, caffeine is the world's most widely consumed drug, known for its capacity to stimulate the central nervous system (CNS)(Martini *et al.*, 2016). Remarkably, recent estimates highlight a substantial global daily consumption of over 50 million cups of coffee, with per capita annual consumption varying from 5.4 to 11.4 kg in European nations (Barcelos *et al.*, 2020). It's noteworthy that contemporary epidemiological studies have suggested potential inverse relationships between coffee consumption and several diseases and conditions, such as type 2 diabetes (Ding *et al.*, 2014), cardiovascular (Ding *et al.*, 2015), and neurodegenerative disorders (Madeira *et al.*, 2017) Caffeine acts via non-selective antagonist of adenosine receptors as presented on (Figure 7).

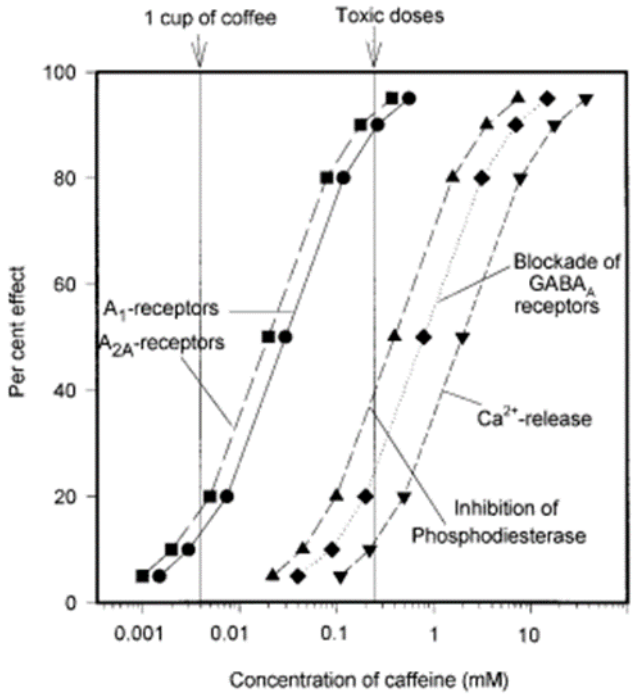

Figure 7. Effects of different concentrations of caffeine on its receptors and targets in humans:

Aat A1 and A2A adenosine receptors, on blockade of GABBAA receptors, on Ca2+ release and on the inhibition of phosphodiesterase. Comparison between the concentrations of caffeine present on coffee cups and its mechanism of action. Image from (Fredholm et al.*, 1999)*

Due to its structural similarity to purines, caffeine and its metabolites operate through mechanisms associated with adenosine receptors, playing crucial roles in cellular energy regulation and inflammation pathways (Svenningsson *et al.*, 1999; Jacobson & Gao, 2006*b*; Guarino *et al.*, 2013*a*; Conde *et al.*, 2017; Melo *et al.*, 2019*a*; Gutiérrez-Hellín *et al.*, 2023). Stimulation of A2A receptors leads to increased cyclic adenosine monophosphate (cAMP) production, potentially mitigating the inflammatory response in various pathophysiological conditions (Chan *et al.*, 2006; Haskó *et al.*, 2008). Although caffeine lacks selectivity as an A2A antagonist, its influence on adenosine receptors can accentuate acute inflammatory responses (Ohta *et al.*, 2007). Experimental models have illustrated that relatively low circulating caffeine concentrations (below 100 µmol/L, equivalent to 1-3 cups of coffee) suppress adenosine's pharmacological effects in animal brains, with the modulatory effects directly related to caffeine dosage (Meeusen *et al.*, 2013).

The impact of caffeine on the body varies depending on whether it is consumed acutely or chronically, especially concerning insulin resistance and glucose intolerance (Guarino *et al.*, 2013*a*; Sacramento *et al.*, 2015). Acute caffeine intake tends to induce insulin resistance, whereas chronic caffeine consumption has the opposite effect, promoting insulin sensitivity and improved glucose tolerance (Panchal *et al.*, 2012; Sacramento *et al.*, 2015). Additionally, chronic caffeine consumption has been associated with weight loss in both animals and humans (Guarino *et al.*, 2013*a*; Bo *et al.*, 2020), an outcome linked to caffeine's ability to boost thermogenesis, lipolysis, and fat oxidation (Lopez-Garcia *et al.*, 2006; Jeukendrup & Randell, 2011).

Mature adipocytes express a large quantity of A1 adenosine receptors that act to inhibit lipolysis by reducing adenylate cyclase activity, indicating that these receptors could be a focal point in the management of obesity and diabetes (Gharibi *et al.*, 2012). Previous reports have demonstrated the therapeutic potential of caffeine in attenuating hypertension, regulating metabolic parameters, and treating hepatic fibrosis key components of metabolic syndrome. For example, the administration of caffeine at a concentration of 0.1% in drinking water led to reduced plasma levels of glucose and insulin in rats subjected to high-sucrose and high-fat diets, accompanied by a decline in mean arterial pressure in both models (Conde *et al.*, 2012*b*).

Furthermore, caffeine reduced the increase in body weight and visceral adiposity observed in rats on a high-fat diet (Conde *et al.*, 2012*b*). Notably, patients undergoing liver biopsy for clinical reasons who exhibited higher daily caffeine intake were associated with milder fibrosis according to the biopsy results (Modi *et al.*, 2010).

Caffeine demonstrates favorable effects on adipose tissue metabolism, not only by ameliorating insulin resistance (Guarino *et al.*, 2013*a*) but also by enhancing thermogenesis and increasing energy expenditure (Acheson *et al.*, 1980; Lopez-Garcia *et al.*, 2006; Jeukendrup & Randell, 2011). Prior research has indicated that chronic caffeine consumption offers numerous benefits to the body, reducing the risk of type 2 diabetes and metabolic syndrome in humans(Tuomilehto *et al.*, 2004; Ohta *et al.*, 2007). These beneficial outcomes were similarly observed in Wistar rats exposed to high-fat (45% lipid-rich diet) and high-sucrose (35% sucrose in drinking water) diets, where chronic caffeine intake prevented the development of insulin resistance and hypertension (Guarino *et al.*, 2013*a*). Interestingly, the prevention of insulin resistance was linked to a significant reduction in circulating catecholamines, including adrenaline and norepinephrine, suggesting that chronic caffeine consumption may counteract the overactivation of the sympathetic nervous system associated with metabolic disorders (Egan, 2003; Guarino *et al.*, 2013*a*).

II. Hypothesis and Objectives

Adenosine is involved in the regulation of white (WAT) and brown (BAT) adipose tissues and implicated in obesity and related diseases, however the specific contribution is still unknow. Moreover, the therapeutics for obesity and type 2 diabetes are scarce and not completely effective.

Therefore, the present work aimed to:

- Investigate if dysmetabolic states are associated with alterations in adenosine receptors and adenosine metabolism in WAT and BAT and the mechanisms behind.
- Explore the impact of chronic caffeine, an antagonist of adenosine receptors, treatment on these mechanisms.

III. Materials and Methods

3.1 Animals and experiments

In this experimental setup, the animals used were male wistar rats (*Rattus norvegicus* L. (Wistar, Crl:WI(Han)), aged 2 months (8-10 months old) and weighting from 200g to 300g acquired to Charles River laboratory and kept in the vivarium of NOVA Medical School | Faculdade de Ciências Médicas (NMS|FCM) of Universidade Nova de Lisboa which is licensed by the Direcção Geral de Alimentação e Veterinária (DGAV).

Animals were kept under controlled conditions namely: temperature and humidity (21 ± 1 °C; 55 ± 10% humidity); a regular 12-hour light-dark rhythm, respectively (08.00–20.00 h) light, (20.00–08.00 h) dark; and food and water available ad libitum.

To determine the sample size a Power analysis was performed, considering a 5% significance criterion and 90% power with a 5% coefficient of variation. Therefore, in this study were used two cohorts of 13 plus 22 animals.

Initially all animals were kept in the same control conditions for the first 9 weeks. Following, these were randomly selected and dived into 3 groups, each group remaining with 4-4-5 in the first cohort or 6-8-8 animals in the second cohort for about 7 months.

The first group consisted of animals that were submitted to a normal chow diet (CTL) (standard diet 7.4% fat+75% carbohydrate (4% sugar) + 17% protein, SDS diets RM1, Probiológica, Portugal). In the second and third groups animals each were fed with a high-fat high-sucrose diet (HFHSu), enriched in 60% of lipids (61.6% fat + 20.3% carbohydrate + 19.1% protein, Test Diets, Missouri, USA) together with the administration of 35% wt./vol. sucrose (Enzymatic, SA, Portugal) in drinking water. After the first 14 weeks one of the HFHSu groups was administered 1g/L caffeine (Sigma - Aldrich Quimica S.L.) supplemented in drinking water (HFHSu+CAFF) for 11 weeks. The HFHSU model mimics the development of obesity, insulin resistance and glucose intolerance and chronic caffeine treatment has been shown to ameliorates whole body insulin resistance (Guarino *et al.*, 2013*b*; Melo *et al.*, 2019*b*). Animals were maintained during the experimental period for 25 weeks (fig.8).

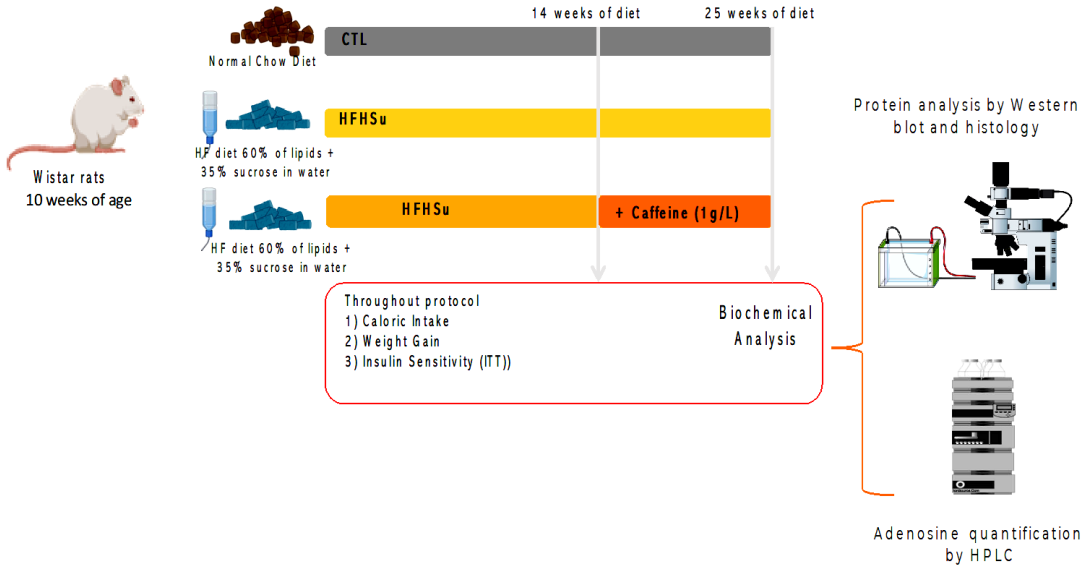

Figure 8. Schematic representation of the protocol of the study.

In all 3 groups caloric and liquid intake were monitored daily and animal behavioural changes were assessed twice per week while handling the animals and food supplying. Additionally metabolic parameters were assessed throughout the experimental protocol: weight gain by body weight, insulin sensitivity and glucose tolerance by an insulin tolerance test (ITT) and an oral glucose tolerance test (OGTT), respectively.

At 25 weeks of diet/protocol, end of experimental period testing, the animals were anesthetized with pentobarbital (60mg/kg i.p.) and blood samples were collected by a cardiac puncture, to analyze insulinemia and other metabolic biomarkers.

Moreover, brains as well as insulin-sensitive tissues - liver, skeletal muscle, and adipose tissues - were collected and weight. Samples for post-mortem analysis were either frozen in liquid nitrogen and stored at -80 °C and processed for High-performance liquid Chromatography (HPLC) and Protein detection or saved in fixative 4% PFA solution to be stored and later processed for histological procedures.

Animals were cared in accordance with the Portuguese law (Decreto-Lei 113/2013) and the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU), also respecting the recommendations of the FELASA (Federation of European Laboratory Animal Science Associations) regarding the health status of laboratory animals. Experimental protocols were approved by the Ethics Committee of the NMS- NOVA Medical School.

3.2 In vivo metabolic profiling analysis

3.2.1 Insulin tolerance test (ITT)

Overall insulin sensitivity was measured through an ITT in conscious animals (Monzillo & Hamdy, 2003; Guarino *et al.*, 2013*c*). The ITT consisted in the intravenous administration of a standard dose of insulin (Humulin® R 100IU/ml, Lilly) bolus of 0.1 U/kg body weight in one of the tail veins, after an overnight fast (approx. 16 hours) and recording basal glucose levels, followed by frequent (every minute) sampling of plasma glucose concentration consequent decline, using the modified tail snip technique, a glucometer (Precision Xtra Meter, Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Portugal) over a 15 minute period (Conde *et al.*, 2012*c*; Ribeiro *et al.*, 2013).

The plasma glucose disappearance rate (KITT) was calculated using the formula $0.693/t^{1/2}$ in which glucose half time ($t^{1/2}$) corresponds to the slope of the least square analysis of plasma glucose concentrations during the linear decay phase (Sacramento *et al.*, 2017).

3.2.2 Oral glucose tolerance test (OGTT)

Glucose tolerance was evaluated through an oral glucose tolerance test. For that, animals were fasted overnight (approx. 16hours) and a bolus of glucose (2g/kg, in a 10 ul/g body weight volume (Sigma, Madrid, Spain)) was administered by oral gavage after establishing basal glycemia at time point 0 and glucose levels ascertained at 15, 30, 60, 120, and 180 minutes post gavage. Blood samples were collected by modified tail snip and glucose plasma levels using a glucometer (Precision Xtra Meter, Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Portugal).

To compare glycemic responses from the plasma glucose oscillation total area under the curve was calculated using the minimum squares method or the trapezoidal method (Trinh *et al.*, 1998; de Moura *et al.*, 2009).

3.3 Ex vivo analysis

3.3.1 HPLC analysis of adenosine in the adipose tissue

Adenosine was quantified in the adipose tissue by HPLC with UV detection at 254 nm as previously described by (Conde & Monteiro, 2004). For that adipose tissue samples were homogenized in PCA 3M and then centrifuged at 13000, 4ºC for 10 min. Supernatant were then neutralized with KOH/Tris and centrifuged again at the same conditions. The HPLC system consisted of a LC 9-A solvent delivery pump (Shimadzu Corporation, Kyoto, Japan), an 7725i injector (Shimadzu Corporation), an SPD-6 AV UV-VIS wavelength detector (Shimadzu Corporation) and Shimadzu Class VP software to analyse the chromatograms. The analytical column was a Lichrospher 100 RP-18 (125 · 4 mm, i.d., particle size 5 lm, Merck, Rahway, NJ, USA) protected by LichroCART 4–4 guard columns (Merck). The columns and guard-columns were incorporated in the HPLC system through a ManuCART (Merck). Isocratic elution was used: the mobile phase consisted of a solution of KH2PO4 100 mM with 15% of methanol, pH 6.5 run at a flux of 1.75 mL/min. Standards were prepared under the same conditions as the biological samples. Calibration curve performed in duplicates was used. The identification of the peaks in the biological samples was made by comparison with the retention times of the standards(Conde *et al.*, 2012*a*).

3.3.2 Western Blot (WB) analysis

3.3.2.1 Adipose Tissue lysate preparation for WB analysis

Visceral WAT (100 mg) and BAT (75 mg) depots was used for WB analysis of protein levels on the adipose tissue. For adipose tissue lysate preparation 1ml of lysis buffer (25 mM Tris-HCL, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1mM EGTA, 50 mM NaF, 25 mM β-glycerolphosfatec, 2.5 mM Pyrophosphate, 2 mM Na3VO4, 1% Triton X-100, 10 mM PMSF, 20ul commercial protease inhibitor cocktail) were added to 300mg of visceral white adipose tissue on an eppendorf tube with a metallic beed. Samples were macerated with TissueLyser (Qiagen) for 10min at 4ºC. Protein extracts were centrifuged twice sequentially for 15-20 minutes at 14.000 xg at 4º C, collecting

supernatant between centrifuges. Also, supernatants were collected, avoiding cellular remains, tissue fibers and lipidic layer. Concerning the difficulty of accurately quantifying adipose tissue proteins, protocol was previously optimized for loading a final sample volume between 15-20ul, although the sample protein concentration would be unknown. Aliquots were stored after addition of 4xLaemmli Buffer. Finally, samples were boiled at 95ºC for 4min and quickly pulse sonicated before loading the western blot gel.

3.3.2.2. Immunobloting

Western Blot (WB) technique is used to relatively quantify specific protein levels extracted from biological samples, cells, and whole tissues. The separation of proteins by SDS-Page electrophoresis is achieved by differences on their molecular weights, where neutral charge and linearized protein structure is attained, and running protein extract through a polyacrylamide electrophoresis gel. Subsequently, the stratified proteins trapped in the matrix gel are transferred to a porous membrane using electroblotting. Later membranes can be probed by stains or highly specific antibodies allowing for the detection of proteins of

interest. Finally, the results are visualized using a chemiluminescence method.

Protein densitometry was performed using ImageLab System, BioRad (version 6.0.1).

3.3.2.3 Polyacrylamide gel electrophoresis (PAGE)

For the Western blot analysis, 16ul of tissue extract were separated by SDS-PAGE using a Tetra cell (Bio-Rad; Hercules, CA, USA), in 10% polyacrylamide separation gel and a 4% polyacrylamide stacking gel, applying a constant voltage of 120 V.

3.3.2.4 Protein blotting, blocking and probing

Bis acrylamide gel separated proteins were transferred to nitrocellulose membranes, using standard wet transfer cassette and tank (Bio-Rad; Hercules, CA, USA), and transfer buffer. To avoid unspecific bonds, membranes were incubated with blocking solution (5% milk) in 1x TBST (20 mM Tris, 136 mM NaCl, 10% Tween 20, pH 7.6) at room temperature for 1 hour 30 min. Primary antibody incubations were carried out overnight (ON) at 4ºC, using concentrations described in table.

Then, membranes were washed and incubated with specie specific secondary antibodies tagged with a Horseradish peroxidase, at the concentration of 1/2000 for 1.5 hours. Detection procedures were carried on according to ECL system (GE Healthcare, Life Sciences; Little Chalfont, UK), and the signal detected using a ChemiDoc™ Imaging Systems (Bio-Rad, Hercules, CA, USA). Membranes were reintubated or cut for the detection of loading control namely Calnexin.

Table 2. Antibodies and its concentrations used for western blot quantification of the proteins of interest.

Protein of interest	Primary Antibody	Secundary antibody
A1 R (37kDa)	Anti-Adenosine A1-R (1:200) (HA1) monoclonal IgM mouse antibody (santacruz: sc-66193)	ECL Goat Anti-Mouse (1:2000) IgG (H + L)-Horseradish Peroxidase Conjugate (Bio-Rad, code: 1706516)

A2A R (42kDa)	Anti-ADO-R A2A (1:200) polyclonal IgG goat antibody (sicgen: AB0318)	ECL Rabbit Anti-Goat (1:2000) IgG (H+L)-Horseradish Peroxidase Conjugate (Bio-Rad, code: 1721034)
A2B R (45kDa)	Anti- Adenosine A2B-R (H-40) (1:200) polyclonal IgG rabbit antibody (santa cruz: sc-28996)	ECL Goat Anti-Rabbit (1:2000) IgG (H + L)-Horseradish Peroxidase Conjugate (Bio-Rad, code: 1706515)
ENT1 (55KDa)	Anti-ENT1 (F-12) (1:100) monoclonal IgG mouse antibody (Santa cruz: sc-377283)	ECL Goat Anti-Mouse (1:2000) IgG (H + L)-Horseradish Peroxidase Conjugate (Bio-Rad, code: 1706516)
CD73 (63KDa)	Anti-CD73 (1:200) polyclonal IgG rabbit antibody (abcam: ab175396)	ECL Goat Anti-Rabbit (1:2000) IgG (H + L)-Horseradish Peroxidase Conjugate (Bio-Rad, code: 1706515)
Calnexin (90kDa)	Anti-CANX (1:1000) polyclonal IgG Goat antibody (sicgen: AB0041)	ECL Rabbit Anti-Goat (1:2000) IgG (H+L)-Horseradish Peroxidase Conjugate (Bio-Rad, code: 1721034)

3.4 Data analysis

Data was analyzed using GraphPad Prism Software, version 8.0.2 (GraphPad Software Inc., San Diego, CA, USA) and results presented as mean values with the standard error of the mean (SEM). The significance of the differences between the groups was calculated by one- way ANOVA followed bypost-hoc Tukey or Sidak multiple comparison tests. Differences were considered significant with 95% confidence interval ($p \leq 0.05$). Experimental groups number of animals was n=6-8.

IV. Results

4.1 Body weight, metabolic profile, and adipose tissues (WAT and BAT): Effects of chronic caffeine treatment in animals fed a hypercaloric diet

Figure 9 shows body weight, basal glycemia, area under the curve of the glycemic curve and insulin sensitivity measured by ITT and expressed as the constant rate of glucose disappearance (K_{ITT}) of CTL, HFHSu or HFHSuCAF animals. We observed that, as expected, HFHSu animals showed a higher body weight gain compared to CTL (CTL: 222.17 ± 24.95 vs. HFHSu: 311.75 ± 18.35 g; **Figura 9A**), and that chronic treatment with caffeine prevented body weight gain (HFHSuCAF: 234.37 ± 13.65 g; **Figure 9A**). Regarding fasting glycemia and the glucose tolerance assessed by the OGTT, the HFHSu group presented higher baseline fasting glycemia (CTL: 86.83 ± 1.8 vs. HFHSu: 104.9 ± 5.66 mg/dL; **Figure 9B**) and greater area under the curve (CTL: 13489 ± 256.7 vs. HFHSu: 17409 ± 482.4 mg/dL*min; **Figure 9C**) compared to CTL. Chronic caffeine treatment for 11 weeks did not change basal glycemis (**Figure 9B**) and attenuated in a non-significant way glucose tolerance (HFHSuCAF: 16.464 ± 379.8 mg/dL*min; p = 0.23) Insulin sensitivity assessed by ITT demonstrated that HFHSu animals exhibit insulin resistance compared to CTL animals (CTL: 4.04 ± 0.23 vs. HFHSu: 1.85 ± 0.11% glucose/min; **Figure 9D**). Caffeine treatment improved insulin sensitivity (HFHSuCAF: 3.56 ± 0.30; **Figure 9D**), although no significant effects were observed on fasting blood glucose (HFHSuCAF: 108.0 ± 1.86; **Figure 9B**) and AUC (16.464 ± 379.8 mg/dL*min; **Figure 9C**).

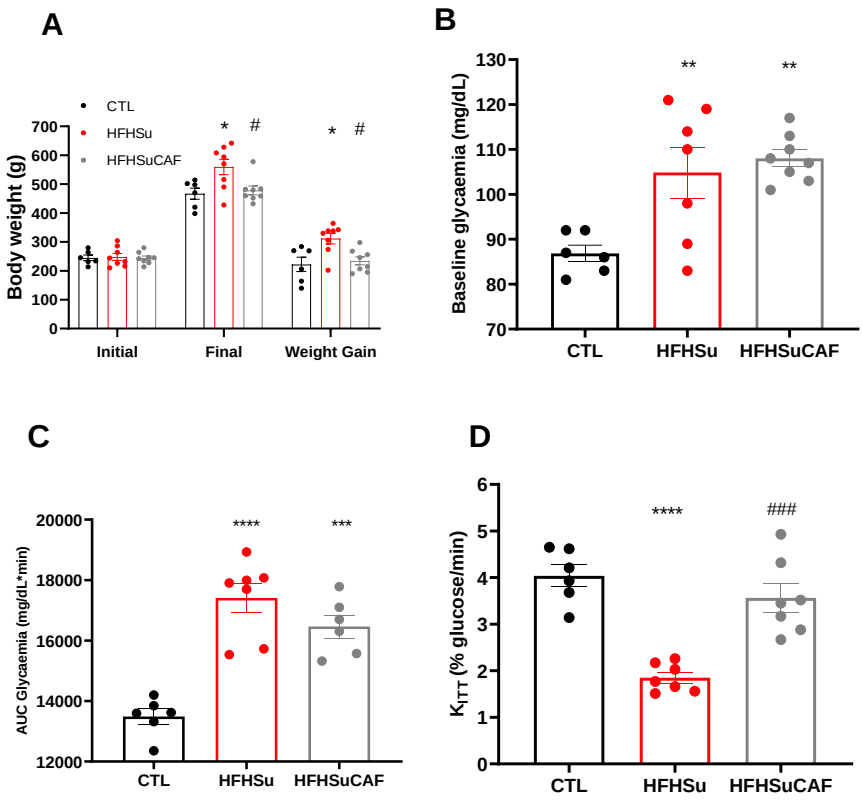

Figure 9. Effects of the hypercaloric HFHSu diet and chronic caffeine treatment (1g/L) on metabolic variables.

*A) shows the impact of chronic caffeine on body weight (A); fasting plasma glucose (B); glucose tolerance (C), evaluated by the oral glucose tolerance test (OGTT); and insulin sensitivity (D), assessed by the insulin tolerance test (ITT) and expressed as the constant rate of glucose disappearance (KITT). Values are represented as mean ± SEM. One-way ANOVA with Tukey's multiple comparison tests; *p <0.01, *p <0.001, ***p <0.0001, ****p <0.0001 vs. CTL group; #p <0.01, ###p <0.0001 vs. HFHSu group.*

The effects of chronic caffeine treatment on white adipose tissue (WAT) and brown adipose tissue (BAT) in HFHSu or CTL animals are shown in **Figure 10**. As expected, animals fed the high-calorie diet showed increases in visceral adipose tissue (CTL: 15.98 ± 1.4 vs. HFHSu: 23.95 ± 1.6 g/kg; **Figure 10A**) and perinephric (CTL: 30.89 ± 1.36 vs. HFHSu: 55.15 ± 4.8 g/kg; **Figure 10A**), resulting in a higher total WAT (CTL: 75.86 ± 4.0 vs. HFHSu: 118.2 ± 8.90 g/kg; **Figure 10A**). Interestingly, the HFHSuCAF group showed lower visceral (15.91 ± 0.85 g/kg; **Figure 10A**) and perinephric (41.77 ± 1.8 g/kg; **Figure 10A**) fat deposits after chronic caffeine treatment, resulting in a lower total WAT (HFHSuCAF: 85.18 ± 5.66 g/kg; **Figure 10A**). No significant effects on BAT were observed between groups (CTL: 1.14 ± 0.1 vs. HFHSu: 1.45 ± 0.15 vs. HFHSuCAF: 1.19 ± 0.11 g/kg; **Figure 10B**).

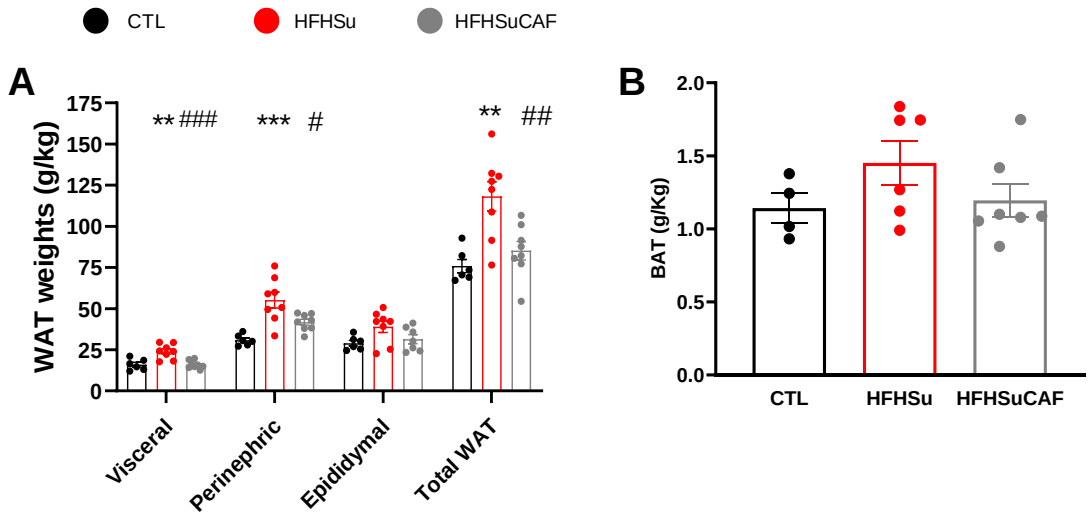

Figure 10. Effects of the hypercaloric HFHSu diet and chronic caffeine treatment (1 g/L)

*On white adipose tissues (WAT) (A) and brown adipose tissues (BAT) (**B**) weights. Values are represented as mean ± SEM. One-way ANOVA with Tukey's multiple comparison tests; *p <0.01, **p <0.001, ***p <0.0001, ****p <0.0001 vs. CTL group; #p <0.05; ##p <0.001; ###p <0.0001 vs. HFHSu group.*

4.2 Biochemical analysis of adenosine tissue content, adenosinergic signaling and its metabolism: Effects of chronic caffeine treatment on in animals fed a hypercaloric diet

Adenosine content in visceral WAT and BAT are shown in **Figure 11**. Animals fed the hypercaloric diet show reduced ADO levels in WAT (CTL: 1.03 ± 0.11 vs. HFHSu: 0.54 ± 0.10 nmol/g tissue; **Figure 11A**), which were restored by chronic caffeine treatment (HFHSuCAF: 0.95 ± 0.12 nmol/g tissue; **Figure 11A**). Additionally, caffeine treatment also increased ADO levels in BAT (HFHSuCAF: 6.68 ± 0.72 nmol/g tissue; **Figure 11B**).

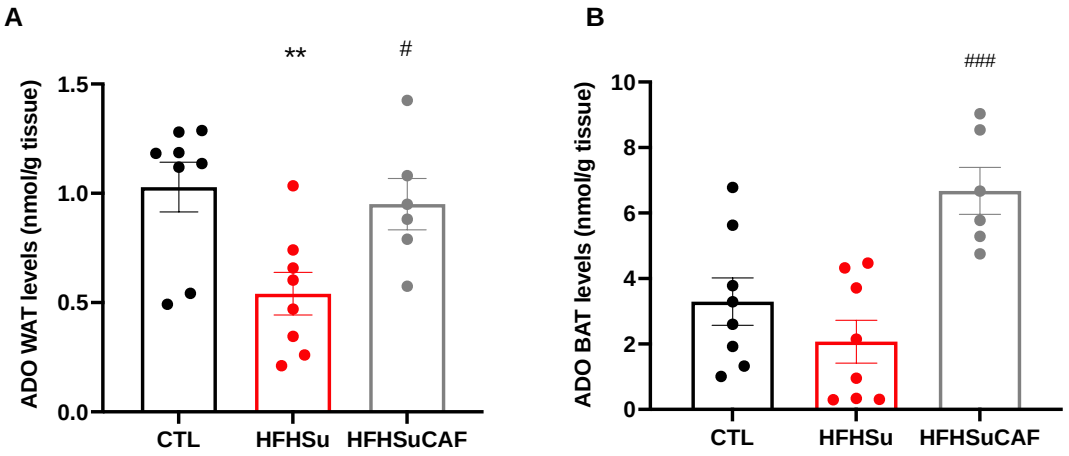

Figure 11. Effects of the hypercaloric diet and chronic caffeine treatment (1g/L) on adenosine (ADO) levels, measured by HPLC:

*In visceral white adipose tissue (WAT) visceral (**A**) and brown adipose tissue (BAT) (**B**). Values are represented as mean ± SEM. One-way ANOVA with Sidak's multiple comparison tests; **p <0.001 vs. CTL group; ##p <0.001; ###p <0.0001 vs. HFHSu group.*

The adenosine signaling pathway in visceral WAT and BAT was evaluated from the quantification of the levels of A_1R, $A_{2A}R$, $A_{2B}R$, ENT1 and CD73 and are shown in **Figures 12** and **13**, respectively. In visceral WAT, HFHSu animals showed a reduction in $A_{2A}R$ levels (CTL: 100.0 ± 6.0 vs. HFHSu: 66.26 ± 8.57%, **Figure 12B**), with no impact on A_1R, ENT1 and CD73 levels (**Figures 12A**, **12D** and **12E** respectively). However, $A_{2B}R$ levels appear to be non-significantly reduced in HFHSu animals compared to the CTL group (CTL: 100.0 ± 10.58 vs. HFHSu: 67.80 ± 7.42%; p = 0.061; **Figure 12C**). Caffeine restored the levels of A2A and A2B adenosine receptors, although without statistical significance (HFHSuCAF$A_{2A}R$ levels: 90.76 ± 13.54, p = 0.18; $A_{2B}R$ levels: 100.3 ± 13.40, p =0.99) and without affecting A_1R levels. Caffeine treatment increased significantly CD73 levels by 42% (HFHSuCAF: 142.4 ± 9.99%. **Figure 12E**), although it did not change the levels of ENT1 (**Figures 12A**, **12B**, **12C** and **12D**, respectively).

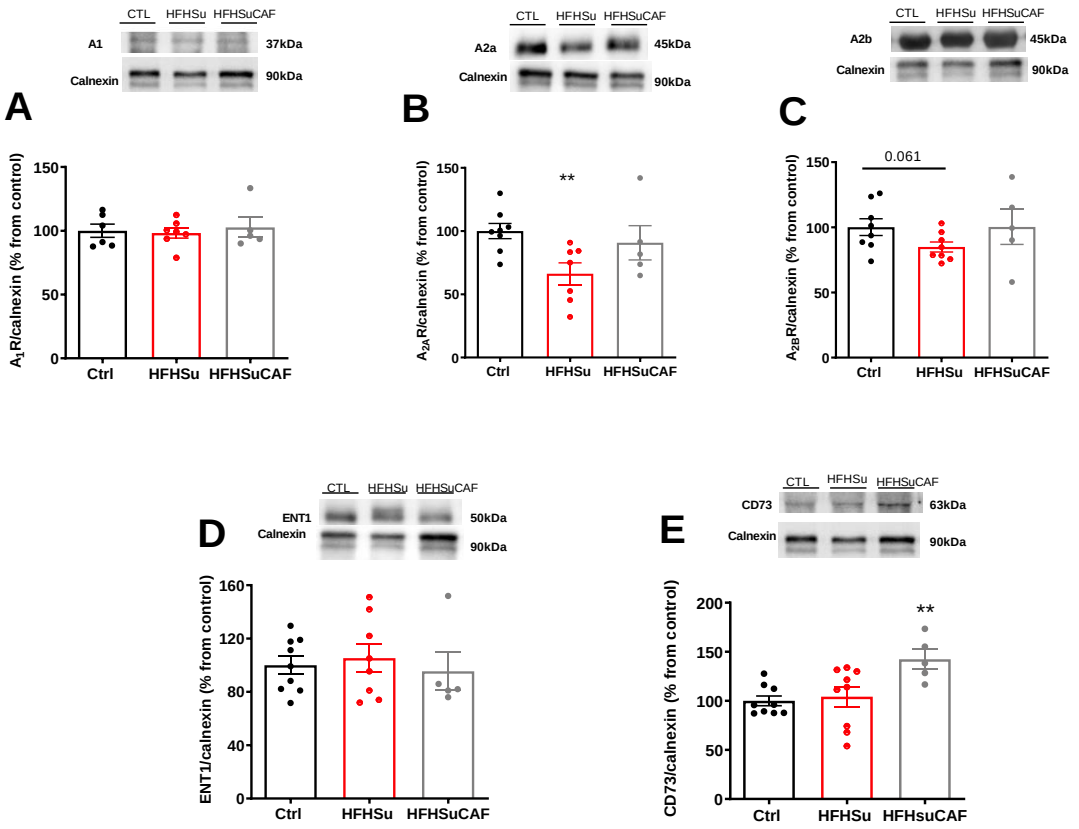

Figure 12. Effects of the hypercaloric HFHSu diet and chronic caffeine treatment (1g/l) on protein levels of receptors

*A_1 (**A**), A_{2A} (**B**), A_{2B} (**C**); equilibrative nucleoside transporter (ENT1) (**D**); cluster of differentiation 73 (CD73) (**E**) in white adipose tissue (WAT) visceral. Values are represented as mean ± SEM. One-way ANOVA with Sidak's multiple comparison tests; **p <0.001vs. CTL group.*

Regarding the adenosinergic system in BAT, animals in the HFHSu group showed increases in the levels of A_2AR (CTL: 100.0 ± 7.24 vs. HFHSu: 169.5 ± 17.68%; **Figure 13B**), ENT1 (CTL: 100.0 ± 11.34 vs. HFHSu : 159.4 ± 17.93%; **Figure 13D**) and CD73 (CTL: 100.0 ± 1.87 vs. HFHSu: 195.1 ± 20.38; **Figure 13E**), and a reduction in $A_{2B}R$ levels (CTL: 100.0 ± 10.58 vs. HFSu: 67.80 ± 7.42%; **Figure 13C**), without significant changes in A_1R levels (**Figure 13A**). Chronic caffeine treatment res the levels of $A_{2A}R$ (HFHSuCAF: 106.7 ± 14.25%; **Figure 13B**), ENT1 (HFHSuCAF: 69.78 ± 11.05%; **Figure 13D**) and CD73 (HFHSuCAF: 123.1 ± 16.34; **Figure 13E**). Additionally, caffeine treatment increased $A_{2B}R$ levels (HFHSuCAF: 109.8 ± 15.78%; Figure 5C), without impacting A_1R levels (**Figure 13A**).

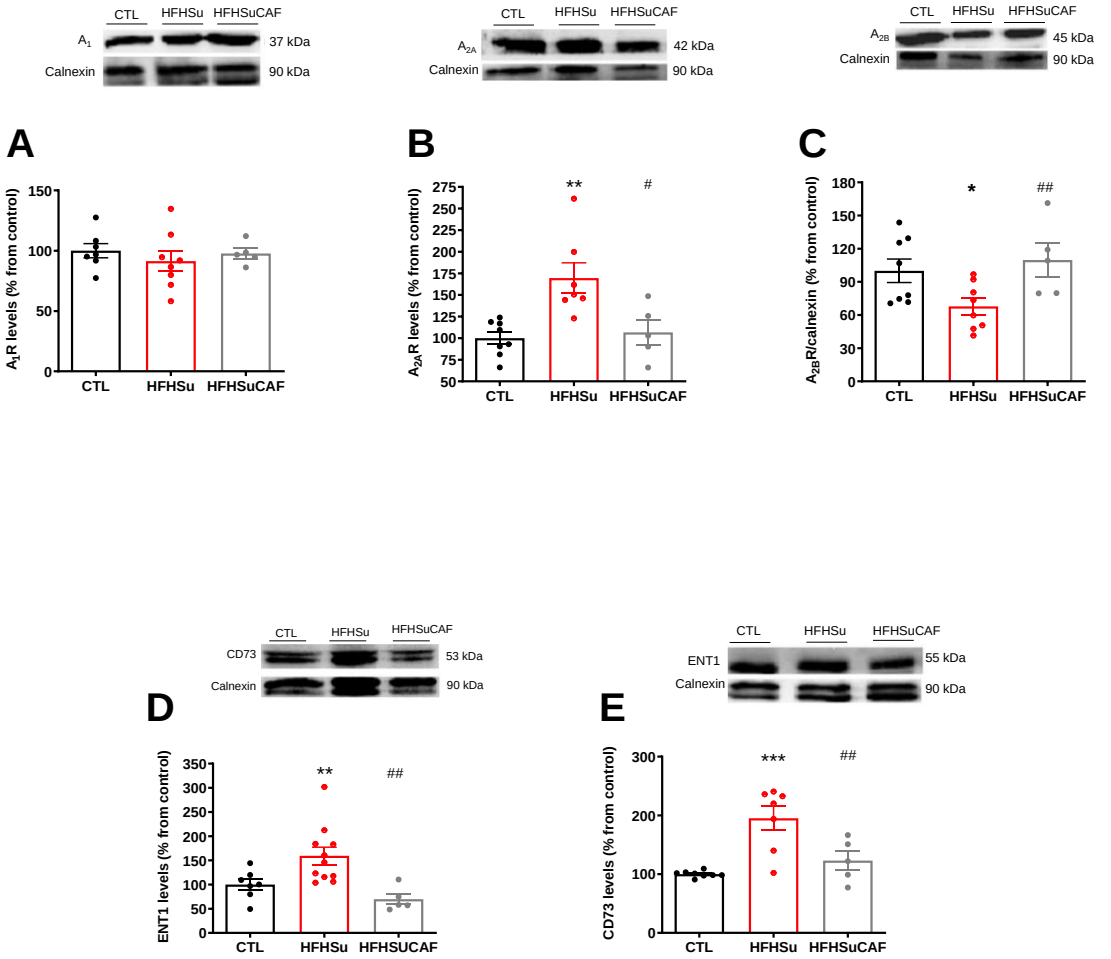

Figure 13. Effects of the hypercaloric HFHSu diet and chronic caffeine treatment (1g/l) on protein levels of receptors

*A_1 (**A**), A_{2A} (**B**), A_{2B} (**C**); equilibrative nucleoside transporter (ENT1) (**D**); cluster of differentiation 73 (CD73) (**E**) in brown adipose tissue (BAT). Values are represented as mean ± SEM. One-way ANOVA with Sidak's multiple comparison tests; **p <0.001vs. CTL group.*

V. Discussion

Herein we found that hypercaloric diets promote metabolic dysfunction, transduced in an increase in weight gain, insulin resistance and glucose intolerance as well as an increase in adipose tissue depots, effects that are associated with profound changes in the adenosinergic system. Namely, we found that hypercaloric diet promoted a huge decrease in adenosine content of WAT and BAT as well as changes in the levels of A2 and A2B adenosine receptors and in key enzymes and transporters of adenosine metabolism. In agreement with this key role of adenosynergic system dysfunction to metabolic diseases chronic treatment of caffeine was able to ameliorate glucose metabolism and totally reverse insulin resistance and weigh gain by affecting adenosinergic systems in WAT and BAT.

5.1 Impact of hypercaloric diets and of chronic caffeine consumption on glucose metabolism and insulin action

Considering the importance of metabolic disorders, several studies have been conducted with different animal models to elucidate the mechanisms underlying these diseases (Kasiske *et al.*, 1992; Winzell & Ahrén, 2004; Peterson *et al.*, 2015; Melo *et al.*, 2019*a*). Previous data of our group demonstrated that hypercaloric diet enriched in 60% of lipids and 35% of sucrose for 25 weeks, increases the body weight, mean blood pressure, glycemia, deposition of lipids in the liver and promotes insulin resistance in wistar rats (Melo *et al.*, 2019*a*). The present study corroborates (Melo et al., 2019), as we demonstrate herein that the same hypercaloric diet was able to promote weight gain, the increase in the different adipose tissue depots, insulin resistance and glucose intolerance. Moreover, we show herein that chronic caffeine treatment was able to ameliorate or even reverse these pathological features.

The link between caffeine consumption and insulin resistance (IR) has been documented in a wide-range population studies (Jain & Jacobson, 2021); (van Dam *et al.*, 2004; Shi *et al.*, 2016). Previous works concerning the link between caffeine and IR have indicated that acute caffeine consumption is connected with heightened insulin

resistance (Keijzers *et al.*, 2002; Arnlöv *et al.*, 2004; Emami *et al.*, 2019), whereas habitual coffee consumption has been associated with improved insulin sensitivity and a safeguarding effect against the risk of type 2 diabetes (Tuomilehto *et al.*, 2004; Bhupathiraju *et al.*, 2013).

These effects of chronic caffeine treatment are not new, as the of caffeine consumption on insulin resistance has been widely described in animal models and in humans. For example, (Guarino et al., 2013) demonstrated that the chronic treatment with 1g/L of caffeine in drinking water for 15 days was able to improve the insulin sensitivity in old wistar rats with 12 and 24 months of age. Additionally, caffeine treatment for 2 weeks prevented insulin resistance, hypertension and decreased visceral fat induced by high sucrose and high fat diet in male and female rats (Conde *et al.*, 2012*b*). Moreover, several studies in humans have showed that coffee consumption improves glucose metabolism (Reis *et al.*, 2019; Hou *et al.*, 2022)X). Therefore, this study reinforces the existing literature's findings regarding the positive impact of caffeine on.

5.2 Effects of hypercaloric diets and chronic caffeine intake on white and brown adipose tissue depots and adenosinergyc system

Here we show that hypercaloric diets as expected increase the depots of adipose tissue adenosine rHerein we showed that chronic caffeine intake reduced both overall body fat mass and abdominal fat deposits, which is consistent with the decrease in weight herein observed. Caffeine acts as an antagonist at A1 and A2 adenosine receptors, which are abundant in adipose tissue. By blocking these receptors, caffeine inhibits the suppressive effects of adenosine on lipolysis. Several studies have demonstrated caffeine's role in enhancing lipolysis, as evidenced by increased plasma concentrations of non-esterified fatty acids (NEFA) after caffeine consumption (Acheson *et al.*, 1980; Arciero *et al.*, 1995).

The adipocyte plays a pivotal role in the regulation of overall metabolism, whereby white adipocytes release a variety of substances that control eating habits, systemic

insulin sensitivity, and the body's response to inflammation (Hill, 2015; Kahn *et al.*, 2019). Unlike white adipocytes, which possess a single large lipid droplet, brown and beige adipocytes contain multiple smaller droplets and a high concentration of mitochondria. This mitochondrial abundance enables the dissipation of energy through uncoupled mitochondrial respiration, a characteristic that has the potential to be harnessed in the fight against obesity(Cypess & Kahn, 2010; Nedergaard *et al.*, 2011).

One of the key observations in this study is that caffeine effectively reversed the increase in total WAT weight induced by the HFHSu diet. This reversal was primarily driven by reductions in visceral and perinephric adipose tissue depots, which are considered critical contributors to metabolic syndrome and its associated complications (Nedergaard *et al.*, 2011).

(Acheson *et al.*, 1980) conducted a study on both normal-weight and obese individuals, investigating the effects of caffeine on metabolic rate and substrate utilization. They found that caffeine intake led to an increase in metabolic rate and enhanced substrate utilization, indicating a potential role of caffeine in influencing energy expenditure and fat utilization in adipose tissue. Furthermore, (Arciero *et al.*, 1995) examined the impact of caffeine ingestion on fat oxidation and energy expenditure in younger and older men. Their findings demonstrated that caffeine increased fat oxidation, suggesting a potential role in promoting the breakdown of stored fats within WAT.

These studies collectively suggest that caffeine may modulate metabolic processes in WAT, making it a subject of interest in the context of weight management. Additionally, recent studies have explored purinergic signaling in obesity, highlighting the relevance of adenosine receptors in adipose tissue and their potential as therapeutic targets(D'Antongiovanni *et al.*, 2021). The reduction in visceral adiposity is especially significant, as it is strongly linked to insulin resistance (Guarino *et al.*, 2013*a*) and may contribute to explaining the capacity of caffeine to reverse IR observed in our study.

Very recently, there have been reports indicating that adenosine receptors may have a central role in addressing obesity and metabolic disorders(de Oliveira *et al.*, 2020*b*; D'Antongiovanni *et al.*, 2021). Activation of A2A and A2B receptors using specific agonists has been shown to enhance lipolysis and induce thermogenesis in BAT, thereby

providing protection against diet-induced obesity in mice (Gnad *et al.*, 2014*b*). Increasing thermogenesis through the metabolic activity of BAT is being considered as a potential pharmacological approach to combat the energy imbalance that contributes to weight gain and obesity (Gnad *et al.*, 2014*b*).

5.3 Role of Adenosine Receptors in Weight

The effect of adenosine and its receptors in weight and body fat deposition are not consensual. On one hand some studies show that the loss of adenosine A1 and A2B receptors promote an increase in weight gain and fat deposition (Faulhaber-Walter *et al.*, 2011; Csóka *et al.*, 2014). However in in this study the pharmacological inhibition of the adenosine receptors presents as beneficial, restoring weight gain to control values consistent with studies that associate long term caffeine intake with weight loss both in humans and rats. (Greenberg *et al.*, 2006)

From previous research in our lab (Sacramento *et al.*, 2020), we showed that in control diet animals treated with selective Ado-R antagonists a paradoxical effect occurs when comparing female and male rats. In females the use of A1 inhibitor promotes weight loss, however in males the inhibition of A2B receptor promotes weight gain.

In this current study we cannot directly correlate which individual Ado-R is responsible for the blockage of weight gain. A1 is unlikely to be correlated as there are no alterations in protein levels consistent with the studies that showed higher impact of this subtype specifically in females. Concerning A2 we only know that protein levels are restored to control values with caffeine treatment as no possible comparison can be made from the use of selective inhibitor in control animals vs. HFHSu diet fed males treated with caffeine. These last showing disrupted protein levels induced by diet.

Therefore, a possible selective inhibition of the Ado-R should clarify the specific effects of the receptor without the confounding changes of the diet used by (Sacramento *et al.*, 2020), another dysmetabolic model.

VI. Concluding remarks

We can conclude that alterations in Ado receptor levels and Ado metabolism in adipose tissues are associated with metabolic dysfunction and that the modulation of Ado receptors with chronic caffeine intake can be useful to improve dysmetabolic states. Although further, more exhaustive, research must be conducted in the future.

References

Acheson KJ, Zahorska-Markiewicz B, Pittet P, Anantharaman K & Jéquier E (1980). Caffeine and coffee: their influence on metabolic rate and substrate utilization in normal weight and obese individuals. *Am J Clin Nutr* **33,** 989–997.

ADA (2023). "American Diabetes Association." Available at: https://diabetes.org/diabetes [Accessed September 1, 2023].

ANAND BK & BROBECK JR (1951). Hypothalamic Control of Food Intake in Rats and Cats. *Yale J Biol Med* **24,** 123.

Anon (2021*a*). IDF Diabetes Atlas, 10th edn. International Diabetes Federation, Brussels, Belgium. Available at: https://www.diabetesatlas.org [Accessed September 2, 2023].

Anon (2021*b*). Obesity and overweight. Available at: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight [Accessed September 1, 2023].

Anon (2022). World Obesity Atlas 2022. World Obesity Federation, London. Available at: https://s3-eu-west-1.amazonaws.com/wof-files/World_Obesity_Atlas_2022.pdf [Accessed September 1, 2023].

Anon (2023). *World Obesity Atlas 2023*. World Obesity Federation. Available at: https://www.worldobesity.org/resources/resource-library/world-obesity-atlas-2023 [Accessed September 1, 2023].

Antonioli L, Blandizzi C, Csóka B, Pacher P & Haskó G (2015). Adenosine signalling in diabetes mellitus--pathophysiology and therapeutic considerations. *Nat Rev Endocrinol* **11,** 228–241.

Arciero PJ, Gardner AW, Calles-Escandon J, Benowitz NL & Poehlman ET (1995). Effects of caffeine ingestion on NE kinetics, fat oxidation, and energy expenditure in younger and older men. *Am J Physiol* **268,** E1192-8.

Arnlöv J, Vessby B & Risérus U (2004). Coffee consumption and insulin sensitivity. *JAMA* **291,** 1199–1201.

Arocha Rodulfo JI (2019). Sedentarismo, la enfermedad del siglo xxi. *Clínica e Investigación en Arteriosclerosis* **31,** 233–240.

Barcelos RP, Lima FD, Carvalho NR, Bresciani G & Royes LF (2020). Caffeine effects on systemic metabolism, oxidative-inflammatory pathways, and exercise performance. *Nutrition Research* **80,** 1–17.

Bhupathiraju SN, Pan A, Malik VS, Manson JE, Willett WC, van Dam RM & Hu FB (2013). Caffeinated and caffeine-free beverages and risk of type 2 diabetes. *Am J Clin Nutr* **97,** 155–166.

Blüher M (2019). Obesity: global epidemiology and pathogenesis. *Nat Rev Endocrinol* **15,** 288–298.

Bo S, Fadda M, Fedele D, Pellegrini M, Ghigo E & Pellegrini N (2020). A Critical Review on the Role of Food and Nutrition in the Energy Balance. *Nutrients*; DOI: 10.3390/nu12041161.

Borea PA, Gessi S, Merighi S, Vincenzi F & Varani K (2018). Pharmacology of Adenosine Receptors: The State of the Art. *Physiol Rev* **98,** 1591–1625.

Bugianesi E, McCullough AJ & Marchesini G (2005). Insulin resistance: a metabolic pathway to chronic liver disease. *Hepatology* **42,** 987–1000.

Burnstock G (2007). Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* **87,** 659–797.

Caprio S, Daniels SR, Drewnowski A, Kaufman FR, Palinkas LA, Rosenbloom AL & Schwimmer JB (2008). Influence of Race, Ethnicity, and Culture on Childhood Obesity: Implications for Prevention and Treatment: A consensus statement of Shaping America's Health and the Obesity Society. *Diabetes Care* **31,** 2211.

Capucho AM, Chegão A, Martins FO, Miranda HV & Conde S V. (2022). Dysmetabolism and Neurodegeneration: Trick or Treat? *Nutrients*; DOI: 10.3390/NU14071425.

Cerf ME (2013). Beta Cell Dysfunction and Insulin Resistance. *Front Endocrinol (Lausanne)*; DOI: 10.3389/FENDO.2013.00037.

Di Cesare M et al. (2016). Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. *Lancet* **387,** 1377–1396.

Chait A & den Hartigh LJ (2020). Adipose Tissue Distribution, Inflammation and Its Metabolic Consequences, Including Diabetes and Cardiovascular Disease. *Front Cardiovasc Med*; DOI: 10.3389/FCVM.2020.00022.

Chan ESL, Montesinos MC, Fernandez P, Desai A, Delano DL, Yee H, Reiss AB, Pillinger MH, Chen J-F, Schwarzschild MA, Friedman SL & Cronstein BN (2006). Adenosine A 2A receptors play a role in the pathogenesis of hepatic cirrhosis. *Br J Pharmacol* **148,** 1144–1155.

Chatterjee S, Khunti K & Davies MJ (2017). Type 2 diabetes. *Lancet* **389,** 2239–2251.

Chooi YC, Ding C & Magkos F (2019). The epidemiology of obesity. *Metabolism* **92,** 6–10.

Conde S V. & Monteiro EC (2004). Hypoxia induces adenosine release from the rat carotid body. *J Neurochem* **89,** 1148–1156.

Conde S V., Monteiro EC, Rigual R, Obeso A & Gonzalez C (2012*a*). Hypoxic intensity: a determinant for the contribution of ATP and adenosine to the genesis of carotid body chemosensory activity. *J Appl Physiol (1985)* **112,** 2002–2010.

Conde S V., Monteiro EC & Sacramento JF (2017). Purines and Carotid Body: New Roles in Pathological Conditions. *Front Pharmacol*; DOI: 10.3389/fphar.2017.00913.

Conde S V., Nunes Da Silva T, Gonzalez C, Mota Carmo M, Monteiro EC & Guarino MP (2012*b*). Chronic caffeine intake decreases circulating catecholamines and prevents diet-induced insulin resistance and hypertension in rats. *Br J Nutr* **107,** 86–95.

Conde S V., Nunes Da Silva T, Gonzalez C, Mota Carmo M, Monteiro EC & Guarino MP (2012*c*). Chronic caffeine intake decreases circulating catecholamines and prevents diet-induced insulin resistance and hypertension in rats. *Br J Nutr* **107,** 86–95.

Csóka B, Koscsó B, Töro G, Kókai E, Virág L, Németh ZH, Pacher P, Bai P & Haskó G (2014). A2B adenosine receptors prevent insulin resistance by inhibiting adipose tissue inflammation via maintaining alternative macrophage activation. *Diabetes* **63,** 850–866.

Cypess AM & Kahn CR (2010). The role and importance of brown adipose tissue in energy homeostasis. *Curr Opin Pediatr* **22,** 478–484.

van Dam RM, Dekker JM, Nijpels G, Stehouwer CDA, Bouter LM, Heine RJ & Hoorn study (2004). Coffee consumption and incidence of impaired fasting glucose, impaired glucose tolerance, and type 2 diabetes: the Hoorn Study. *Diabetologia* **47,** 2152–2159.

D'Antongiovanni V, Fornai M, Pellegrini C, Blandizzi C & Antonioli L (2021). Managing Obesity and Related Comorbidities: A Potential Pharmacological Target in the Adenosine System? *Front Pharmacol*; DOI: 10.3389/fphar.2020.621955.

DeFronzo RA (2004). Pathogenesis of type 2 diabetes mellitus. *Medical Clinics of North America* **88,** 787–835.

Ding M, Bhupathiraju SN, Chen M, van Dam RM & Hu FB (2014). Caffeinated and Decaffeinated Coffee Consumption and Risk of Type 2 Diabetes: A Systematic Review and a Dose-Response Meta-analysis. *Diabetes Care* **37,** 569–586.

Ding M, Satija A, Bhupathiraju SN, Hu Y, Sun Q, Han J, Lopez-Garcia E, Willett W, van Dam RM & Hu FB (2015). Association of Coffee Consumption With Total and Cause-Specific Mortality in 3 Large Prospective Cohorts. *Circulation* **132,** 2305–2315.

Duncan RE, Ahmadian M, Jaworski K, Sarkadi-Nagy E & Sul HS (2007). Regulation of lipolysis in adipocytes. *Annu Rev Nutr* **27,** 79–101.

Egan BM (2003). Insulin resistance and the sympathetic nervous system. *Curr Hypertens Rep* **5,** 247–254.

Emami MR, Khorshidi M, Zarezadeh M, Safabakhsh M, Rezagholizadeh F & Alizadeh S (2019). Acute effects of caffeine ingestion on glycemic indices: A systematic review and meta-analysis of clinical trials. *Complement Ther Med* **44,** 282–290.

Fain JN, Pointer RH & Ward WF (1972). Effects of adenosine nucleosides on adenylate cyclase, phosphodiesterase, cyclic adenosine monophosphate accumulation, and lipolysis in fat cells. *J Biol Chem* **247,** 6866–6872.

Fasshauer M & Blüher M (2015). Adipokines in health and disease. *Trends Pharmacol Sci* **36,** 461–470.

Faulhaber-Walter R, Jou W, Mizel D, Li L, Zhang J, Kim SM, Huang Y, Chen M, Briggs JP, Gavrilova O & Schnermann JB (2011). Impaired Glucose Tolerance in the Absence of Adenosine A1 Receptor Signaling. *Diabetes* **60,** 2578.

Fredholm BB, Bättig K, Holmén J, Nehlig A & Zvartau EE (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol Rev* **51,** 83–133.

Fukagawa NK, Minaker KL, Rowe JW, Goodman MN, Matthews DE, Bier DM & Young VR (1985). Insulin-mediated reduction of whole body protein breakdown. Dose-response effects on leucine metabolism in postabsorptive men. *J Clin Invest* **76,** 2306–2311.

Gaio V, Antunes L, Namorado S, Barreto M, Gil A, Kyslaya I, Rodrigues AP, Santos A, Bøhler L, Castilho E, Vargas P, do Carmo I, Nunes B & Dias CM (2018). Prevalence of overweight and obesity in Portugal: Results from the First Portuguese Health Examination Survey (INSEF 2015). *Obes Res Clin Pract* **12,** 40–50.

Galicia-Garcia U, Benito-Vicente A, Jebari S, Larrea-Sebal A, Siddiqi H, Uribe KB, Ostolaza H & Martín C (2020). Pathophysiology of Type 2 Diabetes Mellitus. *Int J Mol Sci* **21,** 1–34.

Gastaldelli A, Gaggini M & DeFronzo RA (2017). Role of Adipose Tissue Insulin Resistance in the Natural History of Type 2 Diabetes: Results From the San Antonio Metabolism Study. *Diabetes* **66,** 815–822.

Gharibi B, Abraham AA, Ham J & Evans BAJ (2012). Contrasting effects of A1 and A2b adenosine receptors on adipogenesis. *Int J Obes (Lond)* **36,** 397–406.

Gnad T et al. (2014*a*). Adenosine activates brown adipose tissue and recruits beige adipocytes via A 2A receptors. *Nature*; DOI: 10.1038/nature13816.

Gnad T et al. (2014*b*). Adenosine activates brown adipose tissue and recruits beige adipocytes via A 2A receptors. *Nature*; DOI: 10.1038/nature13816.

Golay A & Ybarra J (2005). Link between obesity and type 2 diabetes. *Best Pract Res Clin Endocrinol Metab* **19,** 649–663.

Greenberg JA, Boozer CN & Geliebter A (2006). Coffee, diabetes, and weight control. *Am J Clin Nutr* **84,** 682–693.

Griffin ME, Marcucci MJ, Cline GW, Bell K, Barucci N, Lee D, Goodyear LJ, Kraegen EW, White MF & Shulman GI (1999). Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes* **48,** 1270–1274.

Guarino MP, Ribeiro MJ, Sacramento JF & Conde S V. (2013*a*). Chronic caffeine intake reverses age-induced insulin resistance in the rat: effect on skeletal muscle Glut4 transporters and AMPK activity. *Age (Omaha)* **35,** 1755.

Guarino MP, Ribeiro MJ, Sacramento JF & Conde S V. (2013*b*). Chronic caffeine intake reverses age-induced insulin resistance in the rat: effect on skeletal muscle Glut4 transporters and AMPK activity. *Age (Dordr)* **35,** 1755–1765.

Guarino MP, Santos AI, Mota-Carmo M & Costa PF (2013*c*). Effects of anaesthesia on insulin sensitivity and metabolic parameters in Wistar rats. *In Vivo* **27,** 127–132.

Guilherme A, Virbasius J V., Puri V & Czech MP (2008). Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol* **9,** 367–377.

Guo S (2014). Insulin Signaling, Resistance, and the Metabolic Syndrome: Insights from Mouse Models to Disease Mechanisms. *J Endocrinol* **220,** T1.

Gutiérrez-Hellín J, Aguilar-Navarro M, Ruiz-Moreno C, Muñoz A, Varillas-Delgado D, Amaro-Gahete FJ & Del Coso J (2023). Effect of caffeine intake on fat oxidation rate during exercise: is there a dose–response effect? *Eur J Nutr* **62,** 311–319.

Harland BF (2000). Caffeine and nutrition. *Nutrition* **16,** 522–526.

Haskó G, Linden J, Cronstein B & Pacher P (2008). Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov* **7,** 759.

Heckman MA, Weil J & Gonzalez de Mejia E (2010). Caffeine (1, 3, 7-trimethylxanthine) in foods: a comprehensive review on consumption, functionality, safety, and regulatory matters. *J Food Sci* **75,** R77-87.

Hermida MA, Dinesh Kumar J & Leslie NR (2017). GSK3 and its interactions with the PI3K/AKT/mTOR signalling network. *Adv Biol Regul* **65,** 5–15.

Heymsfield SB & Wadden TA (2017). Mechanisms, Pathophysiology, and Management of Obesity. *N Engl J Med* **376,** 254–266.

Hill BG (2015). Insights into an adipocyte whitening program. *Adipocyte* **4,** 75–80.

Hou C, Zeng Y, Chen W, Han X, Yang H, Ying Z, Hu Y, Sun Y, Qu Y, Fang F & Song H (2022). Medical conditions associated with coffee consumption: Disease-trajectory and comorbidity network analyses of a prospective cohort study in UK Biobank. *American Journal of Clinical Nutrition* **116,** 730–740.

Hu FB (2008). *Obesity epidemiology*. Oxford University Press, Oxford ; New York.

Huang X, Liu G, Guo J & Su ZQ (2018). The PI3K/AKT pathway in obesity and type 2 diabetes. *Int J Biol Sci* **14,** 1483.

Ibrahim MM (2010). Subcutaneous and visceral adipose tissue: structural and functional differences. *Obes Rev* **11,** 11–18.

Jacobson KA & Gao ZG (2006*a*). Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* **5,** 247–264.

Jacobson KA & Gao ZG (2006*b*). Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* **5,** 247–264.

Jain S & Jacobson KA (2021). Purinergic Signaling in Liver Pathophysiology. *Front Endocrinol (Lausanne)* **12,** 718429.

Jaworski K, Sarkadi-Nagy E, Duncan RE, Ahmadian M & Sul HS (2007). Regulation of triglyceride metabolism. IV. Hormonal regulation of lipolysis in adipose tissue. *Am J Physiol Gastrointest Liver Physiol*; DOI: 10.1152/AJPGI.00554.2006.

Jeukendrup AE & Randell R (2011). Fat burners: nutrition supplements that increase fat metabolism. *Obes Rev* **12,** 841–851.

Kahn CR, Wang G & Lee KY (2019). Altered adipose tissue and adipocyte function in the pathogenesis of metabolic syndrome. *Journal of Clinical Investigation* **129,** 3990–4000.

Kahn CR & White MF (1988). The insulin receptor and the molecular mechanism of insulin action. *J Clin Invest* **82,** 1151–1156.

Kasiske BL, O'Donnell MP & Keane WF (1992). The Zucker rat model of obesity, insulin resistance, hyperlipidemia, and renal injury. *Hypertension* **19,** I110-5.

Kautzky-Willer A, Leutner M & Harreiter J (2023). Sex differences in type 2 diabetes. *Diabetologia 2023 66:6* **66,** 986–1002.

Keijzers GB, De Galan BE, Tack CJ & Smits P (2002). Caffeine can decrease insulin sensitivity in humans. *Diabetes Care* **25,** 364–369.

Koepsell H (2020). Glucose transporters in brain in health and disease. *Pflugers Archiv* **472,** 1299.

Kumanyika S & Dietz WH (2020). Solving Population-wide Obesity — Progress and Future Prospects. *New England Journal of Medicine* **383,** 2197–2200.

Lingvay I, Sumithran P, Cohen R V & le Roux CW (2022). Obesity management as a primary treatment goal for type 2 diabetes: time to reframe the conversation. *The Lancet* **399,** 394–405.

Liu J, Wang Y & Lin L (2019). Small molecules for fat combustion: targeting obesity. *Acta Pharm Sin B* **9,** 220–236.

Lizcano JM & Alessi DR (2002). The insulin signalling pathway. *Curr Biol*; DOI: 10.1016/S0960-9822(02)00777-7.

Lonardo A, Ballestri S, Marchesini G, Angulo P & Loria P (2015). Nonalcoholic fatty liver disease: a precursor of the metabolic syndrome. *Dig Liver Dis* **47,** 181–190.

Lopez-Garcia E, van Dam RM, Rajpathak S, Willett WC, Manson JE & Hu FB (2006). Changes in caffeine intake and long-term weight change in men and women. *Am J Clin Nutr* **83,** 674–680.

Madeira MH, Boia R, Ambrósio AF & Santiago AR (2017). Having a Coffee Break: The Impact of Caffeine Consumption on Microglia-Mediated Inflammation in Neurodegenerative Diseases. *Mediators Inflamm* **2017,** 1–12.

Malone JI & Hansen BC (2019). Does obesity cause type 2 diabetes mellitus (T2DM)? Or is it the opposite? *Pediatr Diabetes* **20,** 5–9.

Martini D, Del Bo' C, Tassotti M, Riso P, Del Rio D, Brighenti F & Porrini M (2016). Coffee Consumption and Oxidative Stress: A Review of Human Intervention Studies. *Molecules* **21,** 979.

Matafome P & Seiça R (2017). Function and Dysfunction of Adipose Tissue. *Adv Neurobiol* **19,** 3–31.

Meeusen R, Roelands B & Spriet LL (2013). Caffeine, Exercise and the Brain. In, pp. 1–12.

Melo BF, Sacramento JF, Ribeiro MJ, Prego CS, Correia MC, Coelho JC, Cunha-Guimaraes JP, Rodrigues T, Martins IB, Guarino MP, Seiça RM, Matafome P & Conde S V. (2019*a*). Evaluating the Impact of Different Hypercaloric Diets on Weight Gain, Insulin Resistance, Glucose Intolerance, and its Comorbidities in Rats. *Nutrients*; DOI: 10.3390/NU11061197.

Melo BF, Sacramento JF, Ribeiro MJ, Prego CS, Correia MC, Coelho JC, Cunha-Guimaraes JP, Rodrigues T, Martins IB, Guarino MP, Seiça RM, Matafome P & Conde S V. (2019*b*). Evaluating the Impact of Different Hypercaloric Diets on Weight Gain, Insulin Resistance, Glucose Intolerance, and its Comorbidities in Rats. *Nutrients*; DOI: 10.3390/NU11061197.

Modi AA, Feld JJ, Park Y, Kleiner DE, Everhart JE, Liang TJ & Hoofnagle JH (2010). Increased caffeine consumption is associated with reduced hepatic fibrosis. *Hepatology* **51,** 201–209.

Monzillo LU & Hamdy O (2003). Evaluation of insulin sensitivity in clinical practice and in research settings. *Nutr Rev* **61,** 397–412.

de Moura RF, Ribeiro C, de Oliveira JA, Stevanato E & de Mello MAR (2009). Metabolic syndrome signs in Wistar rats submitted to different high-fructose ingestion protocols. *Br J Nutr* **101,** 1178–1184.

Müller-Riemenschneider F, Reinhold T, Berghöfer A & Willich SN (2008). Health-economic burden of obesity in Europe. *Eur J Epidemiol* **23,** 499–509.

Murray S, Tulloch A, Gold MS & Avena NM (2014). Hormonal and neural mechanisms of food reward, eating behaviour and obesity. *Nat Rev Endocrinol* **10,** 540–552.

Nedergaard J, Bengtsson T & Cannon B (2011). New powers of brown fat: fighting the metabolic syndrome. *Cell Metab* **13,** 238–240.

Ohta A, Lukashev D, Jackson EK, Fredholm BB & Sitkovsky M (2007). 1,3,7-Trimethylxanthine (Caffeine) May Exacerbate Acute Inflammatory Liver Injury by Weakening the Physiological Immunosuppressive Mechanism. *The Journal of Immunology* **179,** 7431–7438.

de Oliveira M, Mathias LS, de Sibio MT, Noronha-Matos JB, Costa MA, Nogueira CR & Correia-de-Sá P (2020*a*). Pitfalls and challenges of the purinergic signaling cascade in obesity. *Biochem Pharmacol*; DOI: 10.1016/J.BCP.2020.114214.

de Oliveira M, Mathias LS, de Sibio MT, Noronha-Matos JB, Costa MA, Nogueira CR & Correia-de-Sá P (2020*b*). Pitfalls and challenges of the purinergic signaling cascade in obesity. *Biochem Pharmacol*; DOI: 10.1016/J.BCP.2020.114214.

Panchal SK, Wong W-Y, Kauter K, Ward LC & Brown L (2012). Caffeine attenuates metabolic syndrome in diet-induced obese rats. *Nutrition* **28,** 1055–1062.

Petersen MC & Shulman GI (2018). Mechanisms of Insulin Action and Insulin Resistance. *Physiol Rev* **98,** 2133.

Peterson RG, Jackson C V, Zimmerman K, de Winter W, Huebert N & Hansen MK (2015). Characterization of the ZDSD Rat: A Translational Model for the Study of Metabolic Syndrome and Type 2 Diabetes. *J Diabetes Res* **2015,** 487816.

Piché ME, Tchernof A & Després JP (2020). Obesity Phenotypes, Diabetes, and Cardiovascular Diseases. *Circ Res* **126,** 1477–1500.

Rahn T, Ridderstråle M, Tornqvist H, Manganiello V, Fredrikson G, Belfrage P & Degerman E (1994). Essential role of phosphatidylinositol 3-kinase in insulin-induced activation and phosphorylation of the cGMP-inhibited cAMP phosphodiesterase in rat adipocytes. Studies using the selective inhibitor wortmannin. *FEBS Lett* **350,** 314–318.

Reaven GM (1988). Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* **37,** 1595–1607.

Reis CEG, Dórea JG & da Costa THM (2019). Effects of coffee consumption on glucose metabolism: A systematic review of clinical trials. *J Tradit Complement Med* **9,** 184.

Ribeiro MJ, Sacramento JF, Gonzalez C, Guarino MP, Monteiro EC & Conde S V. (2013). Carotid body denervation prevents the development of insulin resistance and hypertension induced by hypercaloric diets. *Diabetes* **62,** 2905–2916.

Richard AJ, White U, Elks CM & Stephens JM (2000). *Adipose Tissue: Physiology to Metabolic Dysfunction*.

Roden M & Shulman GI (2019). The integrative biology of type 2 diabetes. *Nature* **576,** 51–60.

Rohm T V., Meier DT, Olefsky JM & Donath MY (2022). Inflammation in obesity, diabetes, and related disorders. *Immunity* **55,** 31–55.

Ruze R, Liu T, Zou X, Song J, Chen Y, Xu R, Yin X & Xu Q (2023). Obesity and type 2 diabetes mellitus: connections in epidemiology, pathogenesis, and treatments. *Front Endocrinol (Lausanne)* **14,** 1161521.

Sacramento JF, Martins FO, Rodrigues T, Matafome P, Ribeiro MJ, Olea E & Conde S V. (2020). A2 Adenosine Receptors Mediate Whole-Body Insulin Sensitivity in a Prediabetes Animal Model: Primary Effects on Skeletal Muscle. *Front Endocrinol (Lausanne)* **11,** 262.

Sacramento JF, Ribeiro MJ, Rodrigues T, Olea E, Melo BF, Guarino MP, Fonseca-Pinto R, Ferreira CR, Coelho J, Obeso A, Seiça R, Matafome P & Conde S V. (2017). Electronic Suplementary materials ESM- Functional abolition of carotid body activity restores insulin action and glucose homeostasis in rats: key roles for visceral adipose tissue and the liver. *Diabetologia* **60,** 158–168.

Sacramento JF, Ribeiro MJ, Yubero S, Melo BF, Obeso A, Guarino MP, Gonzalez C & Conde S V. (2015). Disclosing caffeine action on insulin sensitivity: effects on rat skeletal muscle. *Eur J Pharm Sci* **70,** 107–116.

Saltiel AR & Kahn CR (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414,** 799–806.

Schimmel RJ & McCarthy L (1984). Role of adenosine as an endogenous regulator of respiration in hamster brown adipocytes. *Am J Physiol*; DOI: 10.1152/AJPCELL.1984.246.3.C301.

Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scimè A, Devarakonda S, Conroe HM, Erdjument-Bromage H, Tempst P, Rudnicki MA, Beier DR & Spiegelman BM (2008). PRDM16 controls a brown fat/skeletal muscle switch. *Nature* **454,** 961–967.

Shanik MH, Xu Y, Skrha J, Dankner R, Zick Y & Roth J (2008). Insulin resistance and hyperinsulinemia: is hyperinsulinemia the cart or the horse? *Diabetes Care*; DOI: 10.2337/DC08-S264.

Shao M, Wang QA, Song A, Vishvanath L, Busbuso NC, Scherer PE & Gupta RK (2019). Cellular Origins of Beige Fat Cells Revisited. *Diabetes* **68,** 1874–1885.

Shi X, Xue W, Liang S, Zhao J & Zhang X (2016). Acute caffeine ingestion reduces insulin sensitivity in healthy subjects: a systematic review and meta-analysis. *Nutr J* **15,** 103.

Smith CJ, Vasta V, Degerman E, Belfrage P & Manganiello VC (1991). Hormone-sensitive cyclic GMP-inhibited cyclic AMP phosphodiesterase in rat adipocytes. Regulation of insulin- and cAMP-dependent activation by phosphorylation. *J Biol Chem* **266,** 13385–13390.

Smith KB & Smith MS (2016). Obesity Statistics. *Primary Care: Clinics in Office Practice* **43,** 121–135.

Sørensen TIA, Martinez AR & Jørgensen TSH (2022). Epidemiology of Obesity. *Handb Exp Pharmacol* **274,** 3–27.

Stumvoll M, Goldstein BJ & Van Haeften TW (2005). Type 2 diabetes: principles of pathogenesis and therapy. *Lancet* **365,** 1333–1346.

Suchacki KJ & Stimson RH (2021). Nutritional Regulation of Human Brown Adipose Tissue. *Nutrients* **13,** 1748.

Svenningsson P, Nomikos GG & Fredholm BB (1999). The stimulatory action and the development of tolerance to caffeine is associated with alterations in gene expression in specific brain regions. *Journal of Neuroscience* **19,** 4011–4022.

Tchernof A & Després JP (2013). Pathophysiology of human visceral obesity: an update. *Physiol Rev* **93,** 359–404.

Timper K & Brüning JC (2017). Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity. *Dis Model Mech* **10,** 679–689.

Tokarz VL, MacDonald PE & Klip A (2018). The cell biology of systemic insulin function. *J Cell Biol* **217,** 2273–2289.

Trayhurn P & Beattie JH (2001). Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc Nutr Soc* **60,** 329–339.

Trinh KY, O'Doherty RM, Anderson P, Lange AJ & Newgard CB (1998). Perturbation of fuel homeostasis caused by overexpression of the glucose-6-phosphatase catalytic subunit in liver of normal rats. *J Biol Chem* **273,** 31615–31620.

Tsai AG & Bessesen DH (2019). Obesity. *Ann Intern Med* **170,** ITC33.

Tuomilehto J, Hu G, Bidel S, Lindström J & Jousilahti P (2004). Coffee consumption and risk of type 2 diabetes mellitus among middle-aged Finnish men and women. *JAMA* **291,** 1213–1219.

VAUGHAN M, BERGER JE & STEINBERG D (1964). HORMONE-SENSITIVE LIPASE AND MONOGLYCERIDE LIPASE ACTIVITIES IN ADIPOSE TISSUE. *J Biol Chem* **239,** 401–409.

Weyer C, Bogardus C, Mott DM & Pratley RE (1999). The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *J Clin Invest* **104,** 787–794.

WHO (2023). Diabetes. Available at: https://www.who.int/news-room/fact-sheets/detail/diabetes [Accessed September 1, 2023].

Winzell MS & Ahrén B (2004). The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes* **53 Suppl 3,** S215-9.

World Obesity Federation (2022). *Obesity: missing the 2025 global targets - trends, costs and country reports*. London, UK: World Obesity Federation.

Wu J, Boström P, Sparks LM, Ye L, Choi JH, Giang AH, Khandekar M, Virtanen KA, Nuutila P, Schaart G, Huang K, Tu H, Van Marken Lichtenbelt WD, Hoeks J, Enerbäck S, Schrauwen P & Spiegelman BM (2012). Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* **150,** 366–376.

Wysham C & Shubrook J (2020). Beta-cell failure in type 2 diabetes: mechanisms, markers, and clinical implications. *Postgrad Med* **132,** 676–686.

Ye J (2013). Mechanisms of insulin resistance in obesity. *Front Med* **7,** 14.

Young HC, Park S, Hockman S, Zmuda-Trzebiatowska E, Svennelid F, Haluzik M, Gavrilova O, Ahmad F, Pepin L, Napolitano M, Taira M, Sundler F, Holst LS, Degerman E & Manganiello VC (2006). Alterations in regulation of energy homeostasis in cyclic nucleotide phosphodiesterase 3B-null mice. *J Clin Invest* **116,** 3240–3251.

Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW & Shulman GI (2002). Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* **277,** 50230–50236.

Zakir F, Mohapatra S, Farooq U, Mirza MA & Iqbal Z (2022). Introduction to metabolic disorders. *Drug Delivery Systems for Metabolic Disorders*1–20.

Zheng Y, Ley SH & Hu FB (2018). Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nat Rev Endocrinol* **14,** 88–98.