UNIVERSIDADE D
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Mariana Sá Rocha

THE ROLE OF EXPOSURE TO OBESOGENIC ENVIRONMENTS DURING THE PERINATAL PERIOD IN THE DEVELOPMENT OF INSULIN RESISTANCE.

Dissertação no âmbito do Mestrado em Bioquímica, orientada pelo Professor Doutor Paulo Nuno Centeio Matafome e pelo Professor Doutor Carlos Palmeira, apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra, Departamento de Ciências da Vida.

Julho de 2022

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ACKNOWLEDGEMENTS:

O meu avô era um grande fã dos ABBA. Amo-te.

“Schoolbag in hand, she leaves home in the early morning
Waving goodbye with an absent-minded smile
I watch her go with a surge of that well-known sadness
And I have to sit down for a while
The feeling that I'm losing her forever
And without really entering her world
I'm glad whenever I can share her laughter
That funny little girl
Slipping through my fingers all the time
I try to capture every minute
The feeling in it
Slipping through my fingers all the time
Do I really see what's in her mind?
Each time I think I'm close to knowing
She keeps on growing
Slipping through my fingers all the time
Sleep in our eyes, her and me at the breakfast table
Barely awake, I let precious time go by
Then when she's gone, there's that odd melancholy feeling
And a sense of guilt I can't deny
What happened to the wonderful adventures
The places I had planned for us to go?
(Slipping through my fingers all the time)
Well, some of that we did but most we didn't
And why? I just don't know
Slipping through my fingers all the time
I try to capture every minute
The feeling in it
Slipping through my fingers all the time
Do I really see what's in her mind?
Each time I think I'm close to knowing
She keeps on growing
Slipping through my fingers all the time
Sometimes I wish that I could freeze the picture
And save it from the funny tricks of time
Slipping through my fingers
Slipping through my fingers all the time
Schoolbag in hand, she leaves home in the early morning
Waving goodbye with an absent-minded smile”
- Slipping Through My Fingers by ABBA.

Este projeto, que culmina na entrega e defesa da minha dissertação, marca um ponto de viragem na minha vida e de novos desafios. Iniciei este projeto em Abril de 2019 e ao ver o que já foi conquistado, dá-me imenso prazer ter sido uma parte ínfima do mesmo. Por isso, início estes agradecimentos ao Professor Doutor Paulo Matafome. Sem ele, claramente não teria conseguido. Pela confiança depositada em mim desde o primeiro dia, pelo conhecimento transmitido e pela amizade. Mas principalmente, pela paciência e nunca ter desistido de mim. Aqui lhe exprimo a minha gratidão – sendo que não conheci só um orientador, mas também um amigo. Sei que vais conquistar muito mais do que já conquistaste e espero estar lá para assistir. É o que é.

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Aos meus maiores exemplos, aos meus pais e irmã. Espero deixá-los orgulhosos. Amo-vos muito.

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RESUMO

Introdução e Objetivos: Obesidade, Diabetes e Síndrome Metabólica são cada vez mais prevalentes e têm um elemento em comum que é a resistência à insulina. Quando este estado metabólico se manifesta, leva à inflamação, hiperinsulinemia, hipoxia, acumulação ectópica de gordura e lipodistrofia. O tecido adiposo é um órgão importante para o metabolismo geral, que regula o armazenamento e o gasto de energia, sendo também afetado pela resistência à insulina. O conceito de "programação metabólica" correlaciona o estado metabólico materno (como obesidade e Diabetes) e sua nutrição a possíveis desfechos negativos na descendência, sendo a gestação e a lactação, duas fases proeminentes para a saúde/doença da descendência. O nosso grupo tem como objetivo determinar se a exposição a condições obesogénicas durante o período perinatal afeta o metabolismo de glicose e lípidos no tecido adiposo branco, fígado e pâncreas. Qualquer alteração nesses órgãos pode causar distúrbios metabólicos (e resistência à insulina precoce) que se manifestam precocemente na infância e mais tarde na idade adulta.

Materiais e métodos: Neste projeto, foram utilizados 3 modelos animais: 1) Descendência de fêmeas Sprague-Dawley, alimentadas com dieta hipercalórica , 2) Ratos Wistar recém-nascidos, cujas mães foram alimentadas com dieta standard e tratadas após o nascimento, com um inibidor seletivo de Glyoxalase1-S-p-Bromobenzilgutiona ciclopentil diéster a 5 mg/kg, via intraperitoneal por 6 dias e 3) Ratos Wistar recém-nascidos, cujas mães sofreram redução da ninhada e tratadas com o inibidor da Glioxalase1. Avaliou-se o peso corporal e a qualidade do leite das mães. O peso corporal, parâmetros metabólicos, análise morfológica e níveis de proteína da descendência também foram avaliados.

Resultados: Os nossos resultados mostram que a exposição à obesidade materna causa um aumento na expressão de AMPK no tecido adiposo visceral da descendência masculina, porém na descendência feminina, não houve alterações no WAT e na sinalização da insulina hepática. A glicação materna resultou numa menor sensibilidade à insulina, um aumento da AMPK no tecido adiposo visceral e

uma diminuição no recetor de insulina no fígado na descendência masculina. No entanto, na descendência feminina apresentou menos insulina plasmática e nenhuma alteração no recetor de insulina e AMPK em ambos os tecidos. A descendência masculina da hiperfagia infantil ganhou mais peso, mas a exposição à glicação materna impediu o ganho de peso corporal, levando a baixos níveis de insulina plasmática e hipertrigliceridemia.

Conclusão: Este trabalho demonstrou que a exposição à dieta obesogénica materna durante o período perinatal potencializa alterações distintas no metabolismo lipídico na descendência masculina e feminina. A exposição precoce à glicação materna parece comprometer os mecanismos do metabolismo de lípidos e glicose, que aparentemente não foram alterados pela hiperfagia, mas podem contribuir para o aumento da suscetibilidade à resistência à insulina mais tarde na vida.

Palavras-chave: tecido adiposo, resistência à insulina, descendência, programação metabólica, metabolismo da glicose e lípidos.

ABSTRACT

Introduction and Objectives: Obesity, Diabetes and Metabolic Syndrome are increasingly more prevalent and have a common link which is insulin resistance. When this metabolic state manifests, leads to inflammation, hyperinsulinemia, hypoxia, ectopic accumulation of fat and lipodystrophy. The adipose tissue is an important organ for overall metabolism, regulating energy storage and expenditure, being also affected by insulin resistance. The concept of “Metabolic programming” correlates maternal metabolic state (such as obesity and Diabetes) and her nutrition to possible negative outcomes in the descendance, being the gestation and lactation two prominent contributors for offspring un(health). Our group aims to determine whether being exposed to obesogenic conditions during the perinatal period affects the metabolism of glucose and lipids in white adipose tissue, liver, and pancreas. Any alteration to these organs has the potential to cause metabolic disorders (and early insulin resistance) to manifest both early at childhood and later at adulthood.

Materials and methods: In this project, 3 animal models were used: 1) Offspring of females Sprague-Dawley, fed a hypercaloric diet and 2) newborn Wistar rats, whose dams were fed a standard diet and treated after birth with a selective inhibitor of Glyoxalase1- S-p-Bromobenzylguthione cyclopentyl diester at 5 mg/kg, via intraperitoneal for 6 days after birth and 3) Offspring Wistar rats, whose dams suffer litter reduction and treated with the inhibitor of Glyoxalase1. The body weight and the milk quality of the dams was evaluated. The body weight, metabolic parameters, morphological analysis, and protein levels of the offspring were assessed.

Results: Our results show that an exposure to maternal obesity causes upregulation of AMPK in the visceral adipose tissue of female offspring in male offspring, but no changes on WAT and liver insulin signalling. Maternal glycation resulted in less insulin sensitivity, an upregulation of AMPK in visceral adipose tissue and a downregulation in the insulin receptor in the liver in the male offspring. However, female offspring showed less plasma insulin, and no alterations in insulin receptor and AMPK in both tissues. The male offspring of childhood hyperphagia

gained more weight, but exposure to maternal glycation prevented body weight gain, while leading to low plasma insulin levels and hypertriglyceridemia.

Conclusion: This work demonstrated that the exposure to maternal obesogenic diet during the perinatal period potentiates distinct changes in lipid metabolism in male and female offspring. Early exposure to maternal glycation seems to compromise mechanisms of lipid and glucose metabolism, which were not apparently changed by hyperphagia, but may contribute to an increased susceptibility to insulin resistance later in life.

Keywords: adipose tissue, insulin resistance, offspring, metabolic programming, glucose and lipids metabolism.

ABBREVIATION LIST

AGEs Advanced Glycation End Products
Akt Protein Kinase B
AMPK AMP-activated Protein Kinase
AS Akt Substrate
ASP Acylation Stimulating Protein
AT Adipose tissue
ATP Adenosine 5'-Triphosphate
BAT Brown Adipose Tissue
BBGC S-p-Bromobenzylglutathione Cyclopentyl Diester
BMI Body Mass Index
cAMP Cyclic Adenosine Monophosphate
CD36 Cluster of Differentiation 36
CDV Cardiovascular diseases
CEACAM1 Carcinoembryonic Antigen–related Cell Adhesion Molecule 1
ChREBP Carbohydrate response element binding protein
DHA Docosahexaenoic acid
DMSO Dimethyl Sulfoxide
EGIR European Group for the Study of Insulin Resistance
ER Endoplasmic Reticulum
ERK Extracellular Signal-Regulated Kinase
FA Fatty Acids
FELASA Federation of European Laboratory Animal Science Associations
FFA Free Fatty Acids
FoxO1 Fork head box protein O1
G6P Glucose-6-Phosphate
G6pc Glucose-6-Phosphatase
Gck Glucokinase
GH Growth Hormone
GLO1 Glyoxalase1
GLUT Glucose Transporter
GSK3 Glycogen Synthase Kinase 3
GYS2 Glycogen Synthetase 2
HE Hematoxylin and Eosin
HFHS High-Fat High-Sucrose
i.p. Intraperitoneal
IDE Insulin Degradating Enzyme
IDF International Diabetes Federation
I**gA** Immunoglobin A
IGF-1 insulin-like Growth Factor-1
IR Insulin Receptor
IRS Insulin Receptor-Substrate
ITT Insulin Tolerance Test
JNK c-Jun N-terminal kinase
kITT Glucose decay rate
LPL Lipid Protein Lipase
MAPK Ras-Mitogen-Activated Protein Kinase

MCP Monocyte chemoattractant protein
IL Interleukin
MetS Metabolic Syndrome
MG Methylglyoxal
MHO Metabolically Healthy Obesity
mTORC Mammalian Target of Rapamycin Complex
MUO Metabolically Unhealthy Obesity
NAFLD Non-Alcoholic Fatty Liver Disease
NASH Non-Alcoholic Steatohepatitis
NEFA Non-Esterified Fatty Acids
Pck1 Phosphoenolpyruvate Carboxykinase 1
PDK1 Phosphoinositide-dependent protein kinase 1
PI3K Phosphatidylinositol-3-Kinase
PIP_2 Phosphatidylinositol 4,5-bisphosphate
PIP_3 Phosphatidylinositol-3,4,5-triphosphate
PKA Protein Kinase A
PND Postnatal day
PPAR Peroxisome Proliferator-Activated Receptor
PUFA Polyunsaturated Fatty Acids
RAGE Advanced Glycation End Products Receptor
RDA Recommended Dietary Allowances
ROS Reactive Oxygen Species
SAT Subcutaneous Adipose Tissue
SL Small litter or Litter reduction
SOCS Suppressor Of Cytokine Signaling
SREBP1c Sterol-regulatory element binding protein-1c
T2DM Diabetes Mellitus type 2
TG Triglycerides
TGF transforming growth factor
TNF Tumor necrosis factor
Tsc Tuberous sclerosis complex
UCP1 uncoupling protein-1
VAT Visceral Adipose Tissue
VEGF vascular endothelial growth factor
VLDLP very-low-density lipoprotein
WAT White Adipose Tissue
WHO World Health Organization

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Chapter I

INTRODUCTION

1. WHO CAME FIRST – OBESITY, METABOLIC SYNDROME OR DIABETES MELLITUS TYPE 2?

Metabolic Syndrome (MetS), obesity and Diabetes Mellitus type 2 (T2DM) are diseases that are increasingly prevalent in society and are currently seen as an emerging pandemic.

According to the World Health Organization (WHO) more than 650 million adults were obese in 2016 and according to International Diabetes Federation (IDF), 537 million adults are living with T2DM in 2021 (IDF, 2021; Vaamonde & Álvarez-Món, 2020). There is no data available for MetS, but a quarter of the worldwide adult population is estimated to be affected (Shin et al., 2013). In Portugal, 28.7% of the adult population was obese in 2018 and the prevalence for T2DM was around 13.6% in the same year (Gaio et al., 2018; Sociedade Portuguesa de Diabetologia, 2019).

a. OBESITY:

Obesity is defined as an unhealthy excess of body fat, and it is most typically diagnosed using the body mass index (BMI) - calculated by multiplying the weight by the square of height. Obesity is defined as having a BMI of 30 kg/m^2 or higher. However, BMI doesn't provide an accurate representation of body composition (i.e. fat distribution or fat/lean mass) (Iacobini et al., 2019a) and not all obese individuals have traditional markers of dysfunction, such as insulin resistance and dyslipidemia. Therefore, we can classify two types of obesity: metabolically healthy obesity (MHO), and metabolically unhealthy obesity (MUO), which is characterized by several of the metabolic abnormalities that can correlate with MetS (Elabbassi & Haddad, 2005; Iacobini et al., 2019a). MHO is not a harmless condition but doesn't display the same factors has MUO. MHO are insulin-sensitive and do not develop any disorders such as hypertension, hyperlipidaemia, and cardiovascular diseases (CVD). On the other hand, MUO is linked to abdominal obesity, which is defined by a higher visceral and a lower subcutaneous fat mass, as well as systemic inflammation and ectopic fat accumulation, resulting in insulin resistance (Iacobini et al., 2019a). Obesity is also linked to T2DM , related to MetS, non-alcoholic fatty

liver disease (NAFLD), and CVD (Elabbassi & Haddad, 2005; Koliaki et al., 2019; Malone & Hansen, 2019; S. A. Polyzos et al., 2019). However, obesity by itself increases the risk of metabolic and other types of disorders and some authors believe that MHO is an early stage of MUO that will ultimately lead to insulin resistance and MetS (Mongraw-Chaffin et al., 2018).

b. METS:

MetS is a common metabolic condition and was first reported by WHO in 1988 as the manifestation of insulin resistance and related complications - being the central criteria. The definition has changed in 1999 by European Group for the Study of Insulin Resistance (EGIR), where insulin resistance continued to be the key-factor but requires two additional criteria out of the following: obesity, hypertension, and dyslipidemia. The definition has changed again in 2005 and according to the National Cholesterol Education Program/ Adult Treatment Panel III, the criteria were updated to include at least three of the following factors: abdominal adiposity, hypertriglyceridemia, hypercholesterolemia, hypertension, and/or impaired fasting glucose (Elabbassi & Haddad, 2005). MetS is often related to an increase of CVD (Nsiah et al., 2015) and is a strong predictor to T2DM (Elabbassi & Haddad, 2005). The treatment is based on dietary changes and increased physical activity, as well as proper pharmaceutical management, such as metformin.
Regarding MetS, two frequent questions arise: Is this disease a consequence of insulin resistance or is it the outcome? and whether the definition is correct in terms of the relation between factors?

c. TD2M:

T2DM is a heterogeneous metabolic disease characterized by hyperglycemia due to insulin resistance and pancreatic β-cell exhaustion (Roden & Shulman, 2019a). Genetic and environmental factors such as weight gain, sedentarism, and aging can all contribute to the development and progression of this disease (Glovaci et al., 2019). The development of T2DM can be supported by the following steps: an individual who has a normal glucose tolerance and has a progressive impairment due to insulin resistance. Then, to maintain glucose homeostasis, β-cells increase

their activity and plasma insulin levels remain elevated. When β-cell activity begins to deteriorate, having a reduction in function, a chronic hyperglycemia develops, which is a strong characteristic of T2DM (DeFronzo, 2004). This disease can also be associated with several long-term problems like diabetic kidney disease, nephropathy, diabetic retinopathy and, as mentioned above, CDV (Glovaci et al., 2019).

d. THE KNOWN LINK - INSULIN:

The factor in common for the metabolic disorders mentioned above is insulin. This hormone is produced by pancreatic β-cells. After a meal, glucose levels increase and, due glucose transporter (GLUT)2 - a transporter independent of insulin, and glucokinase in the β-cell membrane, this allows a sharpen-sense of glucose elevation levels (**Figure 1**). When glucose enters the cell via GLUT2, activates a chain of events, promoting glycolysis to form pyruvate, which enters in mitochondrial Krebs's Cycle and ultimately producing adenosine 5'-triphosphate (ATP). This cascade of events leads to cell depolarization and calcium-dependent exocytosis of insulin secretory granules from β-cells, leading to the first insulin release peak (Boland et al., 2017a; Tokarz et al., 2018a). Numerous receptors, channels intracellular Ca^{2+} stores, metabolic signals, and cytoskeletal elements all influence the overall secretory response (Tokarz et al., 2018b). The synthesis and secretion of new molecules of insulin leads to the second release peak (Boland et al., 2017a).

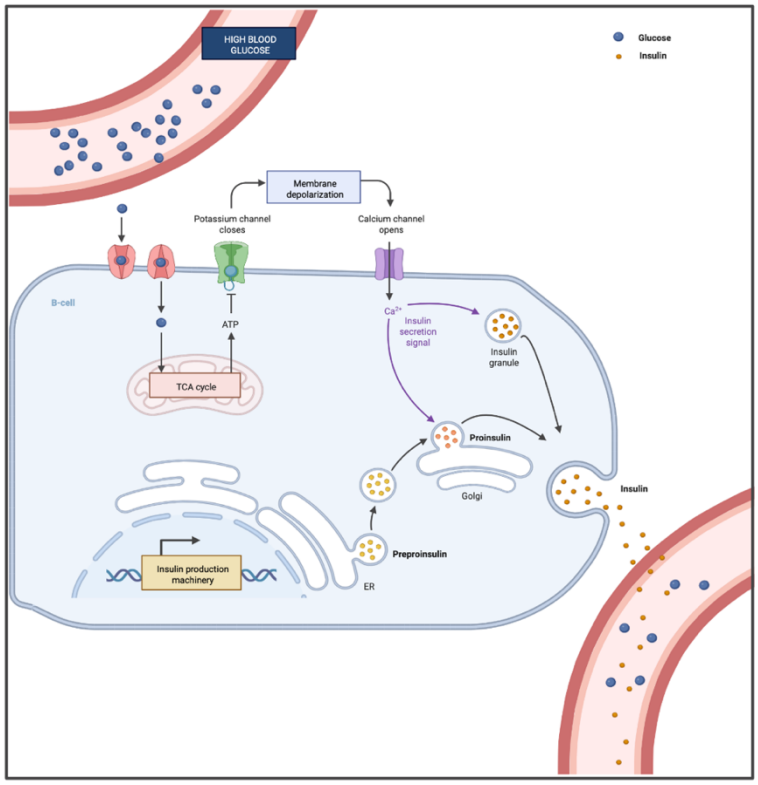

Figure 1 - Insulin production in the pancreatic B-cell. Representative image of Glucose sensing and the pathway of insulin production, until its release.

In physiological conditions, 80% of insulin secreted by the pancreas ends up in the liver through the portal vein, to be further degraded after the first passage. The second time is when insulin enters via hepatic artery. Part of the insulin is modified and redirected to circulation, and the rest suffers degradation. This may seem counterintuitive but it is a “default mechanism” to modulate the insulin concentration for the peripheral organs (Samuel & Shulman, 2016; Tokarz et al., 2018b). A proposed mechanism for this degradation, is when insulin binds the insulin receptor (IR), forms a complex with Carcinoembryonic Antigen–related Cell Adhesion Molecule 1 (CEACAM1), mediating an effective way to insulin endocytosis. Extracellular insulin-degrading enzyme (IDE) partially degrades insulin before internalization (Tokarz et al., 2018c). When insulin enters the cells, additional IDE begins to degrade IR in the endosomes. The degradation products and the remaining insulin are delivered to lysosomes for proteolysis.

Insulin also has a direct effect when binding to hepatic IR and suppresses gluconeogenesis and glycogenolysis, allowing a storage of dietary glucose, which transport is also mediated by GLUT2 (Jones, 2016; Reckzeh & Waldmann, 2020).

Following a meal, insulin binds to the insulin receptor, causing a cascade of phosphorylation and consequent activation of Insulin-receptor substrate (IRS) bind to the phosphorylated IR and activates Phosphatidylinositol-3-Kinase (PI3K), explained below in **Figure 2**. Subsequentially, PI3K phosphorylates the Phosphatidylinositol 4,5-bisphosphate (PIP_2) into Phosphatidylinositol-3,4,5-triphosphate (PIP_3). Protein Kinase B (Akt) must suffer two types of phosphorylation to be fully activated: 1) by 3-phosphoinositide-dependent protein kinase 1 (PDK1) - activated by PIP_3 and 2) mammalian target of rapamycin complex (mTORC)2 must phosphorylate Akt. Insulin can have (as previously stated) a cascade effect, and cause several consequences: 1) Glycogen synthesis, through the inhibition of Glycogen synthase kinase-3 (GSK3) by Akt or by activating glycogen synthetase (GYS)2 with glucose-6-phosphate (G6P); 2) G6P also is involved in glycolysis, across the activation of Sterol-regulatory element binding protein-1c (SREBP1c) and glucokinase (Gck); 3) Akt inhibition of Tuberous sclerosis complex (Tsc) stimulates mTORC1 and with the activation of Carbohydrate response element binding protein (ChREBP) and SREBP1c triggers lipogenesis and 4) Akt inhibits Fork head box protein O1 (FoxO1), which prevents the expression of the proteins glucose-6-phosphatase (G6pc) and phosphoenolpyruvate carboxykinase (Pck)1 - proteins involved in gluconeogenesis, inhibiting this process (Gaio et al., 2018).

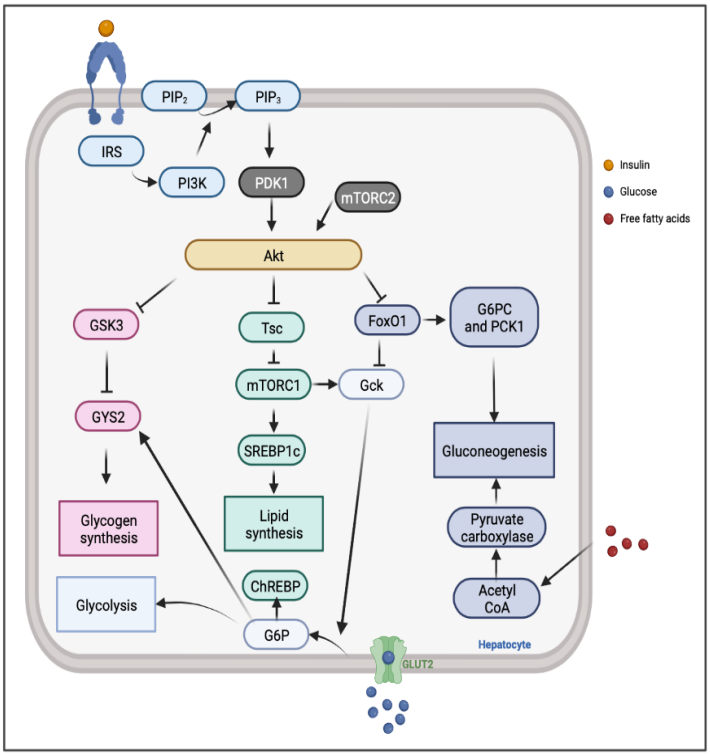

Figure 2- Insulin signaling cascade in the hepatocyte. Insulin binds to IR causing a cascade of phosphorylation, until Akt. This leads to multiple actions by the insulin in the liver: glycogen synthesis, glycolysis, lipid synthesis and gluconeogenesis. Yellow, blue, and red dots are the representation of insulin, glucose and FFA, accordingly. Figure adapted from (Santoleri & Titchenell, 2019)

The skeletal muscle and adipose tissue (AT) (**Figure 3**) are the principal tissues for energy consumption, being stimulated by insulin. When this hormone bind to IR, its tyrosine kinase activity leads to phosphorylation and a structural rearrange of this receptor and consequently the phosphorylation of IRS-1,-2 (Richter & Hargreaves, 2013), allowing the translocation of GLUT4 (Huang & Czech, 2007; Moraes-Vieira et al., 2016) and therefore, glucose uptake.

Two main signal transduction pathways can occur: PI3K-Akt or Ras-Mitogen-Activated Protein Kinase (MAPK) pathway. In the first mentioned pathway, occurs a series of phosphorylation (as reviewed before) of IRS-1/2 by the tyrosine kinase and consequently, PIP_3 and Akt. This pathway is responsible for insulin metabolic functions, such as 1) Phosphorylation of GSK3 increases the activity of glycogen synthase, promotes cellular uptake of glucose and synthesis of glycogen; 2) Phosphorylation of Akt substrate (AS)160 translocate GLUT4 to the cell membrane to absorb glucose. Insulin can also have an impact in lipid metabolism, promoting synthesis and inhibiting the degradation of lipids. Due to enhanced glucose

absorption and the activation of lipid synthesis enzymes such pyruvate dehydrogenase, fatty acid (FA) synthase, and acetyl-CoA carboxylase, glucose is largely stored as lipid and reduces the amount of lipolysis in AT, were insulin acts an inhibitory hormone of Lipid protein lipase (LPL) activity (Lambert et al., 2014; Santoleri & Titchenell, 2019; Schoeler & Caesar, 2019b). Consequently, promotes fatty acids (FA) uptake in the skeletal muscle (Schoeler & Caesar, 2019a),and decreases the rate of FA oxidation. In the liver, insulin stimulates very-low-density lipoprotein (VLDLP) formation and cholesterol synthesis and downregulates FA oxidation (Dimitriadis et al., 2011; Saltiel & Kahn, 2001).

On the other hand, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) interacts with the first pathway, were the activation of MAPK, acts on gene transcription, and controls cell proliferation and differentiation.

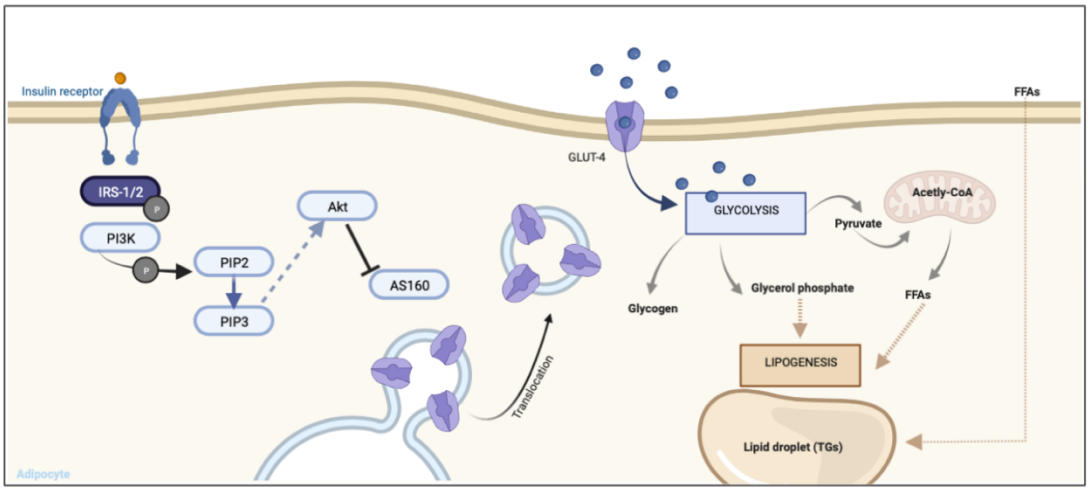

Figure 3 - Insulin signaling cascade in the adipocyte. Insulin binds to IR causing a cascade of phosphorylation in Akt. This leads to multiple actions by the insulin in the AT: Lipogenesis, inhibition of lipolysis and glycolysis. Yellow, blue, and red dots are the representation of insulin, glucose and free fatty acids (FFA), accordingly.

Insulin resistance is associated with mitochondrial dysfunction, chronic inflammation, hyperinsulinemia, hypoxia, ectopic accumulation of fat and lipodystrophy (Wondmkun, 2020). The mitochondria can produce ATP and be a part of several processes such as oxidative phosphorylation and oxidation of substrates. However, when linked to insulin resistance in obese and T2DM patients, characterized by the ectopic accumulation of lipids, the mitochondria reply to cellular stress with a decrease of cell function, an increase in production of reactive species

and an augmented cell death pathway and mitophagy (J. Kim et al., 2008; Montgomery & Turner, 2015). This can also be correlated with a chronic inflammation from the visceral AT. The chronic inflammation is described as an overproduction of inflammatory adipokines (which will be explained further in Chapter II.d) and cytokines that can inhibit insulin signaling by impairing IR (Bugianesi et al., 2005; J. Kim et al., 2008). Adipocytes hypertrophy can lead to hypoxia and stimulate and augmented leptin production, macrophage infiltration, inflammation, adiponectin reduction, stress, and cell death in obese patients (Andrei et al., 2017b).

2. THE ADIPOSE TISSUE:

a. TYPES OF ADIPOSE TISSUE:

The AT is mainly composed by adipocytes and stromovascular cells, essentially mesenchymal progenitor cells, endothelial cells, preadipocytes, pericytes, macrophages and fibroblasts (Kirchner et al., 2009; Sumara et al., 2012). Preadipocytes have their origin in vascular mural cells, pericytes, and/or adventitial fibroblasts and include adipogenic and fibrogenic subtypes (Berry & Rodeheffer, 2013; Vishvanath et al., 2016). Based on location and cell constituents/morphology, AT may be classified in 3 types: white (WAT), brown (BAT) and beige adipose tissue. WAT accounts for more than 95% of AT and adipocytes are characterized as larger cells with a large unilocular lipid droplet, which main function is to store energy as triglycerides (TG) (Driskell et al., 2014).

WAT is subdivided into two categories based on its anatomical distribution: subcutaneous (SAT) and visceral (VAT) AT. The visceral depots are further divided into mesenteric (around the intestine) and omental (extending from the stomach to the ventral abdomen) (Rosendo-Silva & Matafome, 2020). WAT may have different activities and consequences on metabolism. White adipocytes in the dermal layers differ from WAT in the subcutaneous layer in terms of development and have a role in wound healing, hair development, and pathogen resistance (Driskell et al., 2014). On the other hand, BAT accounts for approximately 1-2% of adipose mass and can be found in the cervical, axillary and paraspinal regions. This tissue is highly

vascularized and is densely innervated by the sympathetic nervous system (Mulya & Kirwan, 2017). BAT adipocytes have multilocular droplets and a high mitochondrial density, and they dissipate energy as heat through uncoupled mitochondrial respiration (Kahn et al., 2019). The adipocytes in this tissue are smaller and storage of TG are arranged as small vacuoles. Active BAT was traditionally related with newborns and early childhood, being a natural defense mechanism against hypothermia (Heaton, 1972), but recent studies reported this activation also in healthy adults (Cypess et al., 2009; Nedergaard et al., 2007).
Beige adipocytes feature a central nucleus, multilocular lipid droplets, and are abundant in mitochondria (Harms & Seale, 2013). It is believed to emerge from white adipocytes in response to external factors like sympathetic activity stimulation after prolonged cold exposure, administration of a β3-adrenergic receptor agonist, or exercise (Mulya & Kirwan, 2017). In rodents, these adipocytes can be identified in the WAT depot, and various studies have suggested that human BAT have both brown and beige adipocytes (Lee et al., 2014; Sharp et al., 2012).

b. FUNCTION OF ADIPOSE TISSUE:

AT is a central metabolic organ, responsible for storing energy. After a meal, lipids are emulsified by bile salts and hydrolyzed by pancreatic lipases, being absorbed to the blood in the form of chylomicrons. The chylomicrons bind to plasma membrane cluster of differentiation 36 (CD36) receptor and are subsequently hydrolyzed by LPL and transported as non-esterified fatty acids (NEFA). The adaptor protein complex Ap2 then captures NEFA, preventing it from freely circulating inside the adipocyte. NEFA are converted to TG, which are then deposited in the lipid droplet, that is coated with Perilipin A (Paulo Matafome et al., 2017). FFA are produced from lipid droplets when the organism requires energy. Glucagon, adrenaline, and cortisol induce catabolic activity (lipolysis) through the raise of cyclic adenosine monophosphate (cAMP) levels in adipocytes, which activates protein kinase A (PKA) and causes Perilipin degradation. FFA is released because of this cascade of events. Insulin plays an important regulatory role in adipocyte metabolism, increasing fatty acid synthesis (lipogenesis) and TG esterification while also blocking lipolysis. Insulin, through inhibiting adenylate cyclase, the major enzyme

involved in cAMP synthesis, is responsible for lipolysis suppression (Reilly & Saltiel, 2017).

AT was also identified as an endocrine organ in the control of energy homeostasis (Coelho et al., 2013). However, the adipocytes aren't the only cells to secrete endocrine hormones but the nonadipocyte fraction can also secrete and emphasize this important role (Kershaw & Flier, 2004). Two important factors secreted by the AT are:

Leptin is a short peptide with a molecular weight of 16kDA and 167 amino acids that is released in response to food consumption, and its function is to suppress hunger. Its levels are higher in obese individuals and increase with an excess of nutrients (Coelho et al., 2013). Leptin expression and secretion are regulated by several cofactors such as adrenergic stimulation, cold exposure, growth hormone (GH), thyroid hormone, smoking, and thiazolidinediones (Kersten, 2001; Matsuzawa, 2006). Leptin is also positively regulated by insulin and increases in response to stimulation of this hormone after a meal (Laclaustra et al., 2007). It also regulates glucose metabolism and lipid oxidation, due to the direct action on the peripheral tissues (Galic et al., 2010; Itoh et al., 2011). Leptin also regulates the hypothalamic pathways involved in energy expenditure and intake (Kershaw & Flier, 2004).

Adiponectin circulates in high levels in the blood and prevents insulin resistance by promoting lipid oxidation and anti-inflammatory response (Kubota et al., 2007; Ouchi & Walsh, 2007; Schraw et al., 2008). In adults, there is a negative association between plasma adiponectin levels and fat mass (Schraw et al., 2008). In some studies, adiponectin has been found to enhance insulin sensitivity in genetic and diet-induced obesity models. This was attributed to mechanisms dependent on AMP-activated protein kinase (AMPK) signaling, which stimulates fatty acid oxidation and glucose uptake in skeletal muscle and AT (Kadowaki et al., 2007). Adiponectin levels are low in patients with insulin resistance as a result of obesity or AT dysfunction, and the administration improves metabolic parameters in these individuals (Kershaw & Flier, 2004).

c. BAT'S FUNCTION AS AN ENERGY DISSIPATOR AND THERAPEUTIC ROLE:

The major purpose of BAT, as previously stated, is to dissipate energy in the form of heat, a property triggered by the presence of the mitochondrial uncoupling protein-1 (UCP1), which uncouples mitochondrial respiration. BAT's thermogenic capacity may be vital for heat production in newborns, cold adaptation in adults, and may assist in burning excess calories in overweighted adults.

A process known as "browning" can also be used to induce differentiation of brown-like adipocytes in WAT. Several studies have shown that GLUT4 gene expression is higher in BAT than in WAT (Tang et al., 2008), and that GLUT1 and GLUT4 are more robustly expressed in mice BAT after cold exposure than in other tissues (Handgraaf, 2013), indicating the relevance of glucose for BAT function. According to a prior study (Macotela et al., 2012), many of the genes upregulated in BAT after cold exposure are involved in glucose uptake and catabolism. Cold activates adrenergic signaling, resulting in translocation of GLUT1 and GLUT4 into the plasma membrane of brown adipocytes. These findings imply that BAT could be critical in improving insulin sensitivity and glycemic control (Yamamoto et al., 2010). BAT is thus capable of absorbing glucose without the need of insulin, while thyroid hormones can also induce the glucose uptake and thermogenesis (Carpentier et al., 2018).

Lipids are the preference substrate for oxidation during thermogenesis. FA oxidation is a fundamental pathway in BAT and beige adipocytes, contributing to energy production under acute thermogenic activation and BAT quiescence, maintaining the brown adipocytes phenotype.

In human studies, cold exposure during 2 hours at 10ºC can enhance lipid oxidization by 376% and glucose oxidation by 138% (Haman et al., 2002). Cold exposure for 24 hours can increase lipid oxidation but decreasing glucose oxidation (Haman et al., 2016). Other study confirmed that milder cold exposure increased lipid oxidation by 46% without affecting glucose oxidation (Bakker et al., 2014).

The need for studies regarding BAT activation and WAT browning may lead to a better understanding and the discovery of valuable therapeutic targets for the treatment of obesity and other metabolic diseases (S. H. Kim & Plutzky, 2016).

d. ADIPOSE TISSUE DYSFUNCTION –THE ROLE OF HYPOXIA, INFLAMMATION, NAFLD, AND GLYCATION:

In childhood obesity, WAT mass growth is mediated by hyperplasia (Landgraf et al., 2015), while in adults, the increase in WAT mass is apparently mainly due to adipocyte hypertrophy (Knittle et al., 1979) and it is related to certain complications, such as insulin resistance and T2DM (Gesta et al., 2007a; Item & Konrad, 2012). These two mechanisms can also act simultaneously. Adipocyte hypertrophy impairs its function through inflammation-dependent (secretion of pro-inflammatory cytokines) and independent mechanisms, intensifying insulin resistance and disrupting energy metabolism (Jernås et al., 2006). This increase of pro-inflammatory cytokines leads to the development of insulin resistance (Ye et al., 2007). For hyperplasia, the consequences are not fully understood, and this type of adipose tissue expansion may have beneficial effects against hypertrophy and insulin resistance because inflammation and hypoxia are reduced. Tissue location is also important for the development of metabolic disorders. In obesity, VAT is more susceptive to have hypertrophy, whereas SAT can increase also in number, showing a greater advantage for replication and adipogenesis (Tchkonia et al., 2013) . This can originate different outcomes: an unfavorable metabolic phenotype for VAT and a low degree inflammatory state but generally more benign phenotype for SAT (Marcadenti & de Abreu-Silva, 2015).

Hypoxia and inflammation

Local AT hypoxia is usually observed due to the imbalance of angiogenic factors and deficiency of vasculature, due to the rapid and senseless growth of the adipocyte (Matafome et al., 2015a), and leading as well to insulin resistance. Some factors that could help explaining the hypoxia condition in obesity are: the adipose tissue mass is expanding but the output of the heart and the blood flow to the tissue are not increased; while blood flow to adipose tissue rises postprandially in lean

subjects, it does not increase in the obese; and large adipocytes, that have 150 to 200 μm in diameter, are larger than the normal diffusion distance of O_2 of 100 to 200 μm (Trayhurn, 2013).

Hypoxia can produce reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress (Koumenis et al., 2002), adipocyte death (Ye, 2009), inhibition of adiponectin expression (Chen et al., 2006), leptin hyperproduction (Ambrosini et al., 2002) and initiates the inflammatory response to stimulate angiogenesis and increase blood flow. Studies show that obese individuals with insulin resistance have significantly higher levels of inflammatory factors and macrophage infiltration when compared to equally-obese who are not insulin resistant (Rasouli, 2016).

Hypoxia promotes the secretion of many adipokines related to inflammation and tissue remodeling, such as tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-6 (Andrei et al., 2017a).

TNF-α is a 26 kDA transmembrane protein and is a product of adipocytes and stromovascular cells (Kershaw & Flier, 2004). mRNA expression in obesity have been linked to insulin resistance, since TNF- α can affect the expression of IRS-1 and GLUT4, involved stimulating the uptake of glucose in adipose tissue (Burhans et al., 2019). In a prospect by Akash and colleagues, they highlight the blocking of TNF-α or the signaling to be a successful treatment of insulin resistance and the pathologies associated. Other study has shown that prolonged TNF- α therapy reduces insulin-stimulated glucose absorption in skeletal muscle (Galic et al., 2010).

IL-6 is a cytokine associated to insulin resistance and is expressed by adipocytes and the AT matrix by impairing the phosphorylation of IR and IRS-1 through the induction of Suppressor Of Cytokine Signaling (SOCS)-3 – a known inhibitor of insulin signaling (Kershaw & Flier, 2004). Other factors like angiotensinogen, FFA, acylation stimulating protein (ASP), vascular endothelial growth factor (VEGF), adipsin, glycerol and insulin-like growth factor (IGF-1) and can have an influence with metabolic diseases (Coelho et al., 2013).

NAFLD

Ectopic fat accumulation in insulin-sensitive tissues results in insulin-resistance development. Liver fat is also associated to AT and skeletal muscle insulin-resistance, thereby supporting a central role of the liver and the existence of a crosstalk between the liver and the skeletal muscle in NAFLD patients (S. A. Polyzos

et al., 2017). The hepatocyte is considered to function like an adipocyte when the energy-storage capacity of adipose tissue diminishes (S. A. Polyzos & Mantzoros, 2015). When the adipose tissue is overloaded, lipids are accumulated in the hepatocyte's lipid droplets leading to hepatic steatosis when they occupy more than 5% of cytoplasm. However, NEFA may also accumulate ectopically on the hepatocyte triggering the activation of stress and inflammatory pathways that ultimately lead to inhibition of insulin signaling (Trouwborst et al., 2018). Also, under a dysfunction of the AT, adipokines secretion is changed and they act in favor of a proinflammatory environment. As an outcome, this proinflammatory status contribute to a chronic, low-grade state of inflammation and metabolic disorders (S. A. Polyzos et al., 2016). In the liver, the establishment of this inflammatory environment leads to the development of the following stage of NAFLD - Non-Alcoholic Steatohepatitis (NASH) (Peiseler & Tacke, 2021). NAFLD one billion of the global population and is associated with T2DM and morbidly obese patients (Fazel et al., 2016). Multiple factors contribute to its pathogenesis, including genetical and nutritional factors, and insulin resistance (S. Polyzos et al., 2009).

In the adipose tissue, there is also a crosstalk between adipocytes and immune cells: macrophages are the source of most of adipose-derived cytokines, but their secretory function is affected by inputs from the surrounding adipocytes. Conversely, cytokines produced by immune cells also influence the adipocytes to change their secretory profile (Daemen et al., 2018). This also affects the liver, either directly, since cytokines and adipokines are secreted in the circulation and affect their target-organs, or indirectly via the effect of cytokines and adipokines on AT insulin-resistance, inducing lipolysis and promoting fatty acids efflux to the circulation (S. A. Polyzos et al., 2016). Similar mechanisms occur in the liver, where the production of chemokines and inflammatory infiltration are hallmarks of NASH (Peiseler & Tacke, 2021).

Glycation

Glycation, or non-enzymatic glycosylation, was described by L. Maillard in the 20th century. This non-enzymatic reaction arises in the presence of reducing sugars that could react with the amine groups of various biological molecules, such as proteins, nucleic acids, and lipids (Brownlee, 2005; Goldin et al., 2006; Matafome et al., 2015b). These molecules, when undergoing glycation, change their physical and

chemical properties, as well as their biological function. Methylglyoxal (MG), a reactive glycating agent that reduces carbohydrates to produce advanced glycation end products (AGEs), is produced by the auto-oxidation of glucose. The glycation and oxidation processes occur through a set of complex reactions and subsequent molecular rearrangements, originating from Schiff bases, which transit to Amadori products and which, finally, lead to the formation of AGEs (Matafome et al., 2015b). The accumulation of AGEs can be related to metabolic diseases such as T2DM (Rodrigues et al., 2017) in two ways: through the formation of permanent cross-links with key molecules of the extracellular matrix, altering its structure, and by interacting with its advanced glycation end products receptor (RAGE) on the cell surface. These mechanisms can change the properties of the main matrix proteins (collagen, vitronectin and laminin), binding them to other macromolecules (AGE-AGE bonds), causing their loss of function and greater matrix rigidity. On the other hand, glycation leads to the synthesis of different types of collagens, laminin, and fibronectin, normally associated with an increase in transforming growth factor (TGF)-β expression. Also, intracellular protein can also suffer pathway changes due to induced glycation by MG and be involved in cell response to hypoxia (Bento & Pereira, 2011; Matafome et al., 2017).

The interaction of AGEs with RAGE induces multiple cellular changes, namely in endothelial, muscle and macrophage cells, through the activation of different pathways/mechanisms (Brownlee, 2005; Goldin et al., 2006). At the intracellular level, AGEs form more slowly in the presence of glucose than in the presence of other intracellular sugars, however, contributing to the activation of intracellular pathways of inflammation and stress (Goldin et al., 2006; Matafome et al., 2015b).

The accumulation of AGEs can be related to metabolic diseases and the deterioration of metabolic homeostasis in obesity, which will later contribute to pathologies associated with insulin resistance, such as CVD, liver diseases and diabetes-related complication and diseases including dementia (Rodrigues et al., 2017).

3. WE ARE WHAT WE EAT – MATERNAL NUTRITION AND MILK COMPOSITION:

a. MATERNAL NUTRITION IN GESTATION AND LACTATION:

The gestation period is a time of significant physiological changes and nutritional needs to support foetal growth and development. The fundamental factor on weight gain during pregnancy is the increase of energy intake, which is designed to support both the needs of the mother, with a focus on the 10th and 30th week of pregnancy (with the formation of placenta and amniotic fluid, expansion of the uterus, breast, and AT), and the development of the fetus. Physical activity, pre-pregnancy BMI and metabolic rate are all factors that can influence maternal health. Adequate energy, macro- and micro-nutrient intake is important throughout pregnancy to meet normal mother demands while creating the reserves required for foetal growth and breastfeeding (Williamson, 2006).

Energy and macronutrients: To support foetal growth and development, the energy requirement is also increased, according to the gestation stage. The Italian Recommended Dietary Allowances (RDA) advises more 69kcal/d, 266kcal/d and 496 kcal/d to an adequate energy consumption, in the first, second and third trimester, accordingly (Marangoni et al., 2016). But there is no consensus about these values (Danielewicz et al., 2017; Lowensohn et al., 2016). Protein content is also important, because a low intake can have a negative effect in term of weight and length of birth and an excessive consumption can also affect foetal development (Marangoni et al., 2016). The quality of the protein, in case of animal or vegetable protein is also relevant. The recommended daily intake is about 71g/d, starting in the second trimester (Lowensohn et al., 2016). Fat is also an important requirement for the infant growth and the only recommendation for intake is between 25% and 35% of total calories (Lowensohn et al., 2016). An intake in omega-3 polyunsaturated fatty acids (PUFA)s and docosahexaenoic acid (DHA) should be enhanced, for a proper visual and cognitive development. In some studies, the recommendation intake is more 100 and 200mg/d (Lowensohn et al., 2016; Marangoni et al., 2016).

Micronutrients: Depending on maternal diet and body reserves, vitamins A, B1, B2, B6, B12, D, iron, and iodine are the most relevant micronutrients. Maternal diet isn't always ideal, so multivitamins consumption during lactation may be necessary (Greer, 2001; Victora et al., 2016). Vitamin K levels are usually exceedingly low, regardless of maternal nutrition. Vitamin D is also found in small amounts in tissues specifics to pregnancy such as placenta and decidua, especially when mothers are not exposed to sunlight, as is the case in many cultures across the world (Dawodu et al., 2014). In the first trimester, vitamin D3 is involved in regulation of cytokine metabolism and modulation of the immune system. There is a lack of consensus in different countries, but supplementation is necessary, and the values surround the 600IU/d (Lowensohn et al., 2016; Marangoni et al., 2016). B-complex vitamins are water-soluble vitamins that are required for cell energy generation and release, as well as protein, fat, and carbohydrate metabolism. This complex includes vitamins B1 (thiamine), B2 (riboflavin), B3 (niacin), B6 (pyridoxine), and B12 (cyanocobalamin). B1 deficiency may have an impact on embryonic brain development (Dias et al., 2013), while B2 and B3 deficits have been related to preeclampsia and congenital cardiac defects (Shaw et al., 2010). Folate is a water-soluble B vitamin, which can be found in leafy green vegetables, yeast extract, and citrus fruits. Folate contributes to amino acid metabolism, protein synthesis, and cell division, making it especially important during embryonic and fetal development, when cell division and tissue expansion are fast (De-Regil et al., 2015; Lowensohn et al., 2016). Vitamin A is a fat-soluble vitamin derived from retinoids or carotenoids and has physiological impacts on vision, growth, bone metabolism, immunological function, gene transcription, and antioxidant action. It is particularly important during pregnancy in order to sustain the fetus growth and tissue maintenance, as well as to provide fetal reserves and support the maternal metabolism (Mccauley et al., 2015). Calcium is actively transported to the fetus via placenta and there is a particular need during the 3rd trimester, due to the mobilization from maternal skeleton to a greater efficiency in intestinal absorption and renal retention. WHO recommends 1.5g/d, until 2g/d, in the 20th week until the end (Lowensohn et al., 2016; Marangoni et al., 2016). Iron is important for oxygen transfer to the tissues and the requirement is progressively increasing on course of gestation. An inadequate intake can increase the preterm delivery risk and low birth weight. According to Italian RDA, is necessary 27mg/d (Lowensohn et al., 2016; Marangoni

et al., 2016). Iodine is relevant for glucose metabolism, necessary to produce fetal thyroid hormones and thermogenesis. The intake recommended by WHO is 150ug/d (Lowensohn et al., 2016; Marangoni et al., 2016).

b. MILK COMPOSITION:

Breastfeeding, rather than formula feeding, has been shown to protect against rapid newborn weight gain and susceptibility to adult obesity (Butte, 2009; Owen et al., 2005; Victora et al., 2016). However, the benefits may be influenced by its composition (B. E. Young et al., 2018), genetics, environmental factors and lifestyle (Ballard & Morrow, 2013; Saarela et al., 2005). The content of breast milk is dynamic, varying during a feeding, diurnally, throughout lactation, and between mothers and/or population (Renee et al., 2000).

Colostrum is the first fluid produced after partum, and it differs in amount, appearance, and composition. It is mainly composed by immunologic components such as secretory immunoglobulin (Ig)A, lactoferrin, leukocytes, and developmental factors, including epidermal growth factor (EGF), which is produced in small amounts in the first few days after birth (Castellote et al., 2017; Pang & Hartmann, 2007). It also has a low lactose content, suggesting that its main roles are immunologic and trophic. Salt, chloride, and magnesium levels are higher, while potassium and calcium levels are lower than in mature milk (Castellote et al., 2017). The sodium/potassium ratio decreases, but lactose concentration rises, indicating secretory activity and the formation of transitional milk. Secretory activation (stage II lactogenesis) occurs at different times for different women, although it usually happens within the first several days after delivery. The sodium content, sodium-to-potassium ratio, citrate, and lactose concentration of early milk are all biochemical indicators for secretory activation. Transitional milk has some of the same qualities as colostrum, but it is produced at a higher rate to support the newborn in nutritional and fast-development needs. It is produced between 5 days and 2 weeks following delivery and after that the milk is considered mature. It is considered fully developed 4 to 6 weeks after delivery. In contrast to the substantial changes in composition seen in the 1st month, the composition of human milk usually remains constant during breastfeeding (Wan et al., 2020).

Mature-term milk is estimated to be between 0.9 and 1.2 g/dL, fat content between 3.2 and 3.6 g/dL, and lactose content between 6.7 and 7.8 g/dL. The energy is associated with the fat level, with estimates ranging from 65 to 70 kcal/dL. Casein, alpha-lactalbumin, Immunoglobin A (IgA), lysozyme, and serum albumin are the most abundant proteins (Lönnerdal, 2013). The macronutrient more variable in milk is fat, being palmitic and oleic acids the most abundant fatty acids (Lönnerdal, 2013). The disaccharide lactose is the main sugar while oligosaccharides, which is about 1 g/dL depending on lactation stage and mother genetic variables, are other important carbohydrates (Morrow et al., 2005; Newburg et al., 2005). Protein income in early pregnancy is similar to non-pregnant women, but on the 2nd and 3rd trimester, it is visible an increase of 15% and 25% on protein consumption (Elango & Ball, 2016). These metabolic changes facilitate to maintain maternal homeostasis while responding to fetal needs and preparing for breastfeeding. (Elango & Ball, 2016).

c. NEONATAL METABOLISM OF GLUCOSE AND LIPIDS:

The foetal metabolism can be remodeled by several stimuli, that often leads to alterations in the homeostatic state. The intrauterine environment has a direct effect in foetal organ development and sometimes, compromising the health of the newborn.

During the last trimester, a healthy mother suffers significant changes in their metabolism, such as an accelerated breakdown of fat depot, hypertriglyceridemia and developing insulin resistance. In utero, the placenta supplies glucose - the main carbohydrate transported to the fetus, followed by amino acids, and lastly, lipids (Castillo-Castrejon & Powell, 2017a; Herrera & Amusquivar, 2000; Lager & Powell, 2012). The fetus is capable of gluconeogenesis, but in a low degree (Grilo et al., 2021) and the maternal glucose concentrations is positively correlated with the newborn size and weight (Grilo et al., 2021; Kulkarni et al., 2013a). Glucose crosses the placenta due to a concentration gradient between maternal/fetal plasma and facilitated by specific placental glucose transporters such as GLUT-1, -3 and only 40-50% of maternal glucose is transferred to the fetus, while the remain percentage is converted into lactate (Ward Platt & Deshpande, 2005). Lactate is also important to the fetus because it provides a substrate for oxidative and non-

oxidative metabolism, such as glycogen synthesis (Ward Platt & Deshpande, 2005). In this late stage of gestation, maternal plasma lipoproteins do not cross the placenta and it is mediated by receptors that allows the uptake and release of lipid components for the fetus (Herrera et al., 2006). Also, numerous studies have demonstrated the connection between maternal lipids and newborn weight (Castillo-Castrejon & Powell, 2017b; Kulkarni et al., 2013b).

4. THE FUTURE AHEAD OF US - CORRELATION BETWEEN MATERNAL DIET AND METABOLIC PROGRAMMING OF THE NEWBORN:

When maternal substrate availability exceeds fetal requirements, normal growth patterns can be influenced, and disease outcomes can be granted over the life course. The term "metabolic programming" refers to the phenomenon by which alterations can determine future disease susceptibility, such has metabolic disorders (Hales et al., 1991). Delayed lactogenesis, defined as the initiation of lactogenesis more than 72 hours after delivery, appears to be associated with preterm birth and maternal obesity (Nommsen-Rivers et al., 2012). Although maternal insulin resistance and plasma glucose concentrations are biologically elevated during pregnancy to promote fetal nutrition, there is controversy whether mild maternal hyperglycemia can enhance childhood obesity and diabetes risk (Fall & Kumaran, 2019a). Human studies have shown that hyperphagia, adiposity, hypertension, and insulin resistance develop in offspring between the ages of 3-6 months as a result of maternal over-nutrition (A. M. Samuelsson et al., 2008). The offspring phenotype depends on exposures during the pregnancy and lactation periods (Desai et al., 2014; Habbout et al., 2013).

In recent human studies **(Figure 4)**, maternal obesity results in an increased risk of newborns being large for gestational age, yet they can be growth restricted due to placental insufficiency(Fall & Kumaran, 2019b; Kaul et al., 2019). The newborn has a higher adiposity and BMI, developing most of the times childhood obesity (Bichteler & Gershoff, 2018a) and posterior adult MetS (Catalano & Demouzon, 2015). It is demonstrated that hyperinsulinemia in the offspring (even *in utero* stage) can be a consequence of maternal obesity and high-fat diet consumption (Walsh et al., 2014; Westermeier et al., 2014). Maternal obesity without Diabetes is also

correlated with an increased possibility of developing MetS in children and in adult life, suffer cardiovascular death (Gaillard et al., 2016; Godfrey et al., 2017). Studies to understand the role of intrauterine impact show the effects of maternal bariatric surgery and they demonstrated that this intervention before or during pregnancy can reduce the risk of obesity in the progeny (Godfrey et al., 2017).

It's still up for debate if breastfeeding is advantageous to diabetic women's offspring and whether it has long-term impacts on their glucose metabolism. Nevertheless, breastfeeding is linked to slower postnatal growth and a slimmer phenotype during infancy and adolescence in the general population (Gunderson, 2008). However, studies show that breastfeeding has more advantages than formula feeding (T. Young et al., 2018), preventing metabolic syndrome in adult life. Foetal or neonatal insulin resistance can be induced by gestational diabetes (Dong, Luo, Nuyt, Audibert, Wei, Abenhaim, Bujold, Julien, Huang, Levy, & Fraser, 2018; Luo et al., 2010), due to hyperglycemia exposure. The fetus is also exposed to high concentrations of lipids and amino acids, which cross the placenta and overstimulate the foetal pancreas, leading to hyperinsulinemia and foetal overgrowth. Moreover, insulin resistance was also described in preterm babies without a hyperglycemia exposure (Salis et al., 2017). Fetal insulin resistance has been reported to be conditioned by intrauterine abnormal inflammation, adipokines, and ER stress (Westermeier et al., 2014). Regarding the development of insulin resistance and β-cells dysfunction, studies shows that the environment in utero and postnatal life have can influence to a decrease in cell number and abnormal glucose tolerance in adult offspring (Dabelea et al., 2000).

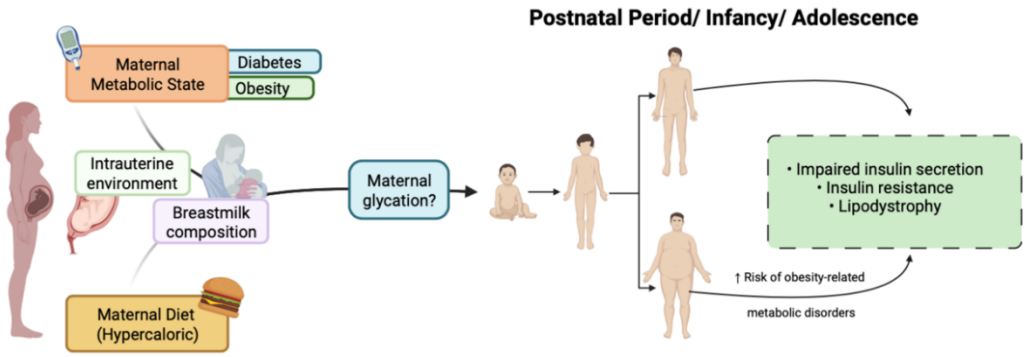

Figure 4- Metabolic insults and the outcome for the offspring.

Animal studies are significantly important to provide insights into possible underlying mechanisms. Studies indicate that nursing of control newborns by obese dams is sufficient to program an increased food intake and offspring obesity, although in a lesser degree than those offspring exposed to maternal obesity also during pregnancy (Monks et al., 2018). This suggests that during lactation, the quality and/or quantity of the milk ingested plays a significant role in the metabolic programming. In several transgenerational studies, maternal high-fat diet induced obesity and insulin resistance in the first- and second-generation offspring (Hanafi et al., 2016).

Other studies using litter-size manipulation after birth have shown that pups raised in small litters are obese at adulthood, due to increased milk availability and intake (Habbout et al., 2013; Xavier et al., 2019). Also the exposure to cafeteria diet leads to permanent changes in the tissue structure, body composition, endocrine response, metabolism of the offspring, ultimately causing diabetes (Fall & Kumaran, 2019a).

These findings evidence the great importance of adequate nutrition in the gestation and lactation and their involvement on the metabolic programming. However, the mechanisms operating during the fetal and newborn periods are still to understand. More studies are needed to ascertain whether breastfeeding and its duration (in case of metabolic disorders) are advantageous to their offspring.

Chapter II

SCIENTIFIC FRAMEWORK AND OBJECTIVES

Scientific framework: A sedentary lifestyle and consumption of Western diets, rich in glycotoxins (AGEs precursors), contribute to the increase in the incidence and prevalence of obesity observed in recent years, predisposing to the development of metabolic pathologies such as obesity, metabolic syndrome and T2DM (Elabbassi & Haddad, 2005; Iacobini et al., 2019b; Roden & Shulman, 2019b). A factor in common of this metabolic disease is insulin resistance, causing adipose tissue dysfunction and impaired glucose homeostasis.

The term "metabolic programming" consists of the early alteration of mechanisms in response to metabolic insults, with the perinatal period being the most susceptible to these changes (Hales et al., 1991), influencing on hyperglycemia, impaired fasting glucose, insulin resistance, inflammation and predisposition of obesity. Western diets, maternal obesity and overnutrition are associated with an increase in adiposity and a greater predisposition to later develop obesity (Bichteler & Gershoff, 2018b; Catalano & deMouzon, 2015).

Glucose homeostasis and lipid storage are processes regulated by various hormones such as insulin. Insulin regulates glucose and lipid metabolism in various organs, decreasing gluconeogenesis and stimulating glycogen synthesis in the liver, and inhibiting lipolysis in adipose tissue. Overnutrition is associated with a pro-inflammatory environment, promoting lipotoxicity, insulin resistance and lipolysis in adipocytes. Consequently, there is hypertrophy of adipocytes, as well as insulin resistance in the liver (Gesta et al., 2007b; Item & Konrad, 2012; S. Polyzos et al., 2009; S. A. Polyzos et al., 2017).

Existing studies highlight the enormous importance of exposure to obesogenic environments in early life and its involvement in metabolic programming. However, the modulation of this mechanisms that occurs during the perinatal period is still poorly understood.

Main objective: This project aims to elucidate if exposure to obesogenic environments in the perinatal period alters glucose and lipid metabolism in WAT, liver, and pancreas. These changes can condition the development of metabolic diseases, both in adulthood and in earlier stages of life, corroborating the increasing incidence of childhood obesity. Thus, the objective of the project is to identify the factors that lead to the development of early insulin resistance.

Specific objectives:

a. To understand the effect of maternal obesity induced by a high-fat diet in the mechanisms of insulin signaling in the liver and adipose tissue of the offspring.

b. To disclose the role of neonatal hyperphagia-induced obesity in regulating peripheral and central mechanisms of insulin signaling.

c. To address the impact of maternal glycotoxins in insulin pathway in normal and hyperphagic newborns.

Chapter III

MATERIALS AND METHODS

1. IN VIVO STUDY:

A. MATERNAL OBESITY MODEL:

The procedures involving Sprague-Dawley were approved by The Ethical Committee of the Institute for Research and Innovation in Health – i3S, University of Porto, and National Government Authority (Direção Geral de Alimentação e Veterinária – No.0421/000/000/2018) approved the experimental protocol, which followed the Guidelines for Care and Use of Laboratory Animals in Research advised by the Federation of European Laboratory Animal Science Associations (FELASA).

42 days-old female Sprague-Dawley rats (150–200 g) were fed with control and high-fat high-sucrose (HFHS) diet (**Figure 5**). At day 91 the dams mated with male rats and fed with standard or HFHS diet. The maternal obesity-inducing diet contained 42% metabolizable energy from fat (vs. 10% in standard), 27% from proteins (vs. 20% in standard), and 31% from carbohydrates (mainly sucrose, vs. 70% in standard with 1% sucrose), with crude fat of 23.1% (vs. 4.1% in standard), high cholesterol content and increased proportion of long-chain fatty acids. After delivering naturally at day 112, litter reduction was performed, reducing to 3 male and 3 female pups to have an equal availability of milk. On day 141, corresponding to postnatal day (PND) 21 in the offspring, weaning was performed, and the pups fed with chow. On PND 42, offspring were euthanized after overnight fasting and WAT and liver were collected for this study. More information of this animal model can be consulted on Stevanović-Silva et al., 2021.

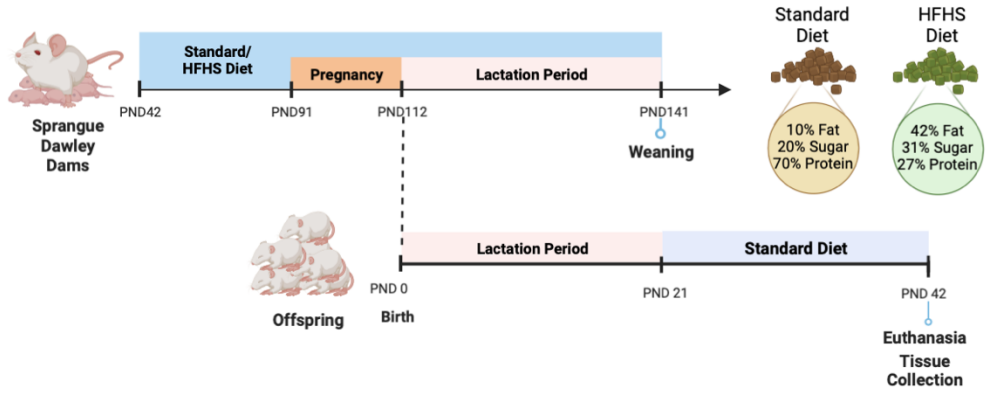

Figure 5 - Schematic representation and timeline of the experimental design of Sprague-Dawley dams, fed with standard or HFHS diet, during gestation and their offspring.

B. MATERNAL GLYCATION MODEL:

The following procedures were approved by the Animal Committee of the Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra. This procedure was followed according to good practices of animal handling, with the consent of the Institutional Animal Care and Use Committee (ORBEA 13/2018) and performed by licensed users by the FELASA, conformed with European Parliament for the Protection of Animals Used for Science Purpose guidelines (2010/63/EU), transposed into the Portuguese law in 2013 (Decreto-Lei 113/2013).

Female Wistar rats from local breeding colonies (Faculty of Medicine, University of Coimbra) were housed in a controlled environment with day-night cycles of 12 h, temperature at 22°C with ventilation, and a relative humidity of 55%.

To understand the effects of maternal glycation (**Figure 6A**), Wistar dams were treated with S-p-Bromobenzylglutathione cyclopentyl diester (BBGC) – a selective inhibitor of Glyoxalase 1 (GLO1) (**Figure 6B**) at 5mg/kg body weight, via intraperitoneal (i.p.) and during the first six days of lactation, whereas control dams were injected with vehicle administration, dimethyl sulfoxide (DMSO) during the same period. The animals had *ad libitium* access to water and standard diet. The caloric and water intake of the dams and their offspring was registered, as well as

their body weight. At the 21st day, occurred the weaning of the offspring, collection of milk samples and euthanasia of the dams and the liver, pancreas and kidneys were collected to histological analysis. At 45th day the offspring was submitted to an insulin tolerance test (ITT) using a Glucometer (Bayer, Germany), triglycerides levels and the sacrifice, were the WAT, liver and pancreas was retrieved to further histological analysis and protein quantification levels with Western Blot.

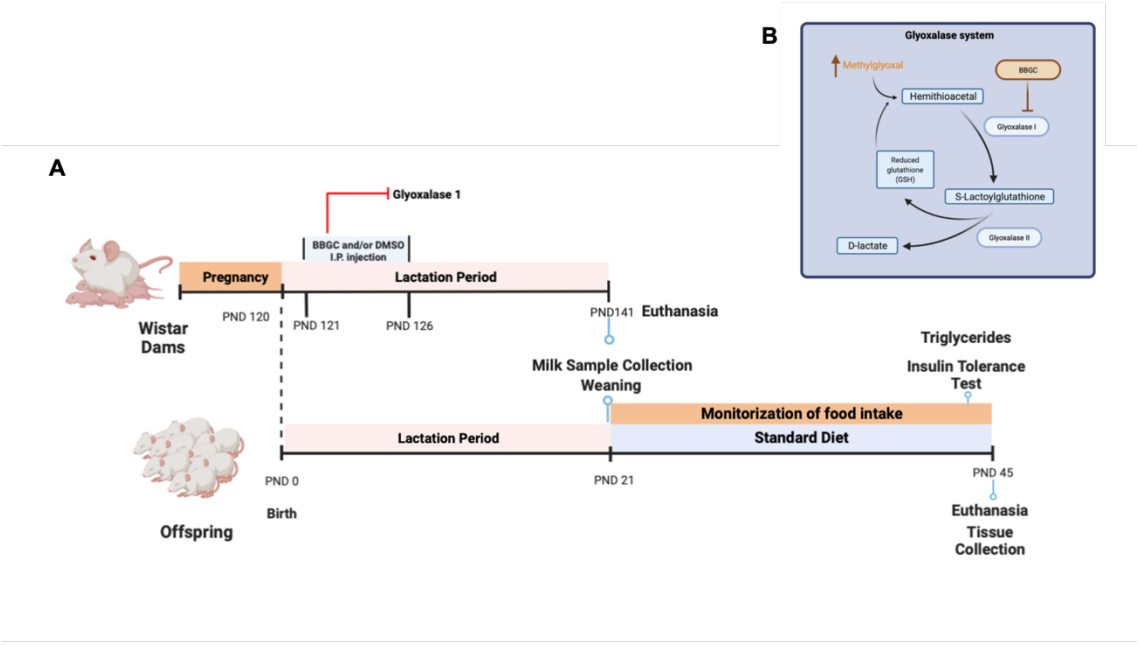

Figure 6 - Schematic representation and timeline of the experimental design of Wistar dams and their offspring (A). Glyoxalase system and BBGC action in this system (B).

C. MATERNAL GLYCATION AND CHILDHOOD HYPERPHAGIA MODEL:

To study the role of neonatal hyperphagia and the correlation with maternal glycation (**Figure 7**), Wistar dams were induced with litter reduction (SL), reducing to 3 male pups at the 3rd day and to correlate the milk availability and quality, the dams were injected with BBGC **(Figure 6B)**, via i.p. and three days after giving birth, when the litter size suffered a reduction to 3 male pups. The animals had *ad libitium* access to water and standard diet. The body weight, caloric and hydric consumption of the dams and the offspring was register. At the 21st day, occurred the weaning of the offspring, collection of milk samples and euthanasia of the dams and the liver,

pancreas and kidneys were collected to histological analysis. At 45th day the offspring was submitted to an ITT using a Glucometer (Bayer, Germany), after a fasting of 6 hours, triglycerides levels and sacrifice, were the liver, WAT, and pancreas was retrieved to further histological analysis and protein quantification levels by Western Blotting.

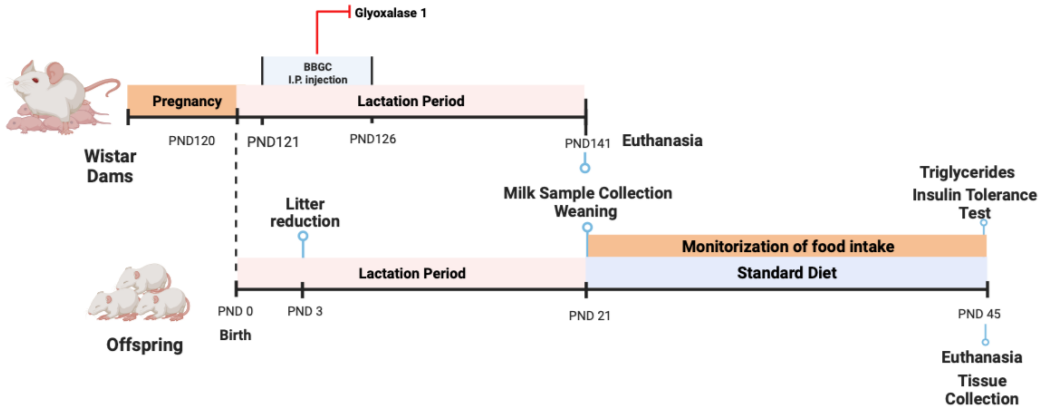

Figure 7 - Schematic representation and timeline of the experimental design of the litter reduction model and the combination with the BBGC.

2. HISTOLOGICAL COLORIMETRIC ASSAYS:

Liver, WAT, and pancreas were fixed in formalin solution (10%), dehydrated in a series of increasing concentrations of alcohol (70% to 100%), cleared in xylene and embedded in histological paraffin. The organs were sectioned in a microtome, in a non-serial section of 4µm thickness and dried at room temperature overnight. tissue sections were then submitted to paraffin removal processes, using xylol (3x3' at room temperature) and subsequently, progressive hydration (EtOH 100%/70%/30% 1x3'/each and Milli-Q water 1x3' at room temperature) and stained with hematoxylin and eosin (HE). After this, tissue sections were washed again, and coverslips were mounted using mounting medium (DAKO, Japan). Finally, images (100X or 40X) were captured in a Zeiss microscope with incorporated camera (Germany).

3. WESTERN BLOTTING:

Liver and WAT were disrupted in lysis buffer (0.25 M Tris-HCl, 125 mM NaCl, 1% Triton-X-100, 0.5% SDS, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 2 mM Na3VO4, 10 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 10 mM phenylmethylsulfonyl, and 40 µL of protease inhibitor, pH 7.7). Afterwards, the tissues underwent 3 cycles/20min of Tissue Lyser II (Qiagen, Germany) and centrifuged at 14000 rpm at 4ºC for 20 minutes. The supernatant was collected, and protein concentration was determined by BSA method (Alfa Aesar, USA). Samples were §added 2x Laemmli buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8), re-sonicated and boiled at 95ºC for 3 minutes. Vertical electrophoresis was carried in 8%-10% polyacrylamide gel with the following composition: resolving (0.75 M Tris-HCl, 0.2% SDS, pH 8.8), stacking (0.25 M Tris-HCl, 0.2% SDS, pH 6.8) plus acrylamide, Milli-Q water, ammonium persulfate and tetramethlyethylenediamine. Electrophoresis system (Bio-Rad, USA) was filled with running buffer (125 mM Tris-base, 480 Mm glycine, 1% SDS, pH 8.8), amounts of protein were loaded, as well as a protein standard (GRiSP, Research Solutions, Portugal), and voltage was kept constant during protein migration (120 V). SDS- polyacrylamide gels were transferred electrophoretically to polyvinilydene difluoride membranes (GRiSP, Research Solutions, Portugal) at 750 mA for 2 hours after being activated in methanol, hydrated in Milli-Q water, and washed 15 minutes in Transfer buffer (50 mM CAPS, 2% NaOH, 10% methanol, pH 11). Afterwards, membranes were blocked for 2 hours in 5% albumin in wash buffer (250 mM Tris, 1.5 mM NaCl pH=7.6 plus 0.5% Tween20), incubated overnight with primary specific antibodies **(Table 1)**. Membranes were revealed through chemiluminescent method using a ECL substrate 1:1 (Advansta, EUA) and the luminescence detection system LAS500 Sotfware (GE Healthcare, United Kingdom). For image data processing, was used Quantity One (BioRad, Hercules, CA, USA) and ImageQuant (GE Healthcare, United Kingdom) software.

Table 1 - Primary antibodies used for Western Blotting.

Protein	Molecular weight (kDa)	Host	Dilution	Manufacturer
Anti-IR	92	Rabbit	1:1000	Cell Signaling
Anti-IR (*phospho Y1361*)	92	Rabbit	1:1000	Abcam
Anti-GLUT4	54	Mouse	1:1000	Cell Signaling
Anti-PPAR gamma	55	Rabbit	1:1000	Cell Signaling
Anti-PPAR alpha	55	Rabbit	1:1000	Cell Signaling
Anti-AMPK	62	Rabbit	1:1000	Cell Signaling
Anti-AMPK (*phospho Y172*)	62	Rabbit	1:1000	Cell Signaling
Anti-GLUT2	54	Rabbit	1:1000	Cell Signaling
Anti-Calnexin	83	Goat	1:2000	Sicgen

4. STATISTICAL ANALYSIS:

The results are presented as mean with standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism 9 (GraphPad Software, Inc, San Diego, USA). The normality of the data was assessed with Shapiro-Wilk normality test. For the maternal obesity group, data were treated with Unpaired-test or Mann-Whitney test. For the maternal glycation model and for neonatal hyperphagia and maternal glycation model, data treated with non-parametric Kruskal-Wallis test or with parametric one-way ANOVA followed by Tukey´s post-hoc test. Differences were significant for $p < 0.05$.

Chapter IV

RESULTS

A. MATERNAL OBESITY MODEL:

1. PROTEIN LEVELS OF TOTAL IR AND AMPK ARE DECREASED IN THE VISCERAL WAT OF MALE OFFSPRING:

As previously stated, in the maternal obesity model, Sprague-Dawley rats were fed with Control and HFHS diet during gestation and with Control diet during lactation and breastfeeding of the offspring. In **Figure 8** we can observe protein levels in the visceral WAT of the male offspring, evaluated by Western Blotting. The expression of IR (**Figure 8A**) and the activation of IR (**Figure 8B**) was similar in the offspring of both groups. Total AMPK (**Figure 8C**) was decreased in the offspring of HFHS group in comparison to the offspring of Control group ($p<0.05$ vs. Control group). However, phosphorylation of AMPK (**Figure 8D**) remains alike in Control and HFHS groups. GLUT4 (**Figure 8E**) was also similar in both groups.

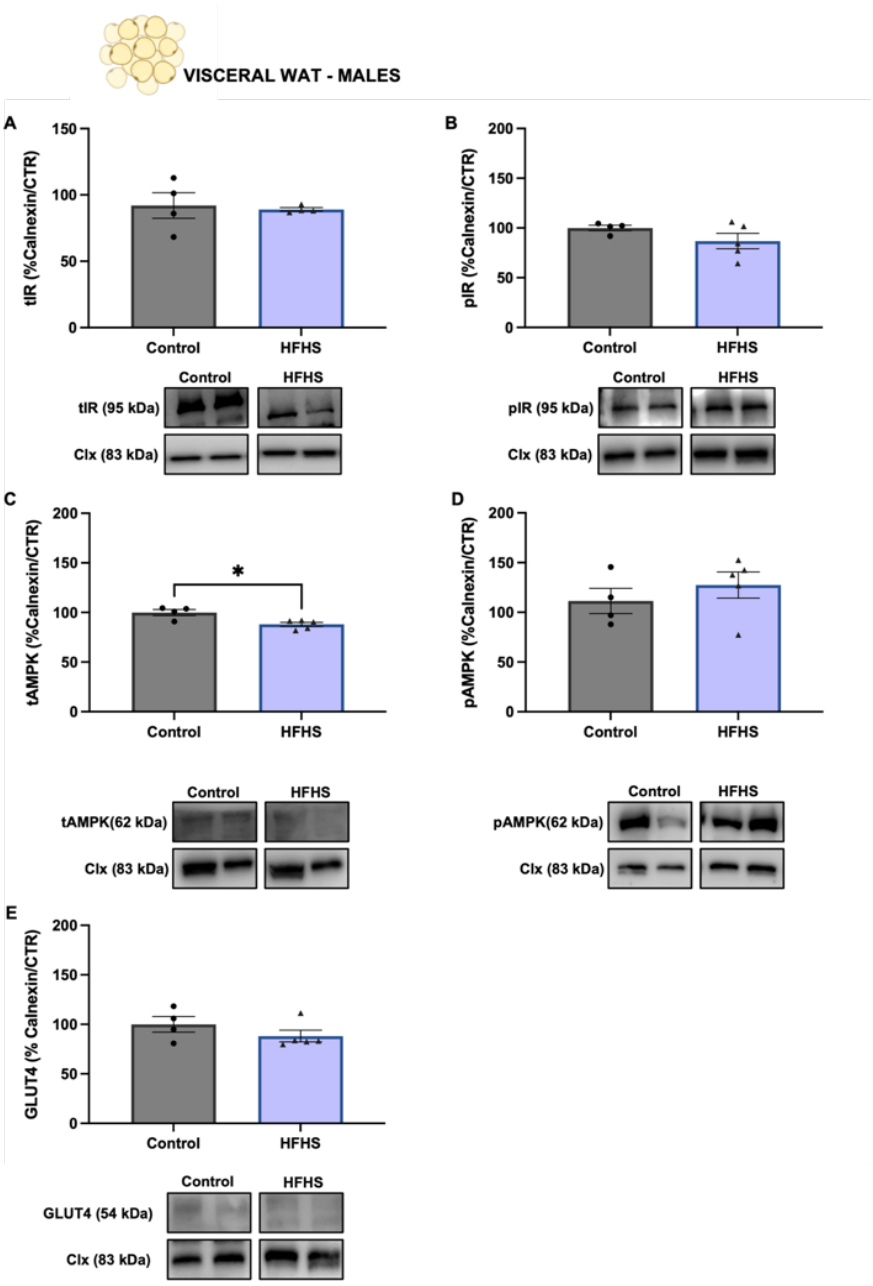

Figure 8 - Protein levels in the visceral WAT of male offspring exposed to maternal obesity. Decreased total AMPK levels in the male offspring of HFHS dams (C). Representative images of Western Blot proteins of interest and loading control (Calnexin or Clx) are shown at the bottom panel. Control – Offspring where the dams were standard diet-fed, HFHS – Offspring where the dams were fed with HFHS diet. Bars represent mean ± SEM and Unpaired t-test or Mann-Whitney comparisons were conducted to compare among the groups. * vs. Control. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

2. PROTEIN LEVELS ARE SIMILAR IN THE LIVER IN MALE OFFSPRING:

Regarding the same model, the liver was also analyzed by Western Blotting. The total IR (**Figure 9A**) is decreased, but with no statistical significance while the activation of IR (**Figure 9B**) is tendentially augmented, with a value of p=0.0779 vs. Control group. Although total AMPK (**Figure 9C**) has a significant decrease compared to the Control group ($p<0.01$ vs Control group), its phosphorylation (**Figure 9D**) is similar in both groups. GLUT2 (**Figure 9E**) and peroxisome proliferator-activated receptor (PPAR)α (**Figure 9F**) levels were also similar in the present groups.

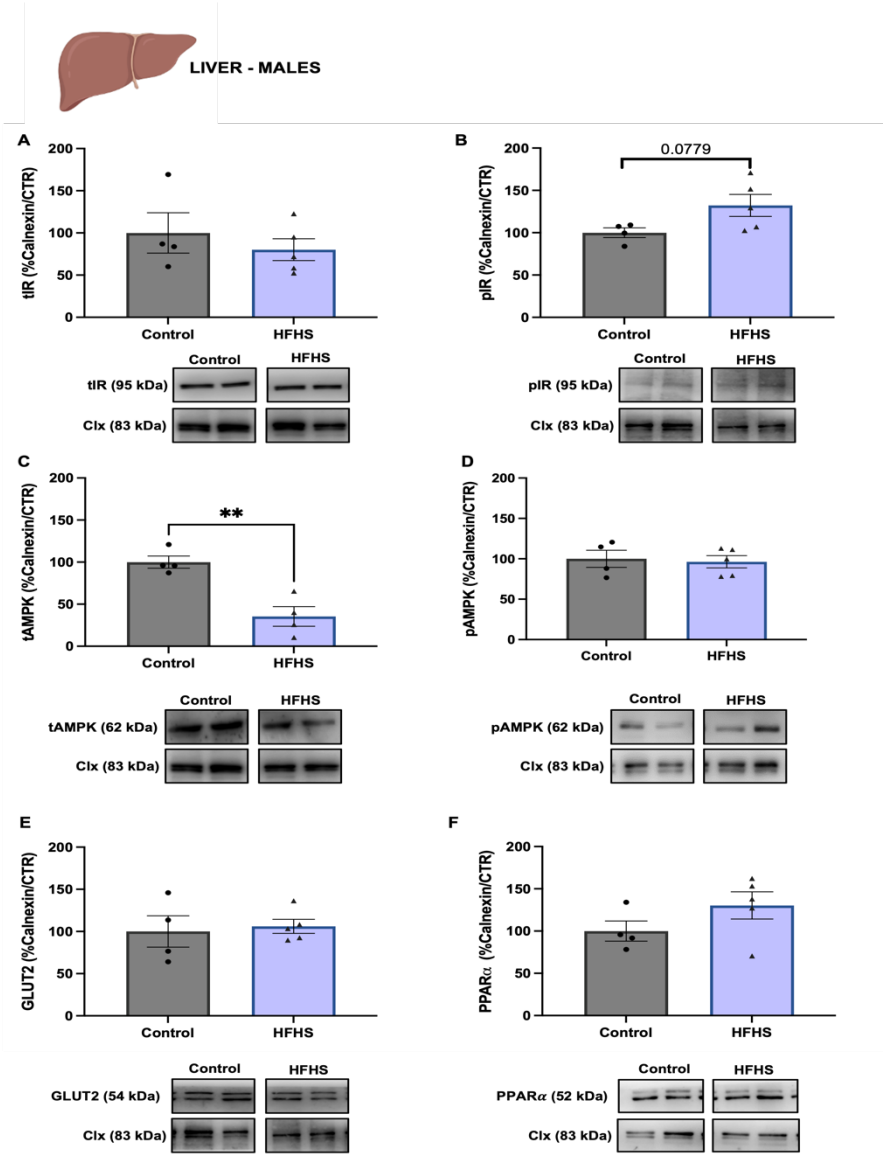

Figure 9 - Protein levels in the liver of male offspring exposed to maternal obesity. Decreased total AMPK levels in the male offspring of HFHS dams (C). Representative images of Western Blot proteins of interest and loading control (Clx) are shown at the bottom panel. Control – Offspring where the dams were standard diet-fed, HFHS – Offspring where the dams were fed with HFHS diet. Bars represent mean ± SEM and Unpaired t-test or Mann-Whitney comparisons were conducted to compare among the groups. * vs. Control. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

3. AUGMENTED LEVELS IN EXPRESSION AND ACTIVATION OF AMPK, IN THE VISCERAL WAT, IN THE FEMALE OFFSPRING:

In the visceral WAT of female offspring (**Figure 10**), according to the same condition stated, we observed no alteration in the IR (**Figure 10A and 10B**). However, the female offspring of HFHS group had an increase of AMPK expression (**Figure 10C**), in comparison to the Control group ($p<0.01$ vs Control group). The same is observed in the levels of phosphorylation of AMPK (**Figure 10D**), compared with the Control group ($p<0.01$ vs Control group). GLUT4 (**Figure 10E**) didn't also show any alterations.

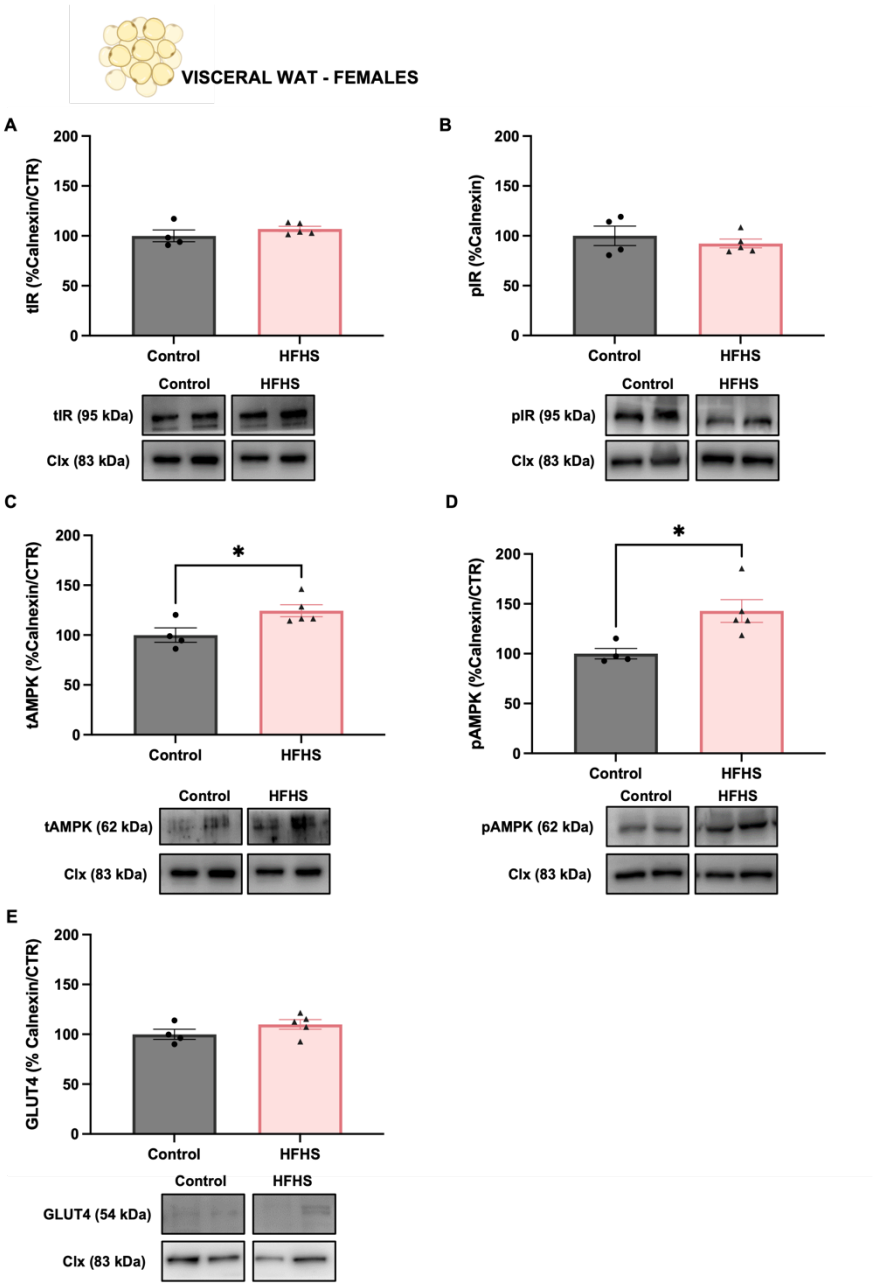

Figure 10 - Protein levels in the visceral WAT of female offspring exposed to maternal obesity. Augmented total and phosphorylated AMPK levels in the female offspring of HFHS dams (C and D). Representative images of Western Blot proteins of interest and loading control (Clx) are shown at the bottom panel. Control – Offspring where the dams were standard diet-fed, HFHS – Offspring where the dams were fed with HFHS diet. Bars represent mean ± SEM and Unpaired t-test or Mann-Whitney comparisons were conducted to compare among the groups. * vs. Control. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

4. THE ACTIVATION OF AMPK IS DECREASED IN THE LIVER OF FEMALE OFFSPRING:

Regarding the protein levels in the liver (**Figure 11**) of female offspring, the activation of AMPK (**Figure 11D**) is decreased in the HFHS group ($p<0.01$ vs Control group). The other proteins studied (IR, GLUT2 and PPARα) had no significant changes.

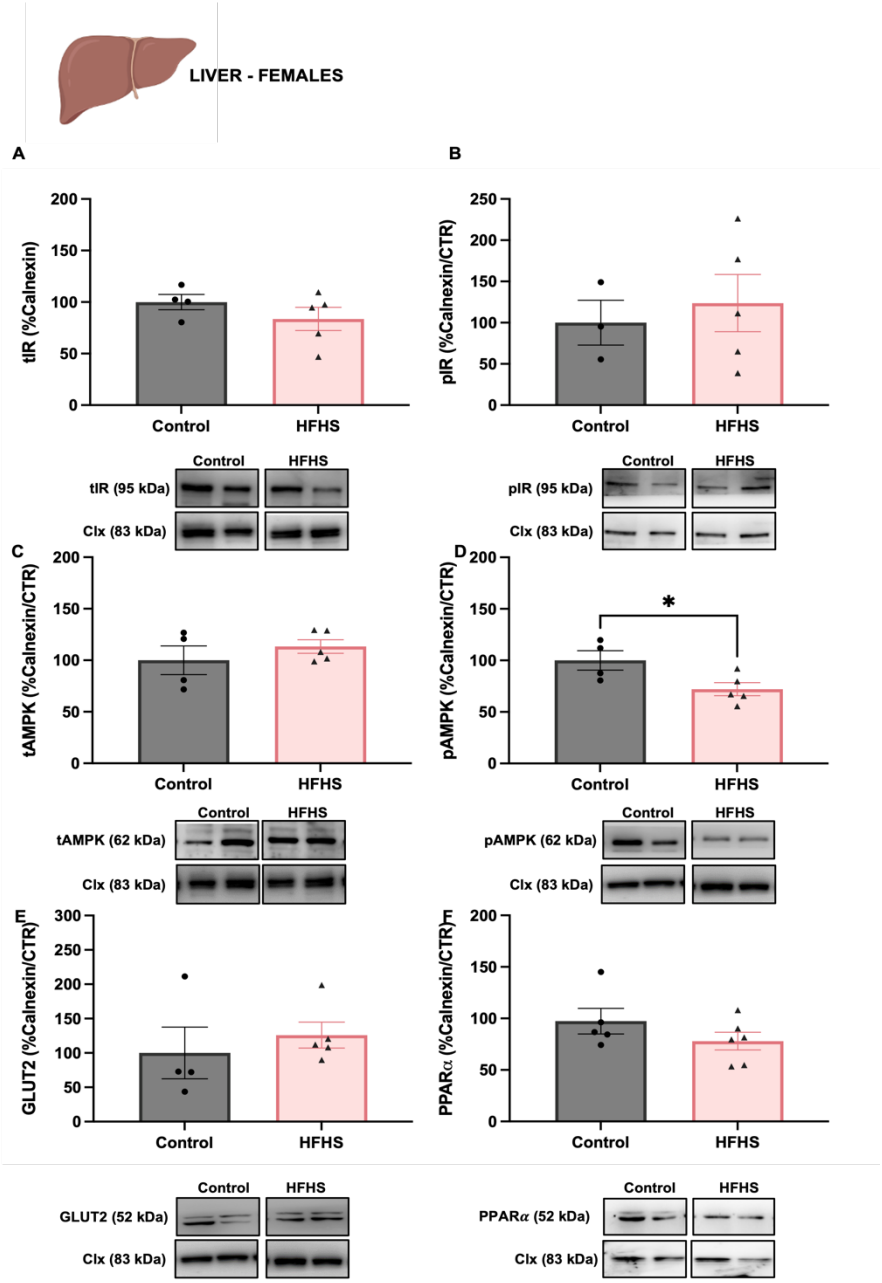

Figure 11 - Protein levels in the liver of female offspring exposed to maternal obesity. Decreased phosphorylated AMPK levels in the offspring of HFHS dams (D). Representative images of Western Blot proteins of interest and loading control (Clx) are shown at the bottom panel. Control – Offspring where the dams were standard diet-fed, HFHS – Offspring where the dams were fed with HFHS diet. Bars represent mean ± SEM and Unpaired t-test or Mann-Whitney comparisons were conducted to compare among the groups. * vs. Control. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

B. MATERNAL GLYCATION MODEL:

1. BBGC ADMINISTRATION CAUSES A DECREASE IN MILK TRIGLYCERIDES AND IN TOTAL ANTIOXIDANT CAPACITY:

Since diets rich in sugars are often also rich in AGEs, we aimed to understand the effects of maternal glycation during breastfeeding- Dams were treated i.p. with BBGC (a known GLO1 inhibitor) during 6 days after offspring birth. The weight of the dams (**Figure 12A**) was evaluated and remained stable, during and after the administration of BBGC. The treatment also didn't cause any alterations in glycemia (**Figure 12B**). In the collected milk, the dams treated with BBGC had a decrease of triglycerides (**Figure 12C**) and the total antioxidant capacity (**Figure 12D**), comparing with the Control dams ($p<0.01$ vs Control group). However, GLO1 activity (**Figure 12E**) had no significant difference. In **figure 12F**, HE staining of hepatic tissue (100X) shows that the morphology remained similar, with no signs of hepatic toxicity.

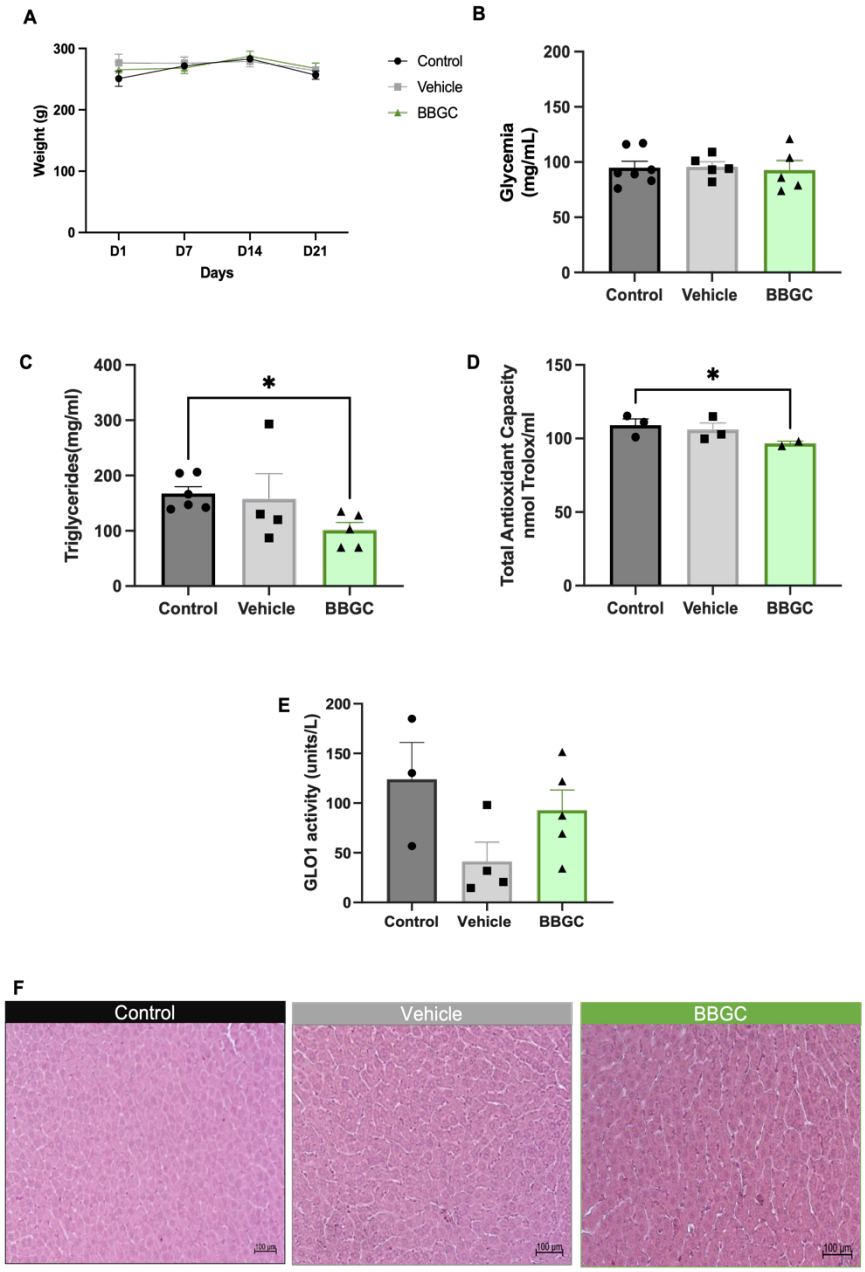

Figure 12 - Weight and glycemia of the dams. Metabolic parameters in the milk and hepatic histological analysis of the liver. Evaluation of the weight of Wistar dams (A) and glycemia (B). In the milk, we evaluated triglycerides (C), total antioxidant capacity (D) and GLO1 activity (E). HE staining (100X) of the hepatic tissue (F) in Control, Vehicle and BBGC groups. Bars represent mean ± SEM and Kruskal-Wallis and Ordinary One-Way ANOVA comparisons were conducted to compare among the groups. * vs Control; $ vs Vehicle. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

2. METABOLIC PROFILE OF MALE OFFSPRING:

The weight of the male offspring was evaluated before (**Figure 13A**) and after (**Figure 13B**) weaning, and we didn't observe any differences. Insulin tolerance test (**Figure 13C**) didn't show us any alteration, an neither the plasma insulin levels (**Figure 13D**).

The glucose decay rate, or kITT (**Figure 13E**) demonstrated that the male offspring of the group BBGC have less insulin sensitivity, when compared to Control and with Vehicle ($p<0.05$). Triglycerides (**Figure 13F**) demonstrated no alteration, while (**Figure 13G**) was lower in the offspring of BBGC group than the Control group ($p<0.01$ vs. Control group).

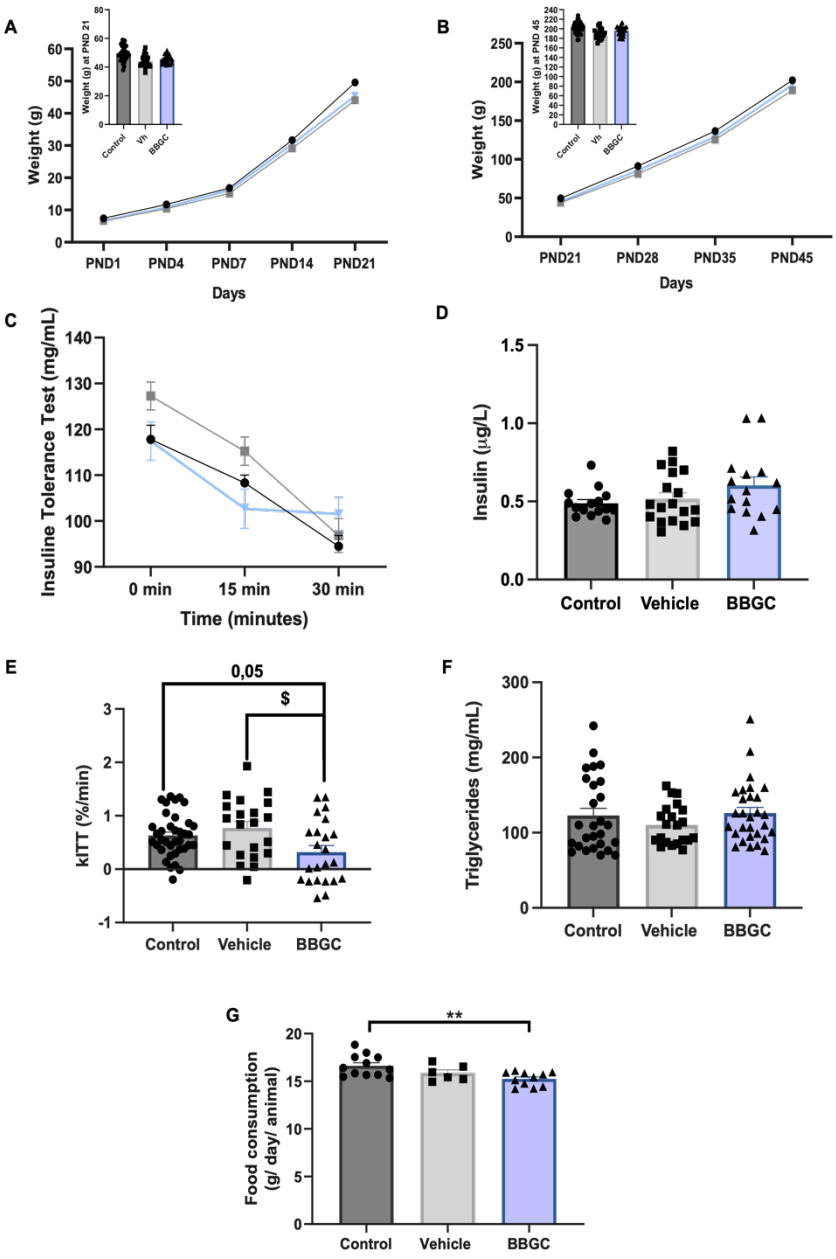

Figure 13 - Metabolic profile of male offspring. Male offspring whose dams were treated with BBGC have less insulin sensitivity. Evaluation of the weight of offspring pre-weaning (A) and pos-weaning(B). Insulin tolerance test (C) showed no alterations. Plasma insulin remained normal (D). kITT (E) demonstrated a lower insulin sensitivity in the BBGC group. Triglycerides (F) was also evaluated but with no modification, However, these animals, namely, BBGC group ate less (G) compared to the Control group. Control – Offspring of dams weren't treated, Vehicle – Offspring of dams were treated with DMSO, BBGC – Offspring of dams were treated with BBGC. Bars represent mean ± SEM and Kruskal-Wallis and Ordinary One-Way ANOVA comparisons were conducted to compare among the groups. * vs. Control; $ vs. Vehicle. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

3. MORPHOLOGICAL ANALYSIS IN THE MALE OFFSPRING:

After the sacrifice, WAT, liver, and pancreas were weighted, examined by HE staining (40X) and further the area of the adipocytes and islets of Langerhans were quantified.

In **figure 14A** the histology of WAT in the offspring of the respective groups are shown. No major alterations were observed in tissue morphology. When comparing the weight of the AT (**Figure 14B**) collected, the values were similar between groups, although a trend for increased adipocyte area (**Figure 14C**) was observed in the offspring of dams treated with BBGC.

The histology of the liver (**Figure 14D**) also didn't show any alteration, nor its weight (**Figure 14E**).

The islets of Langerhans were also examined by HE (**Figure 14F**). The morphology of the islets was altered in the offspring of the BBGC group, although similar islet area (**Figure 14G**) was observed in Control and BBGC groups.

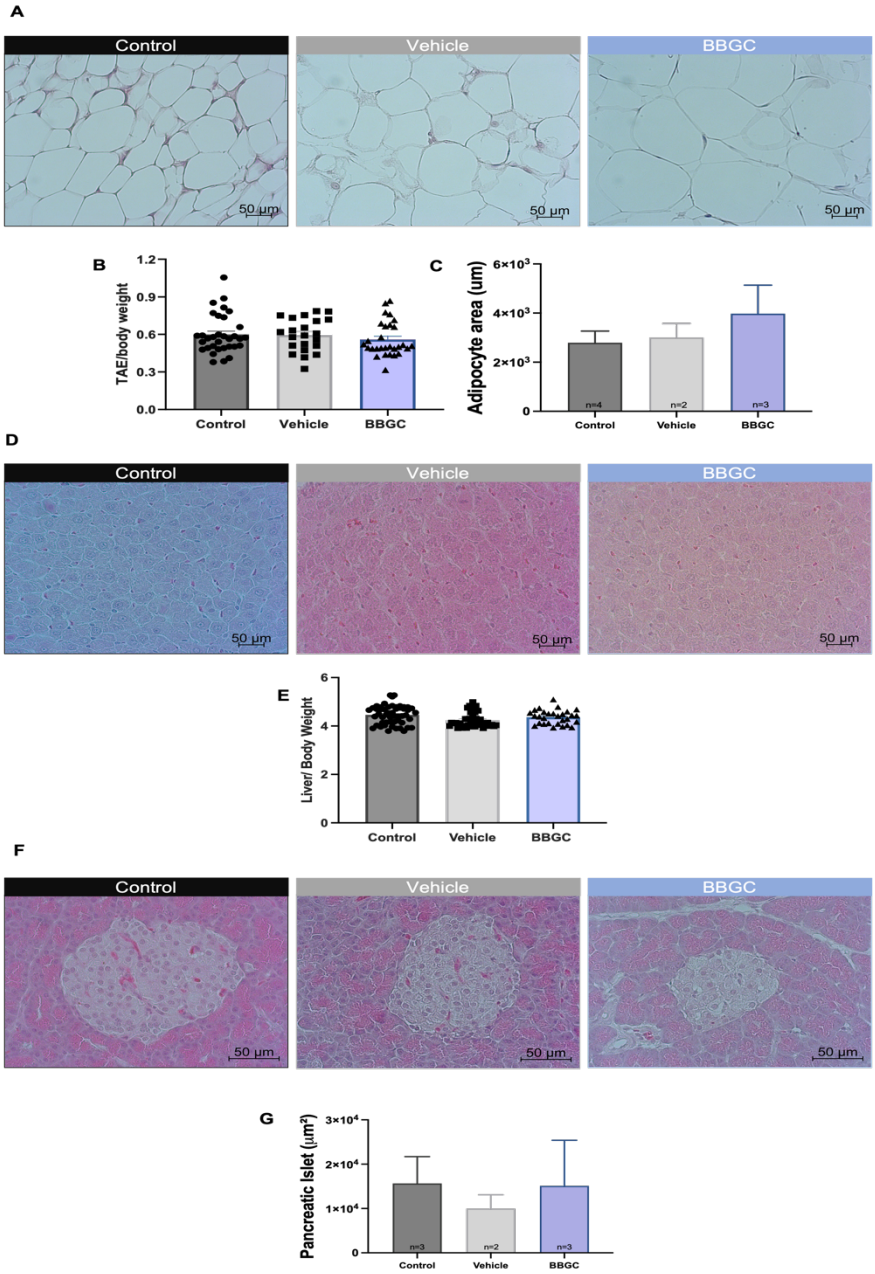

Figure 14 - Morphological analysis of WAT, liver, and pancreas of the male offspring. Histological analysis by HE of the AT (A) shows no alteration in the morphology. The weight of the tissue (B) is similar in the presented groups, yet the area of the adipocytes (C) is bigger in the BBGC group. In the liver (D), the analysis by HE didn't show any modifications and the area of the tissue (E) collected is similar. In the pancreas, HE staining (40X) of the Islet of Langerhans (F) shows a dysmorphic structure in the BBGC group. When comparing to the area of the islets (G), the offspring of dams treated with Vehicle have less area, compared to Control and BBGC conducted to compare among the groups. Control – Offspring of dams weren't treated, Vehicle – Offspring of dams were treated with DMSO, BBGC – Offspring of dams were treated with BBGC. * vs. Control; $ vs. Vehicle. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

4. PROTEIN LEVELS IN WAT OF MALE OFFSPRING:

Figure 15 shows the levels of proteins involved in glucose and lipid metabolism in the visceral WAT of the male offspring, in the maternal glycation group, evaluated by Western Blotting. Regarding total IR (**Figure 15A**), there was no modification of the total protein levels nor its phosphorylation (**Figure 15B**). Total AMPK (**Figure 15C**) was increased in the offspring of BBGC group, comparing to the offspring of Control group ($p<0.05$ vs Control group). The phosphorylation of AMPK (**Figure 15D**) and expression of GLUT4 (**Figure 15E**) was also similar between groups. PPARγ levels (**Figure 15F**) were tendentially diminished in the BBGC group, but with no statistical significance.

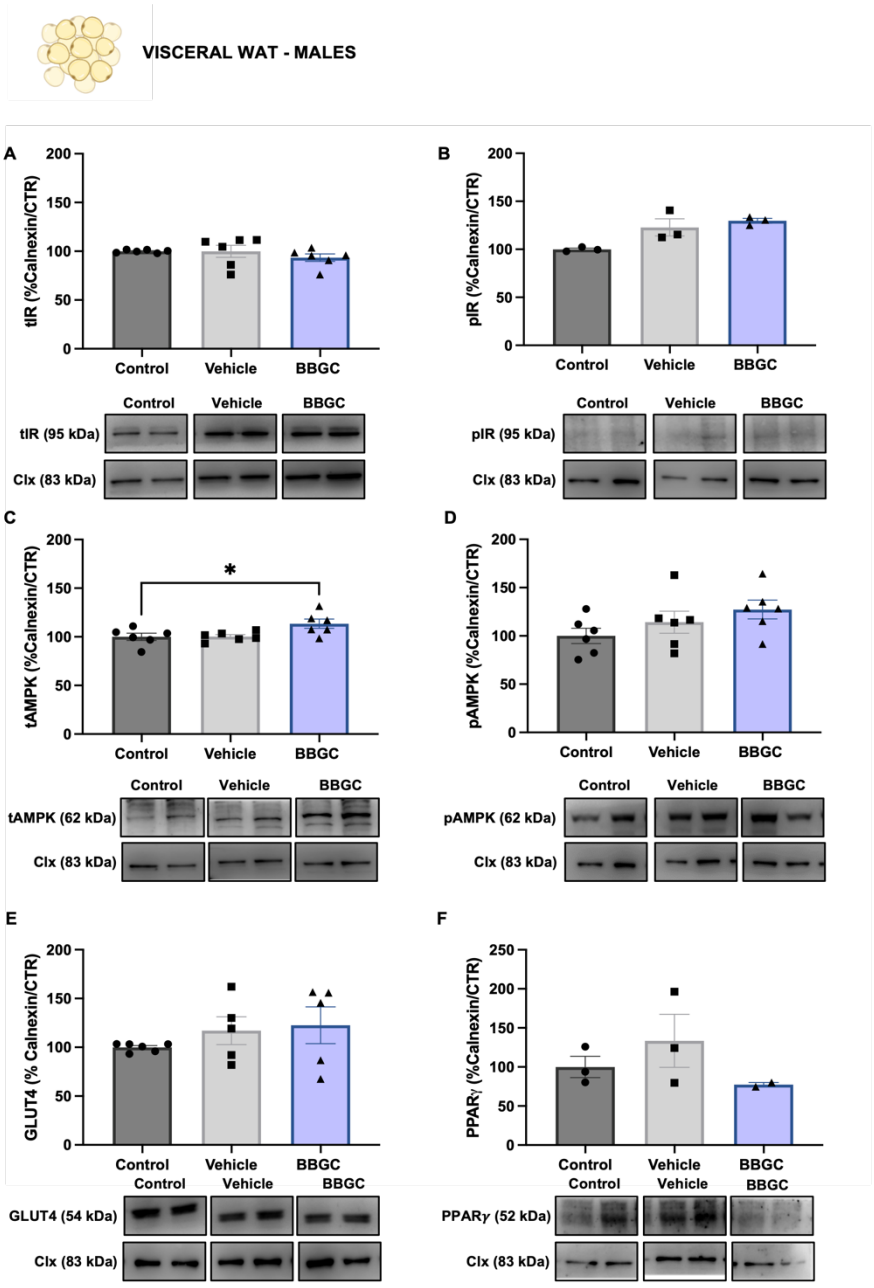

Figure 15 - Protein levels involved in glucose and lipid metabolism in the WAT of male offspring of dams treated with BBGC. Representative images of Western Blot proteins of interest and loading controls (Clx) are shown at the bottom panel. Control – Offspring of dams weren't treated, Vehicle – Offspring of dams were treated with DMSO, BBGC – Offspring of dams were treated with BBGC. Bars represent mean ± SEM and Ordinary-One ANOVA or Kruskal-Wallis comparisons were conducted to compare among the groups. * vs. Control, $ vs. Vehicle. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

5. PROTEIN LEVELS IN LIVER OF MALE OFFSPRING:

When evaluating the levels of protein involved in lipid and glucose metabolism in the liver of the male offspring, the expression of IR (**Figure 16A**) was diminished in the offspring exposed to maternal glycation, compared to the offspring of Vehicle dams ($p<0.001$ vs. Vehicle group). The activation of this receptor (**Figure 16B**) was not changed as well as the expression (**Figure 16C**) and activation (**Figure 16D**) of AMPK. The glucose transporter, GLUT2 (**Figure 16E**), is tendentially increased in maternal glycation's offspring, when compared to Vehicle ($p=0.0543$ vs. Vehicle). The protein level of PPARα (**Figure 16F**) is also alike in the three groups.

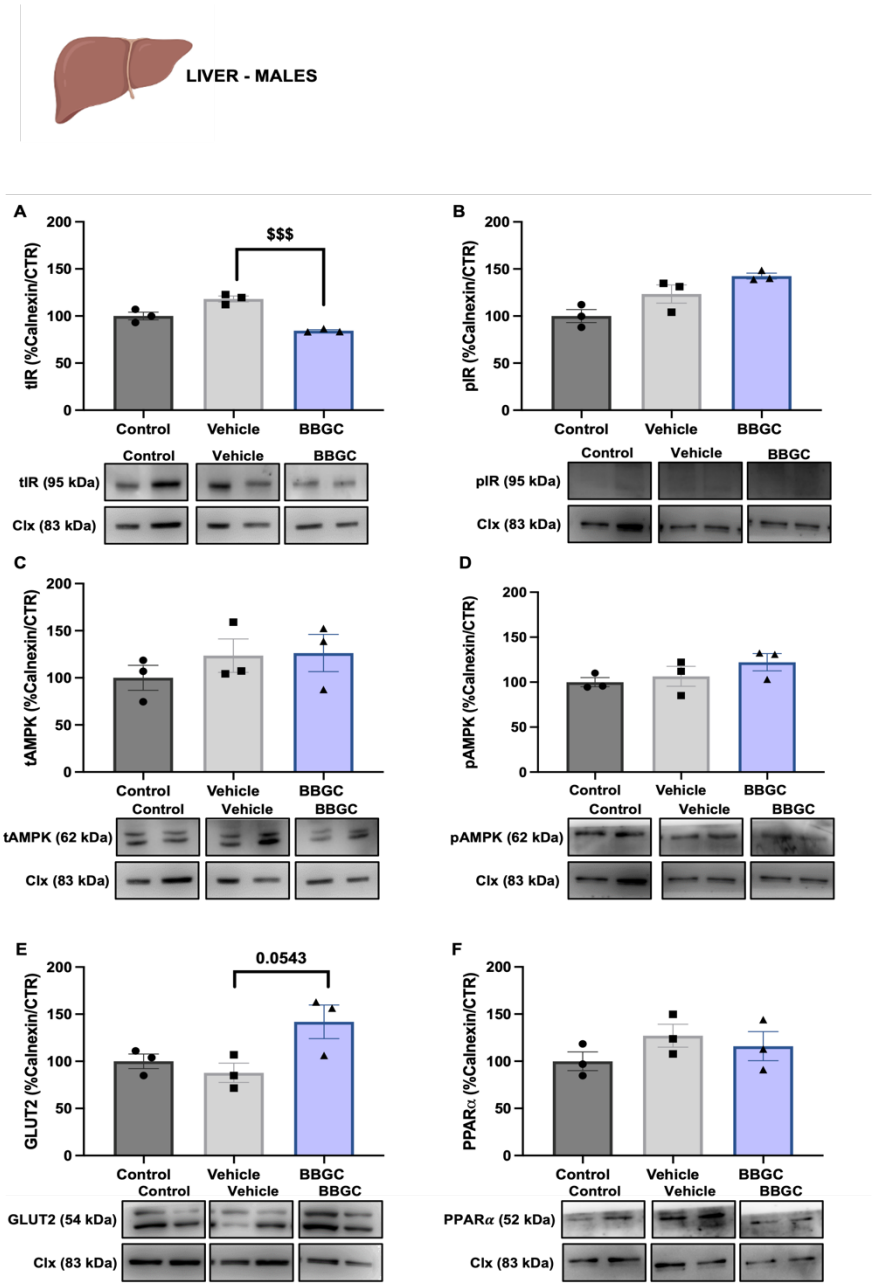

Figure 16 - Proteins levels involved in glucose and lipid metabolism in the liver of male offspring of dams treated with BBGC. Representative images of Western Blot proteins of interest and loading controls (Clx) are shown at the bottom panel. Control – Offspring of dams weren't treated, Vehicle – Offspring of dams were treated with DMSO, BBGC – Offspring of dams were treated with BBGC. Bars represent mean ± SEM and Ordinary-One ANOVA or Kruskal-Wallis comparisons were conducted to compare among the groups. * vs. Control, $ vs. Vehicle. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

6. METABOLIC PROFILE OF FEMALE OFFSPRING:

The weight of the female offspring was evaluated before (**Figure 17A**) and after (**Figure 17B**) weaning, with no alterations between groups. Insulin tolerance test (**Figure 17C**) didn't show any alteration, but plasma insulin levels (**Figure 17D**) were decreased in Vehicle and BBGC group, comparing to the Control ($p<0.01$ vs. Control group). kITT (**Figure 17E**), triglycerides (**Figure 17F**) and food consumption (**Figure 17G**) demonstrated no alteration.

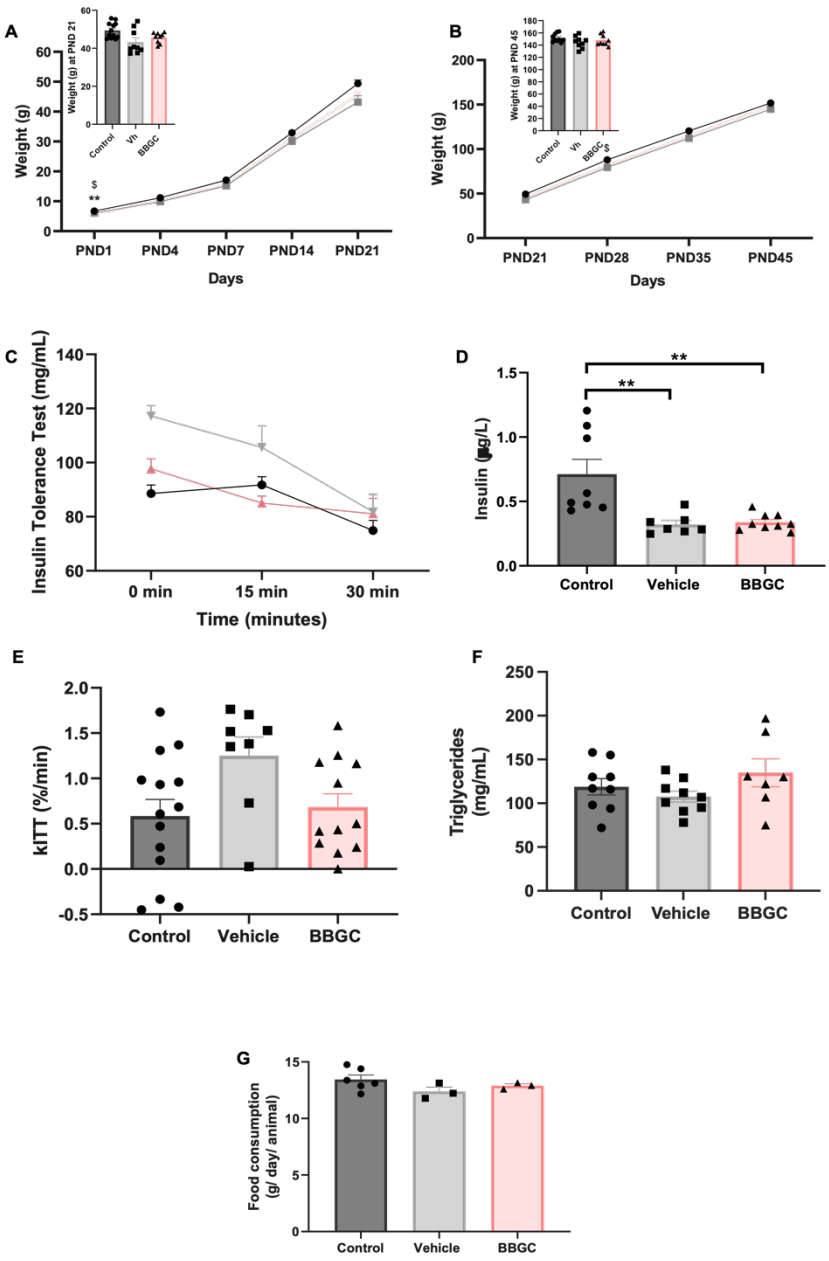

Figure 17 - Metabolic profile of female offspring. Female offspring whose dams were treated with BBGC have less plasma insulin (D). Evaluation of the weight of female offspring pre-weaning (A) and pos-weaning(B). Insulin tolerance test (C) showed no alterations. Plasma insulin is decreased in the offspring of BBGC group (D). kITT (E) was normal. Triglycerides (F) was also evaluated but with no modification, However, these animals, namely, BBGC group ate less (G) compared to the Control group. Control – Offspring of dams weren't treated, Vehicle – Offspring of dams were treated with DMSO, BBGC – Offspring of dams were treated with BBGC. Bars represent mean ± SEM and Kruskal-Wallis and Ordinary One-Way ANOVA comparisons were conducted to compare among the groups. * vs. Control; $ vs. Vehicle. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

7. MORPHOLOGY ANALYSIS OF FEMALE OFFSPRING:

No alterations in the morphology of the adipose tissue in the histological analysis and with HE (40X) (**Figure 18A**) nor in the weight (**Figure 18B**) of the AT. Nevertheless, a significant reduction in adipocyte area (**Figure 18C**) was observed given that females exposed to maternal glycation have a smaller area, when compared to the Vehicle group ($p<0.05$ vs. Vehicle group).

The liver didn't demonstrate any modifications in the morphology (**Figure 18D**), and the weight of the organ (**Figure 18E**) was similar in the three groups. Regarding the pancreatic islets, although their area (**Figure 18G**) was similar between groups, offspring exposed to maternal glycation have shown alteration in morphology, namely islets with irregular shapes (**Figure 18F**).

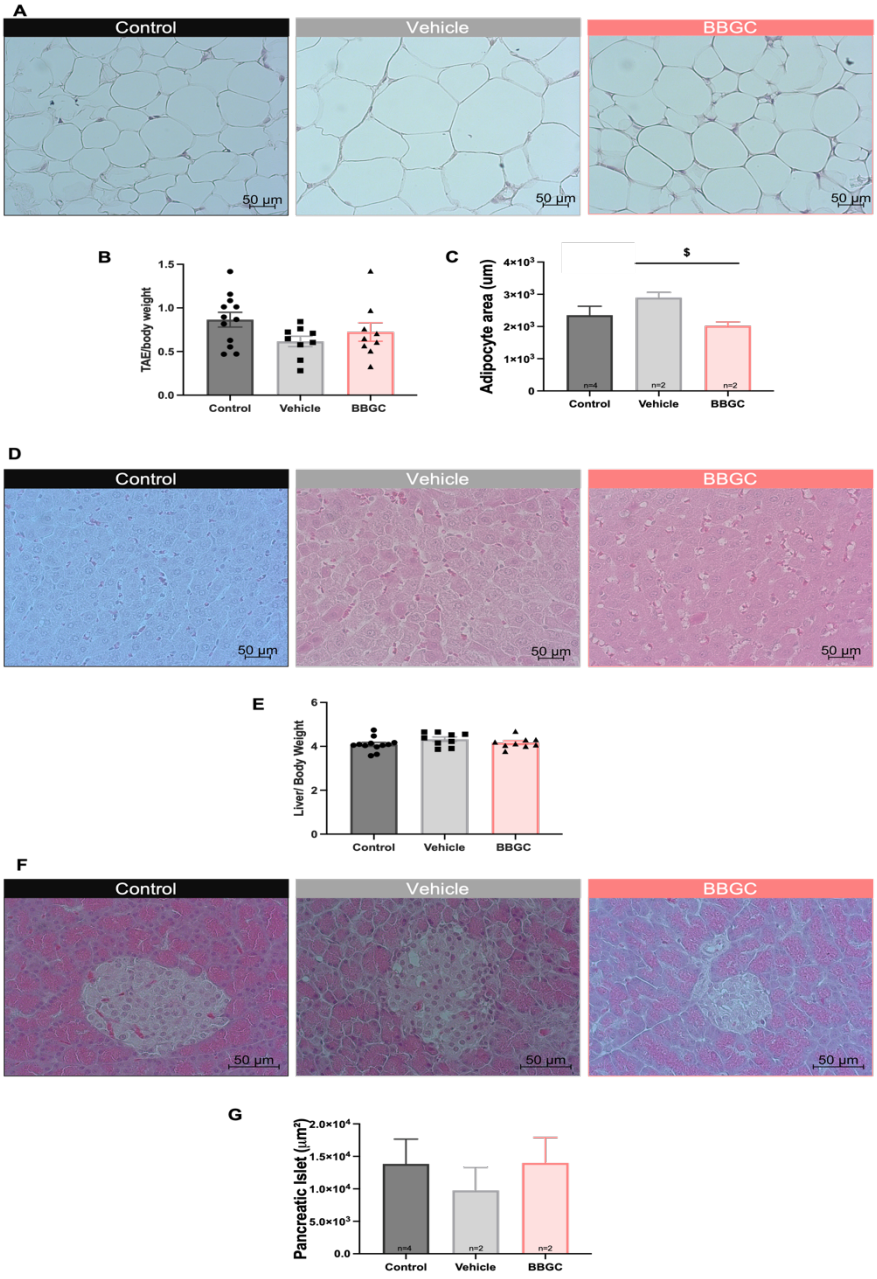

Figure 18 - Morphological analysis of WAT, liver, and pancreas of the female offspring. Histological analysis by HE of the AT (A) shows no alteration in the morphology. The weight of the tissue (B) is similar in the presented groups, yet the area of the adipocytes (C) is smaller in the BBGC group. In the liver (D), the analysis by HE didn't show any modifications and the area of the tissue (E) collected is similar. In the pancreas, HE staining (40X) of the Islet of Langerhans (F) shows a dysmorphic structure in the BBGC group. When comparing to the area of the islets (G), the offspring of dams treated with Vehicle have less area, compared to Control and BBGC conducted to compare among the groups. Control – Offspring of dams weren't treated, Vehicle – Offspring of dams were treated with DMSO, BBGC – Offspring of dams were treated with BBGC. * vs. Control; $ vs. Vehicle. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

8. PROTEIN LEVELS IN THE WAT OF FEMALE OFFSPRING:

The visceral WAT of the female offspring was also evaluated for the proteins involved in lipid and glucose metabolism (**Figure 19**). However, for all the proteins tested, the values were similar between groups.

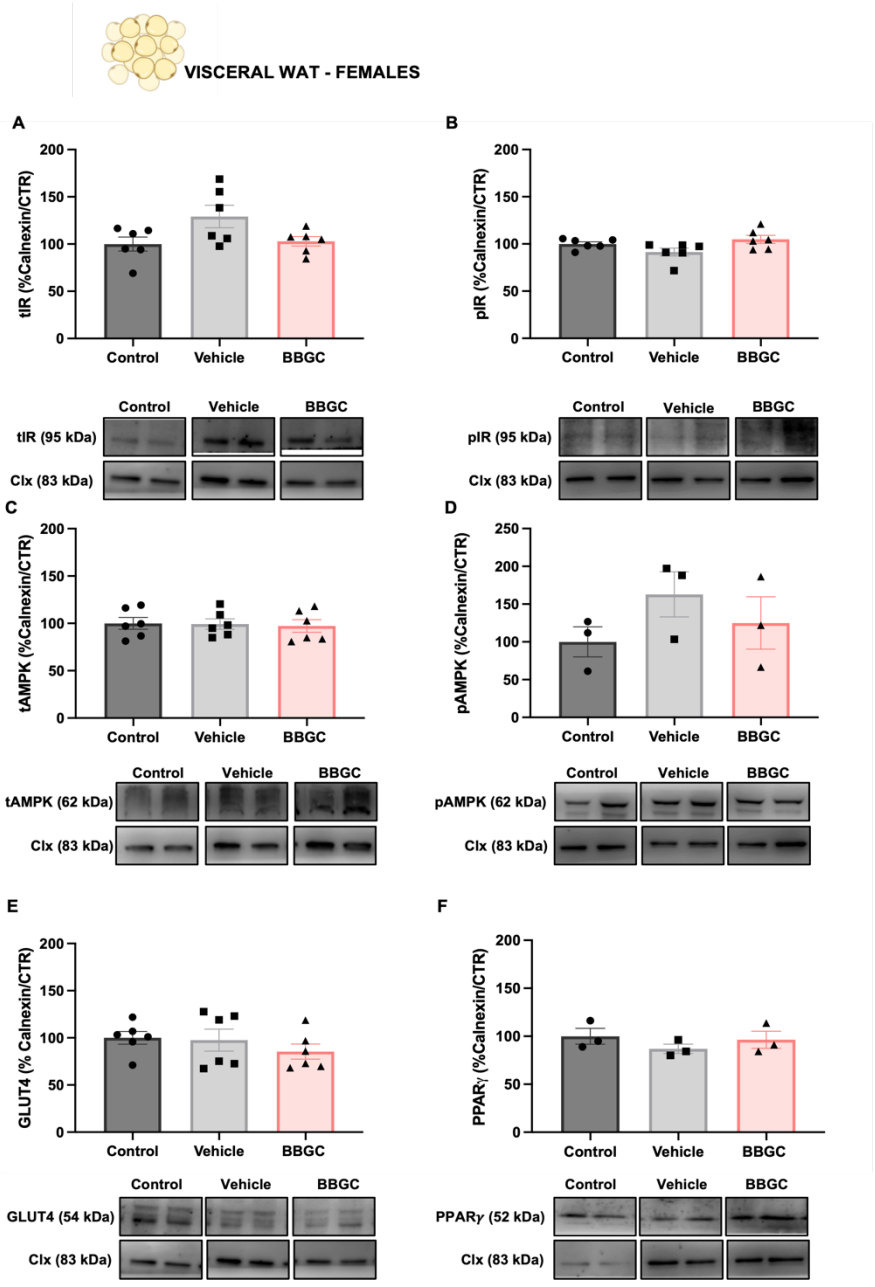

Figure 19 - Protein levels involved in glucose and lipid metabolism in the WAT of female offspring of dams treated with BBGC. Representative images of Western Blot proteins of interest and loading controls (Clx) are shown at the bottom panel. Control – Offspring of dams weren't treated, Vehicle – Offspring of dams were treated with DMSO, BBGC – Offspring of dams were treated with BBGC. Bars represent mean ± SEM and Ordinary-One ANOVA or Kruskal-Wallis comparisons were conducted to compare among the groups. * vs. Control, $ vs. Vehicle. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

9. PROTEIN LEVELS IN THE LIVER OF FEMALE OFFSPRING:

In the liver of the female offspring exposed to maternal glycation (**Figure 20**), the levels of IR (**Figure 20A**) and its activation (**Figure 20B**) were alike in the three groups. The levels of AMPK (**Figure 20C**) and the activation of this protein (**Figure 20D**) wasn't altered. GLUT2 (**Figure 20E**) was slightly augmented in the maternal glycation's offspring, with a $p = 0.0760$ versus Vehicle group. The protein involved in lipid metabolism in this organ, PPARα (**Figure 20F**) is diminished in both the Vehicle and BBGC groups, compared to Control group, with a value of $p<0.01$ and $p<0.001$, correspondingly.

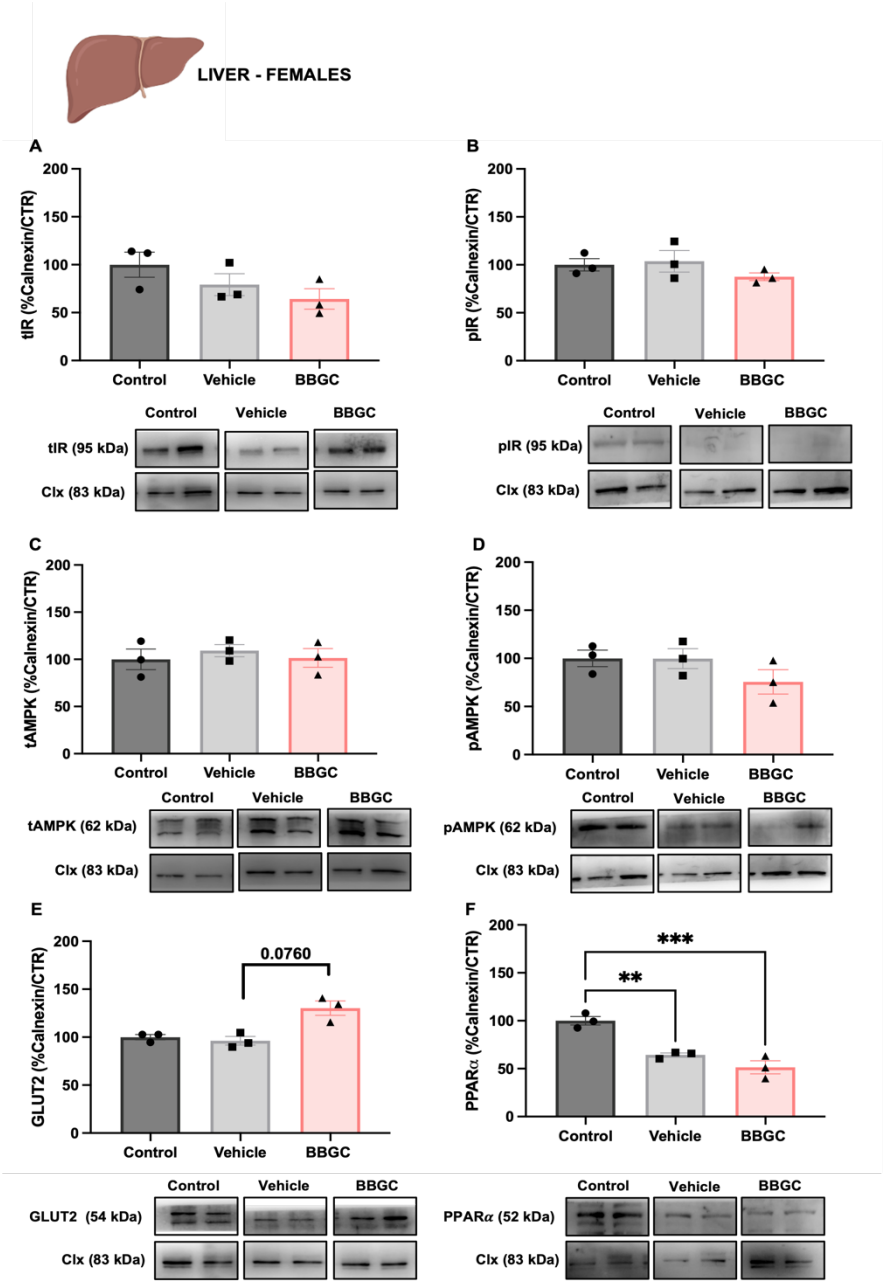

Figure 20 - Proteins levels involved in glucose and lipid metabolism in the WAT of female offspring of dams treated with BBGC. GLUT2 (E) is augmented and PPARa is decreased in BBGC group. Representative images of Western Blot proteins of interest and loading controls (Clx) are shown at the bottom panel. Control – Offspring of dams weren't treated, Vehicle – Offspring of dams were treated with DMSO, BBGC – Offspring of dams were treated with BBGC. Bars represent mean ± SEM and Ordinary-One ANOVA or Kruskal-Wallis comparisons were conducted to compare among the groups. * vs. Control, $ vs. Vehicle. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

C. IMPACT OF MATERNAL GLYCATION IN A MODEL OF OVERWEIGTH INDUCED BY NEONATAL HYPERPHAGIA:

1. DAMS CHARACTHERIZATION:

As mentioned before, after birth, in order to increase milk availability and increased weight gain, we induced litter reduction (n=3 male pups), which were then tested for the effects of maternal glycation. The dams didn't show any fluctuation on weight (**Figure 21A**) and the values of glycemia (**Figure 21B**) also remains constant. Furthermore, in the milk, the triglycerides levels were decreased in the dams subjected to SL or/and BBGC (**Figure 21C**). Dams subjected to litter reduction or dams exposed to the two procedures have less total antioxidant capacity in the milk compared to the Control group ($p<0.05$ vs. Control group) (**Figure 21D**), but there was not statistical significance for GLO1 activity (**Figure 21E**). In the histological analysis of the liver (**Figure 21F**), there was no morphological alterations.

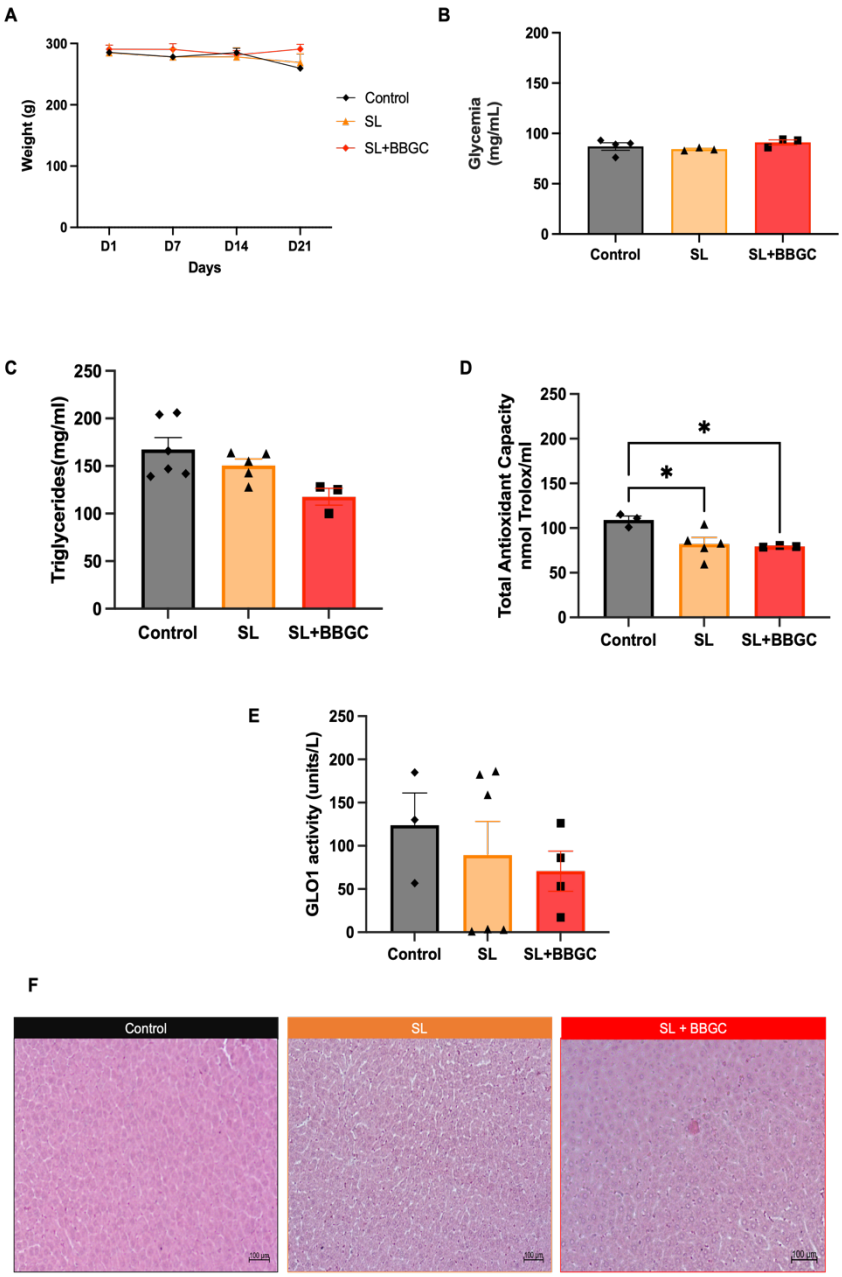

Figure 21 - Dams weight, milk, and tissue characterization. Control –Dams weren't treated, SL –Dams subjected to litter reduction, SL+BBGC – Dams were treated with BBGC and suffered litter reduction. Bars represent mean ± SEM and Ordinary-One ANOVA or Kruskal-Wallis comparisons were conducted to compare among the groups. * vs. Control, # vs. SL. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

2. METABOLIC PARAMETERS OF THE OFFSPRING:

In **Figure 22** is represented the weight and metabolic parameters of the offspring which dams were induced to litter reduction (SL), or litter reduction allied to maternal glycation (SL+BBGC).

The offspring of SL was heavier (**Figure 22A and Figure 22B**) than the Control and SL+BBGC, in every point of the timeline presented, while the maternal glycation reduced such effect (**Figure 22A and Figure 22B**). Insulin tolerance test demonstrated no alterations (**Figure 22C**), however plasma insulin levels (**Figure 22D**) revealed a decrease in the offspring where the dams where inducing to litter reduction, and litter reduction with BBGC administration, compared to the Control group ($p<0.05$ vs. Control group). kITT remained similar between groups (**Figure 22E**). The triglycerides (**Figure 22F**) were significantly increased only in the offspring whose dams were subjected to SL and treated with BBGC, compared to the Control ($p<0.01$ vs. Control group). Food consumption is similar in the groups presented (**Figure 22G**).

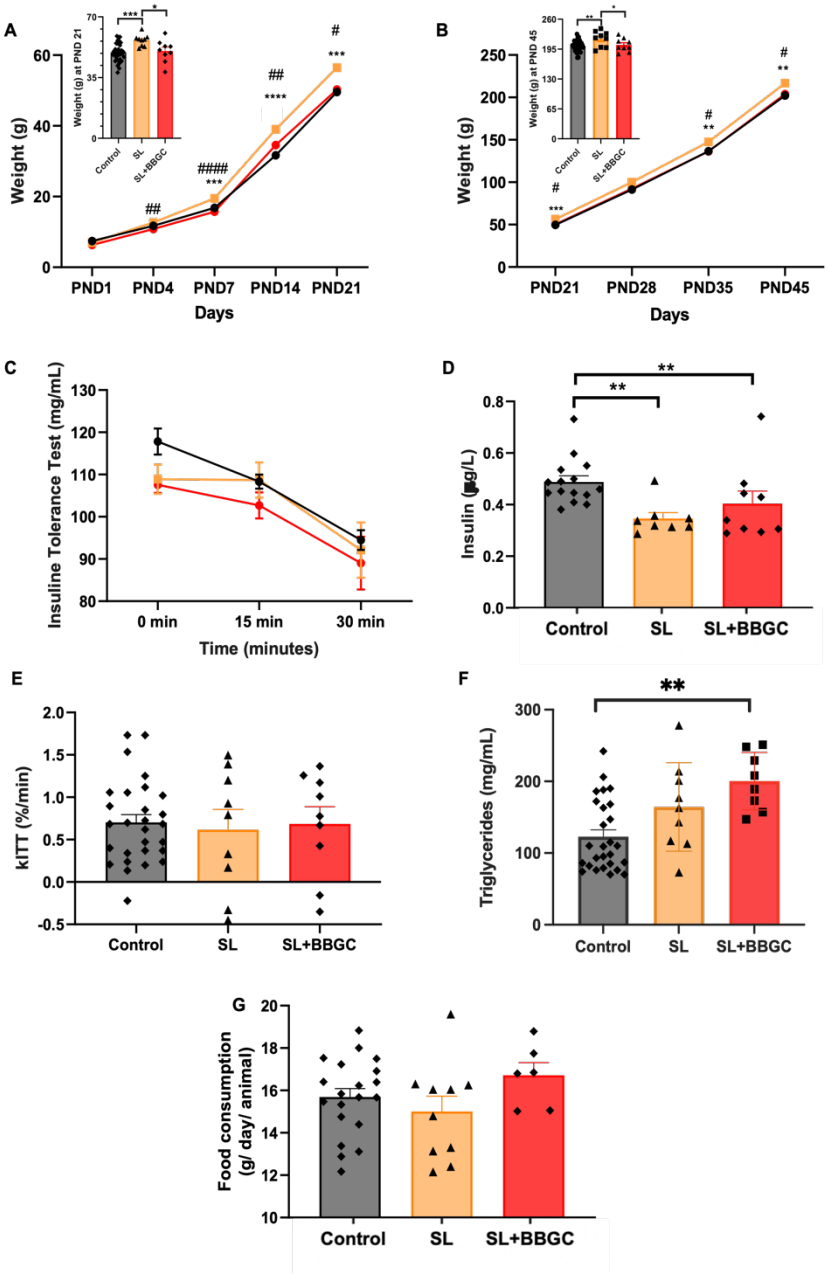

Figure 22 - Metabolic characterization of offspring subjected to litter reduction and maternal glycation. Evaluation of the weight of offspring pre-weaning (A) and pos-weaning(B). Insulin tolerance test(C) shows no alterations, Plasma insulin (D) showed a decrease in SL and SL+BBGC group. kITT (E) remained normal. Triglycerides (F) is higher for the SL+BBGC group. Food consumption (G) was also evaluated but with no modification, Control –Offspring where dams weren't treated, SL –Offspring where dams subjected to litter reduction, SL+BBGC –Offspring of dams treated with BBGC and suffered litter reduction. Bars represent mean ± SEM and Kruskal-Wallis and Ordinary One-Way ANOVA comparisons were conducted to compare among the groups. * vs. Control; # vs SL. 1 symbol p<0.05; 2 symbols p<0.01; 3 symbols p<0.001.

3. MORPHOLOGY ANALYSIS OF THE OFFSPRING:

When performing the histological analysis of AT (**Figure 23A**), there was no modifications in the morphology of the tissue, nor in adipocyte area (**Figure 23C**). However, the offspring of the SL group have more epididymal AT, which was not changed by the maternal glycation (**Figure 23B**).

Also, no alterations were observed in liver histology regarding morphology (**Figure 23D**), and weight of this tissue (**Figure 23E**).

Nevertheless, when analyzing the pancreas, in specific, the Islets of Langerhans (**Figure 23F**), by HE staining, there is a dysmorphic association with the groups SL and SL+BBGC. Furthermore, the area of Islets of Langerhans (**Figure 23G**) is augmented in the offspring whose dams were subject to SL and BBGC. However, none of these results presents statistical significance associated.

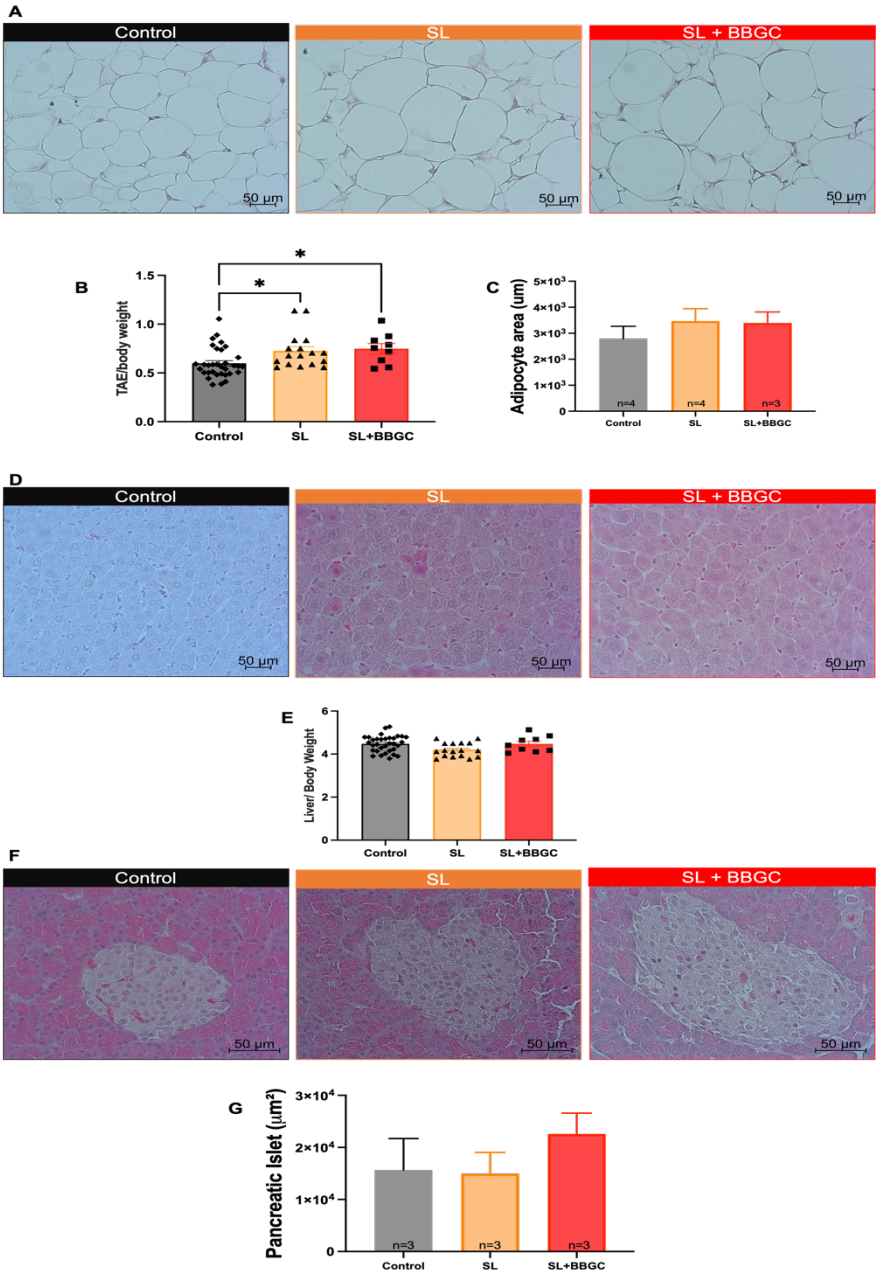

Figure 23 - Morphological analysis of WAT, liver, and pancreas of the offspring. SL and SL+BBGC group have more adipose tissue (B). Histological analysis by HE of the AT (A) shows no alteration in the morphology. The weight of the tissue (B) is augmented in SL and SL+BBGC groups, yet the area of the adipocytes (C) is remained similar. In the liver (D), the analysis by HE didn't show any modifications and the area of the tissue (E) collected is similar. In the pancreas, HE staining of the Islet of Langerhans (F) showed a dysmorphic structure in the SL+BBGC group. When comparing to the area of the islets (G), the offspring of dams treated with Vehicle have less area, compared to Control and BBGC conducted to compare among the groups. Control –Offspring where dams weren't treated, SL –Offspring where dams subjected to litter reduction, SL+BBGC – Offspring of dams treated with BBGC and suffered litter reduction. * vs. Control; # vs. SL. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

4. PROTEIN LEVELS IN WAT OF THE OFFSPRING:

The visceral (in this case epididymal) WAT was analyzed by Western Blotting. The total levels of IR (**Figure 24A**) were augmented in the offspring of SL+BBGC group, when comparing to the Control group ($p<0.01$ vs. Control group), despite no changes were observed for activation (**Figure 24B**)

The expression (**Figure 24C**) and activation (**Figure 24D**) of AMPK remained similar in the three groups, as well as the levels of GLUT4 (**Figure 24E**) and PPARγ (**Figure 24F**).

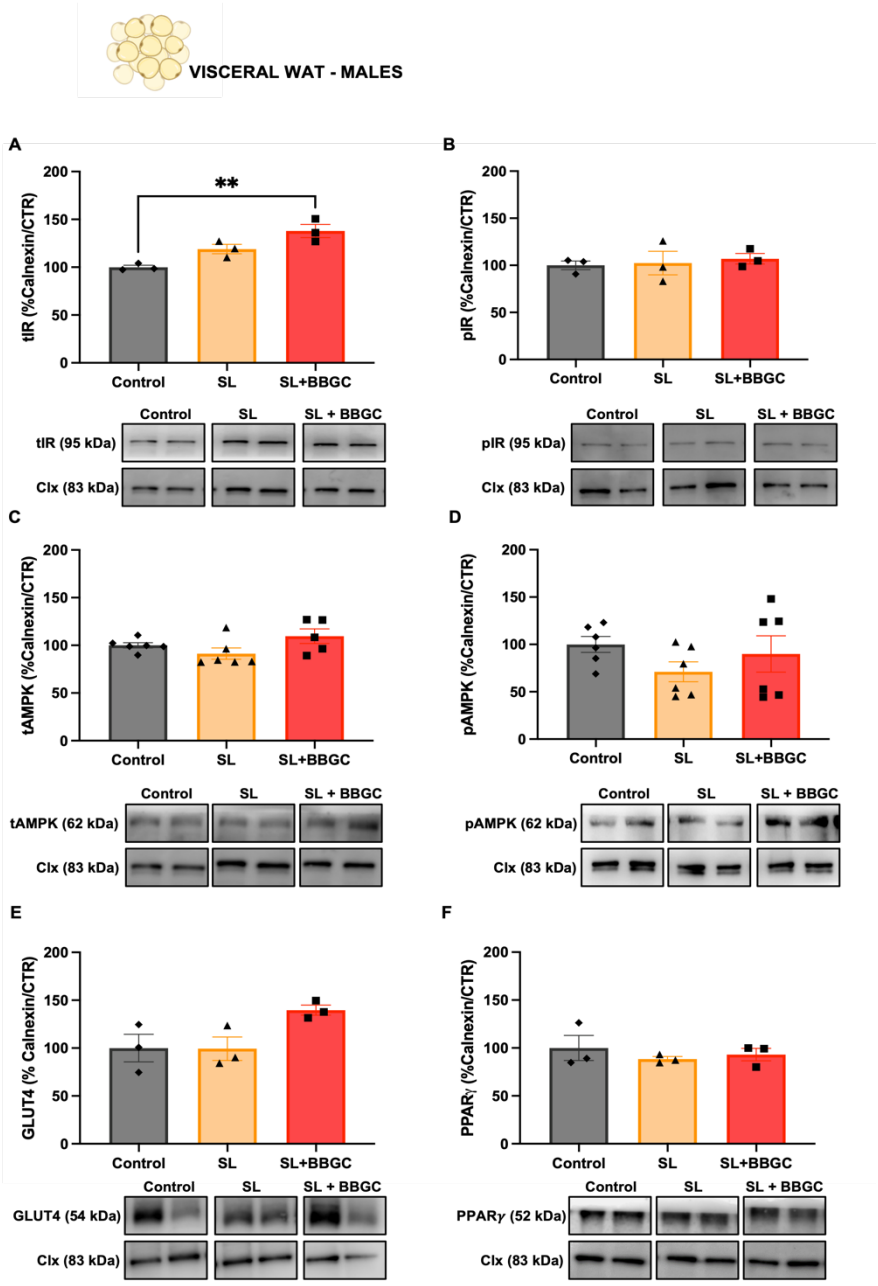

Figure 24 - Protein levels involved in glucose and lipid metabolism in the WAT of male offspring of dams that suffered litter reduction and treated BBGC.Representative images of Western Blot proteins of interest and loading controls (Clx) are shown at the bottom panel. Control – Offspring where the dams weren't treated, Vehicle – Offspring where the dams were treated with DMSO, BBGC – Offspring where the dams were treated with BBGC. Bars represent mean ± SEM and Ordinary-One ANOVA or Kruskal-Wallis comparisons were conducted to compare among the groups. * vs. Control, # vs. SL. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

5. PROTEIN LEVELS IN LIVER OF THE OFFSPRING:

When evaluating the levels of the same proteins in the liver of the offspring (**Figure 25**), no alterations were observed in SL nor SL+BBGC groups.

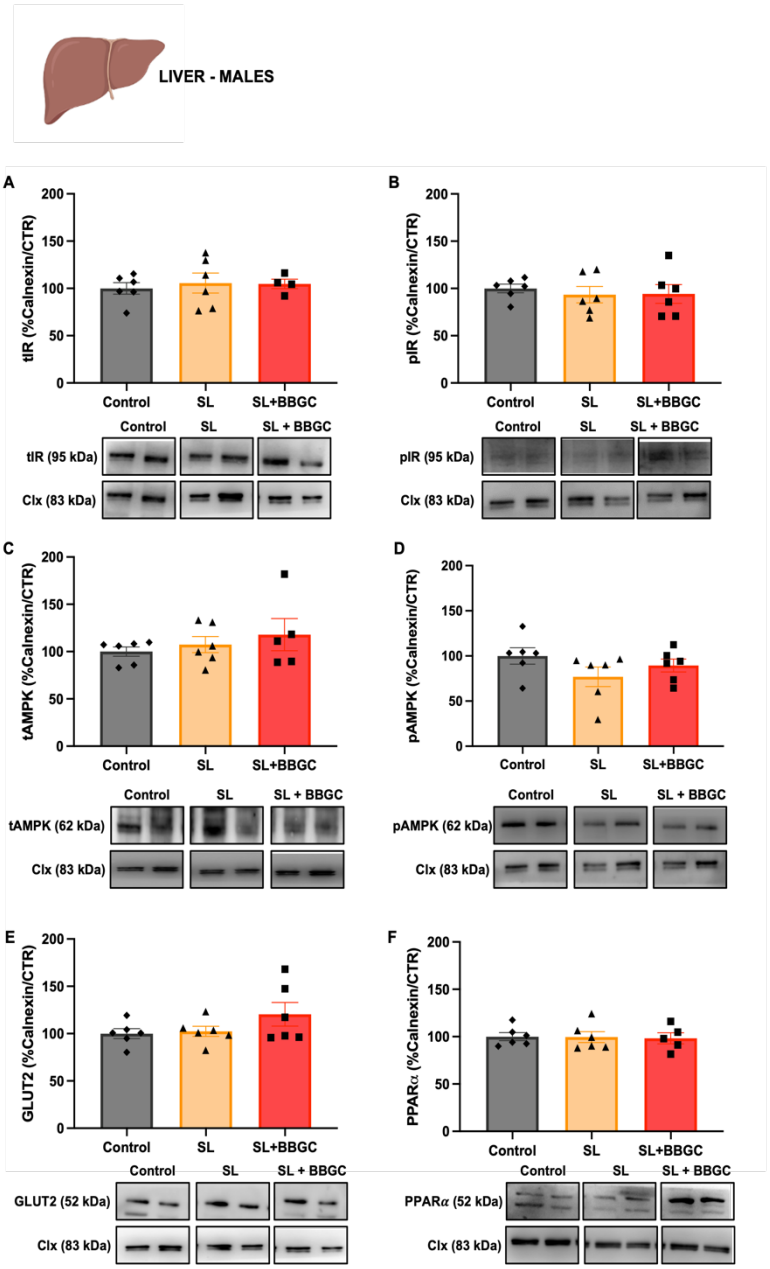

Figure 25 - Protein levels involved in glucose and lipid metabolism in the liver of male offspring of dams that suffered litter reduction and treated BBGC. Representative images of Western Blot proteins of interest and loading controls (Clx) are shown at the bottom panel. Control – Offspring where the dams weren't treated, Vehicle – Offspring where the dams were treated with DMSO, BBGC – Offspring where the dams were treated with BBGC. Bars represent mean ± SEM and Ordinary-One ANOVA or Kruskal-Wallis comparisons were conducted to compare among the groups. * vs. Control, # vs. SL. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

Chapter V

DISCUSSION

The prevalence of metabolic diseases such as obesity, Diabetes and MetS is increasing and affects more than a billion individuals. Normally, this diseases are associated with a sedentary lifestyle and consumption of diets rich in sugars and glicotoxins- known AGEs precursors(Elabbassi & Haddad, 2005; Iacobini et al., 2019b; Roden & Shulman, 2019b). A key-factor present in these diseases is insulin resistance, that is related to AT dysfunction and leads to impaired glucose homeostasis. With the prevalence of obesity and Diabetes, a metabolic imprint during the perinatal period can affect the offspring, promoting hyperglycaemia, insulin resistance, inflammation (Fall & Kumaran, 2019b; Gunderson, 2008; Salis et al., 2017).

In this work, we explored the effect of postnatal obesity on the mechanisms of glucose and lipid metabolism and insulin signalling in the male and female offspring. We studied two different model, maternal obesity induced by a high-fat diet, and neonatal hyperphagia-induced obesity. Moreover, we aimed to disclose the role of glycotoxins in the dysfunction of such pathways.

As a major finding in this work, we highlight that: 1. Exposure to maternal obesogenic diet during the perinatal period potentiates changes in lipid metabolism metabolism that are different in male and female offspring. 2. Early exposure to maternal glycation seems to impair these mechanisms, which may predispose to insulin resistance in the male offspring. The female offspring is apparently more protected. 3. Neonatal hyperphagia dis not induce alterations in insulin signalling and metabolism and didn't increase the effects of glycotoxins.

Analysing the impact of maternal obesogenic diets or maternal obesity on offspring metabolic outcomes is especially pertinent given the rise in obesity among women of childbearing age and the possible outcomes for the offspring. In our study, female Sprague-Dawley rats were fed with HFHS diet during gestation and lactation and the observations were on the offspring with 42 days-old. This exposure didn't result in any alteration in IR, however, causes an upregulation of the activation of AMPK in the visceral WAT in female offspring, which was partially (non-significant)

observed in male offspring. In several studies, the dams were fed with HFHS diet during and after gestation and the observational effects in the offspring was an impairment in the expression in IR (de Almeida Faria et al., 2017; Fernandez-Twinn et al., 2014; A.-M. Samuelsson et al., 2008), possible indicating the strong role of maternal diet during lactation. A previous study also stated insulin resistance in the offspring by maternal diet-induced obesity (Fernandez-Twinn et al., 2014). Regarding AMPK, it is not consensual in the literature, where a study indicates that the expression of AMPK has a small increase, while the activation is decreased (Borengasser et al., 2013). AMPK activation results in a decrease of glucose uptake and lipogenesis, while an increase in lipid and mitochondrial oxidation. Also, AMPK leads to adipocyte enlargement, obesity and metabolic dysfunction (Wu et al., 2018). In a human study with obese mothers, lower expression and activation of AMPK was demonstrated in the umbilical cord tissue (Boyle et al., 2017), plasma (Arab Sadeghabadi et al., 2018) and subcutaneous adipose tissue (Xu et al., 2015). Maternal obesity can also increase the risk of hepatic metabolic dysfunction in the offspring. IR was shown to be diminished in obese male offspring (Meier et al., 1995), which can be a compensatory mechanism for lower insulin signalling. AMPK leads to a stimulation of FA oxidation and inhibition of lipogenesis and glucose production. There is a strong correlation between the ratio pAMPK/AMPK and liver lipid content. A study by Mennitti et al. showed that the hepatic fat accumulation in the offspring can be explained by the effects of AMPK (Mennitti et al., 2022). It has also been stated that patients with obesity, diabetes, or NAFLD have lower levels of hepatic AMPK (Zhao & Saltiel, 2020) and is described in the literature that maternal obesity causes a downregulation in the hepatic AMPK, leading to metabolic disturbances in the newborn offspring (Simino et al., 2021). These low levels of AMPK in AT and in the liver, probably create a pro-inflammatory status that can lead to insulin resistance, metabolic diseases and progression to NAFLD. Accordingly, of model also developed lower activation of AMPK in the liver, being more evident in female offspring. A study demonstrated that AMPK signalling pathway tended to be higher expressed in the female offspring fed from lean dams when fed a HF diet, but lower in the female offspring from obese dams fed with the same diet(Savva et al., 2022). However, female offspring is poorly studied in animal models, and it requires more elucidation. On the other hand, AMPK activation was augmented in the visceral WAT of female offspring, which may denote a peripheral compensatory

mechanism for lipid oxidation with unknown mechanisms. Females from obese dams accumulate more subcutaneous WAT, contrarily to the males, which accumulates more visceral WAT (Savva et al., 2021). In the same mentioned study, lipolysis is augmented in visceral WAT, while FA synthesis and oxidative phosphorylation is diminished in female offspring (Savva et al., 2021). It is hypothesized that AMPK can have a therapeutic role against obesity and acts like a protector from diet-induced obesity (Pollard et al., 2019).

These results suggest that maternal obesity controls differently the phenotype in lipid and glucose metabolic pathways in a sex-specific manner and through this analysis, we were able to conclude that the offspring have alterations in FA oxidation, which may predispose to future insulin resistance.

Given that westernized diets are rich in refined sugars, we aim to study the specific effects of the exposure of maternal glycation during the early life in offspring's glucose and lipid metabolic pathways. For that, we treated the dams with BBGC – a selective inhibitor of GLO1, in order to impair the glyoxalase cycle and ultimately, increase the levels of MG, which is an AGEs precursor. There were no effects on the weight of liver and kidney histology of the dams (Csongová et al., 2019; Merhi et al., 2020), indicating that the treatment with this inhibitor is not provoking cytotoxicity and an overexposure to MG. Breastmilk is crucial for newborn, not only for development, but for protection against metabolic diseases. While assessing the milk quality by quantifying triglycerides and the total antioxidant capacity, we noticed that they were both diminished in dams treated with BBGC. A study by Francisco et al., demonstrated that the milk has a higher content in triglycerides, however the dams were treated by gavage with MG, a more aggressive approach that can cause metabolic dysregulation and thus cause other modifications in the milk (Castelhano et al., 2020; Francisco et al., 2018; Kawaharada et al., 2018).

We also observed a similar weight (lean phenotype) of the offspring, which was different from other studies (Csongová et al., 2019; Francisco et al., 2018). This can be due to the short-term treatment performed in the dams - only six days after birth and at 5mg/kg. The fasting insulin levels were also normal while, as said before, the more aggressive approach increase plasma insulin y (Francisco et al., 2018).

Interestingly, kITT or glucose decay rate was decreased on the offspring exposed to enhanced glycation, suggesting a reduction in insulin sensitivity or at least a predisposition for that later on. Rodent and human studies demonstrated that high concentration of AGEs present in the diet could affect insulin sensitivity and signalling, leading to T2DM (Uribarri et al., 2011; Wang et al., 2022). In the male offspring we also noticed a less food consumption of standard diet. This result is demonstrated also in Csongová et al., where a possible explanation is a modulation in food preferences by the offspring when exposed to certain detrimental environments (Amati et al., 2019). In the histology of the AT, we noticed a trend to augmented adipocyte area. This can be specific to tissue location, because male gender has more visceral WAT than females (Chang et al., 2018), but it can also be the beginning in a hypertrophy, indicating AT dysfunction (Rodrigues et al., 2017). In the histology of the pancreas, the area of the pancreatic islets was normal, but the offspring from the BBGC group presented several dysmorphic pancreatic islets, which may be correlated with impaired insulin secretion. In fact, the perinatal exposure to high-AGEs diet was shown to lead to β-cell dysfunction (Borg et al., 2018).

The idea that AGEs impair insulin action is supported by *in vivo* rodent studies using dietary manipulations to increase AGE content, showing an impairment in insulin sensitivity in AT (Hofmann et al., 2002). Lower biological activity of glycated insulin may result from reduced affinity for the insulin receptor or from reduced insulin signalling because of enhanced exposure to glycation (Walke et al., 2021). Insulin resistance is associated with reduced AMPK levels, which was not observed in offspring exposed to maternal glycation. The treatment in the dams for only 6 days couldn't impose metabolic dysregulation in the offspring, although lower insulin receptor levels were observed in the liver of male offspring. Interestingly, this was associated with an apparently compensatory increase of GLUT2 levels. Rodent and human studies demonstrated T2DM manifests insulin resistance in the liver, characterized by a failed suppression of gluconeogenesis and a continuous activation of lipogenesis, leading to hyperglycaemia and steatosis (Biddinger et al., 2008; Brown & Goldstein, 2008). Kubota and colleagues demonstrated similar results in obese diabetic mice, also proposing a selective insulin resistance in this organ. In the hepatic periportal zone, insulin signalling was impaired despite the

hyperinsulinemia and in contrast, in the hepatic perivenous zone, had an increased lipogenesis and developed steatosis (Kubota et al., 2016).

The female offspring didn't present any alterations in birth weight. Regarding metabolic parameters, the ITT was normal, however plasma insulin was drastically decrease. The ability of DMSO to potentiate lipolysis due to agents such as glucagon (a counterregulatory hormone of insulin) could be a possible explanation for this result, also seen in Wieser's study. The histology of VAT demonstrated a decrease in the area the adipocyte. Although females have more SAT rather than VAT, the mechanism for such observation is unknown. Similar results were obtained in adult models exposed to glycation, which was related with impaired expandability, hypoxia, inflammation and metabolic dysregulation (Rodrigues, 2017). However, is not known whether this is true is this model and how it is related with higher predisposition to metabolic dysregulation. We didn't observe any alteration in protein levels of glucose and lipid metabolism mediators. A plausible explanation is the female gender is generally more protected from metabolic disorders (having more insulin sensitivity), rather than male gender (Tramunt et al., 2020). In the liver, we also stated no alterations in the insulin receptor, but tendentially augmented levels in the GLUT2. Due to the lack of studies in female rodent models, we couldn't take any explanation for this result.

To assess the role of maternal glycation in context of neonatal hyperphagia-induced overweight, the litter was established at 3 male pups. The milk of the dams suffers a decay in total antioxidant capacity when we induced litter reduction and with the administration of BBGC. There is no data to speculate about this result, but the stress induced in the mother could influence biochemical changes. However, the litter reduction has an augmented weight, due to milk availability and no competition for suckling (Souza et al., 2022). The administration of BBGC during 6 days in the dams, postnatally, decreased the weight of the offspring, which may be linked with its role in impairing WAT expandability. Regarding metabolic parameters, plasma insulin was decreased, and triglycerides were increased, suggesting a more susceptibility to metabolic dysregulation. Habbout and colleagues showed that hyperphagia can induce hyperinsulinemia and elevated serum triglycerides (Habbout et al., 2013). Hyperphagia in early-life, specifically during breastfeeding,

can predispose to hyperphagia in the infancy and adult life (Heymsfield et al., 2014), although in our study food intake was similar after weaning.

The WAT weight was also increased due to (Chang et al., 2018), which can lead to hypertrophy (AT dysfunction) and insulin resistance. Although the area of the islets of Langerhans didn't differ among groups, they were observed to lost the traditional morphology in the offspring whose dams suffer litter reduction and administration of BBGC, while the offspring that suffer litter reduction remain similar. In fact, early postnatal overnutrition can cause pancreatic changes in mice, by increasing β-cell mass and reducing islet content, suggesting an impairment in insulin secretion. Several studies indicate that adult SL offspring secrete more insulin when glucose concentration is elevated, comparing to normal litter offspring (de Souza Rodrigues Cunha et al., 2009; Previate et al., 2020). This could be a compensatory mechanism to maintain glucose homeostasis and be further aggravated by exposure to glycation In the literature, adult SL offspring have an impairment in this pathway, due to the downregulation of IR and GLUT4, leading to a deteriorating metabolic status (Souza et al., 2022). However, in our study, SL offspring didn't present changes in the protein levels. A possible explanation is the fact that the offspring had 45 days-old, and this phenotype can be revealed in long time. Accordingly, the SL and BBGC offspring exhibit changes in the insulin signalling pathway, being the expression of the insulin receptor augmented. In several models, this increased may be understood as a compensatory mechanism for lower insulin signalling, denoting a higher risk for impaired insulin signalling (Neves, 2019).

Chapter IV

CONCLUSION AND FUTURE PERSPECTIVES

Metabolic disorders, such as obesity, diabetes and MetS are connected by a key-factor: insulin resistance - associated to inflammation, hyperinsulinemia, hypoxia, ectopic accumulation of fat and lipodystrophy (Wondmkun, 2020). AT is a multifactorial organ, controlling overall metabolism. However, AT dysfunction is also a prominent contributor in the disorders mentioned above (Matafome et al., 2015b). The term "metabolic programming" is a new concept that interconnects maternal metabolic state and/or nutrition with the progeny susceptibility to future diseases (Bichteler & Gershoff, 2018b; Catalano & Demouzon, 2015; Dong, Luo, Nuyt, Audibert, Wei, Abenhaim, Bujold, Julien, Huang, Levy, Fraser, et al., 2018) – such as metabolic disorders, in the intrauterine environment and breastfeeding. It is urgent to understand these mechanisms, given the rise in obesity among women of childbearing age and the possible outcomes for the offspring.

This study aimed to clarify if the exposure to obesogenic environments in the perinatal period alters glucose and lipid metabolism in several organs, such as WAT, liver, and pancreas. Any modification in these organs can lead to the development of metabolic diseases, earlier stages of life and later, at adulthood. Also, we want to identify the factors that lead to the development of early insulin resistance.

We found that maternal high-fat diet-induced obesity during gestation and lactation, which may denote a compensatory mechanism to the nutritional changes of breastmilk. Similar mechanisms were not observed in the SL model, which gained more weight due to more breastmilk availability, but was not subjected to nutritional changes of the mother. It would also be interesting to study if the same happens during the lactation window and the impact of maternal obesity in the lipid and glucose metabolism of the BAT and skeletal muscle of the offspring and observe the dichotomy male/female.

Regarding maternal glycation, both male and female offspring showed subtle alterations that denote a higher predisposition to insulin sensitivity, although in female offspring some effects could be attributable to the vehicle. This model was specifically design for lactation stage. It would be interesting to extend the treatment with BBGC and study the effect in long-term offspring. Also, BAT and skeletal muscle is crucial to understand the bigger picture in the effects of maternal glycation.

Insulin resistance can lead mitochondrial dysfunction, therefore mitochondrial DNA quantification would be interesting to observe.

We observed some evidences that maternal glycation may further impair insulin sensitivity in male offspring subjected to hyperphagia. The male offspring has less plasma insulin and more triglycerides, being associated with an upregulation of IR and enlarged pancreatic islets. In the near future, we plan to observe the female offspring, as well BAT and skeletal muscle.

These models are crucial to understand the mechanism behind the beginning of the disorders and to possibly prevent these outcomes later in life.

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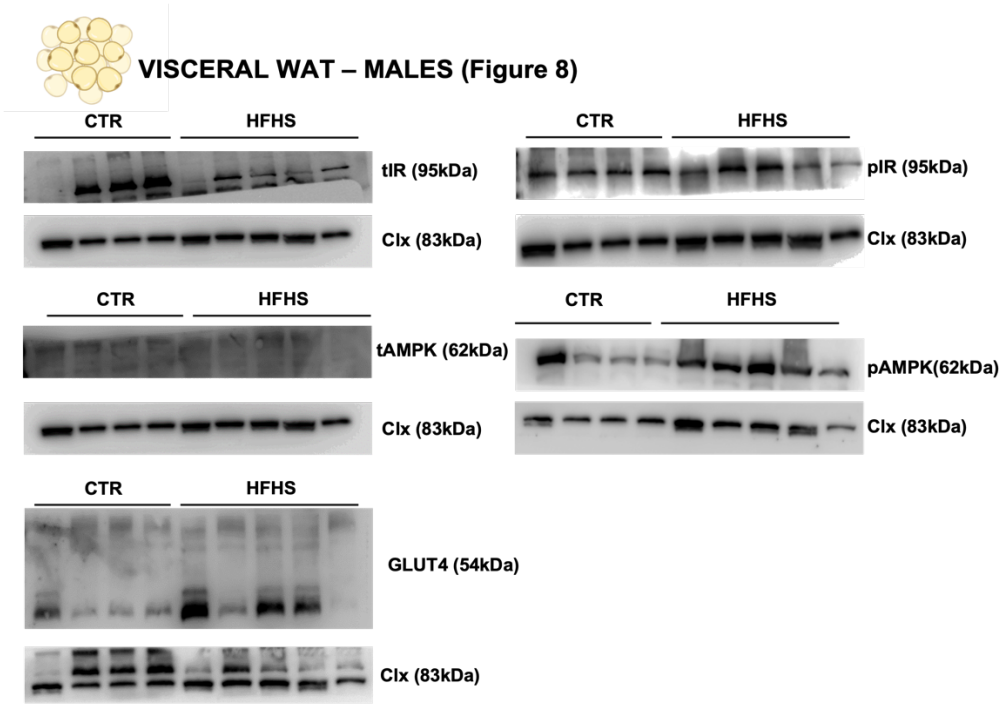
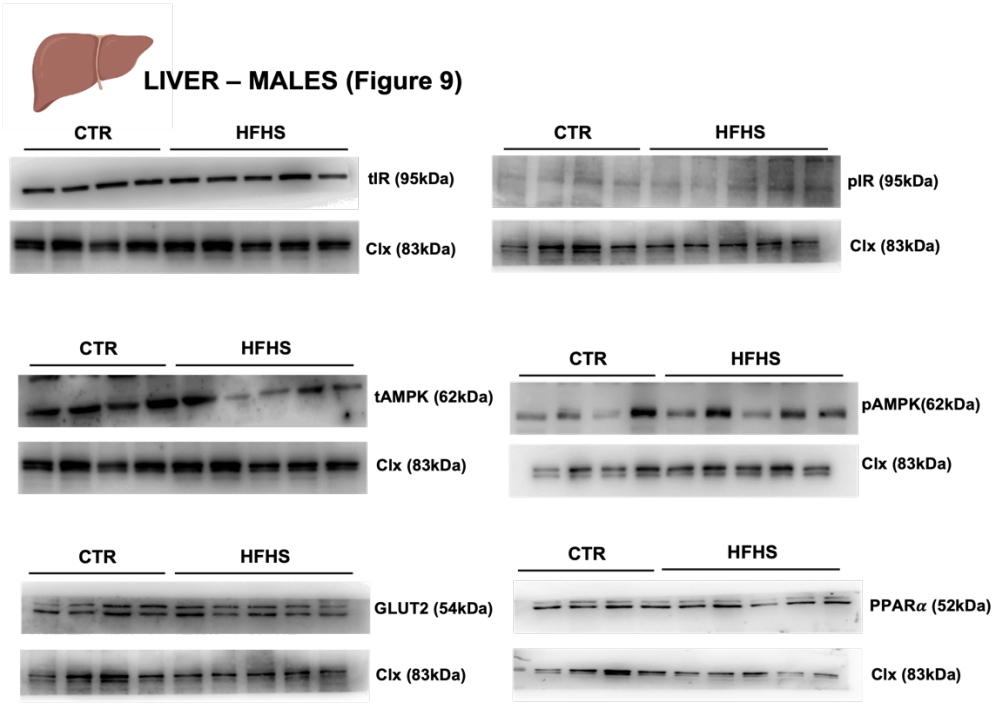
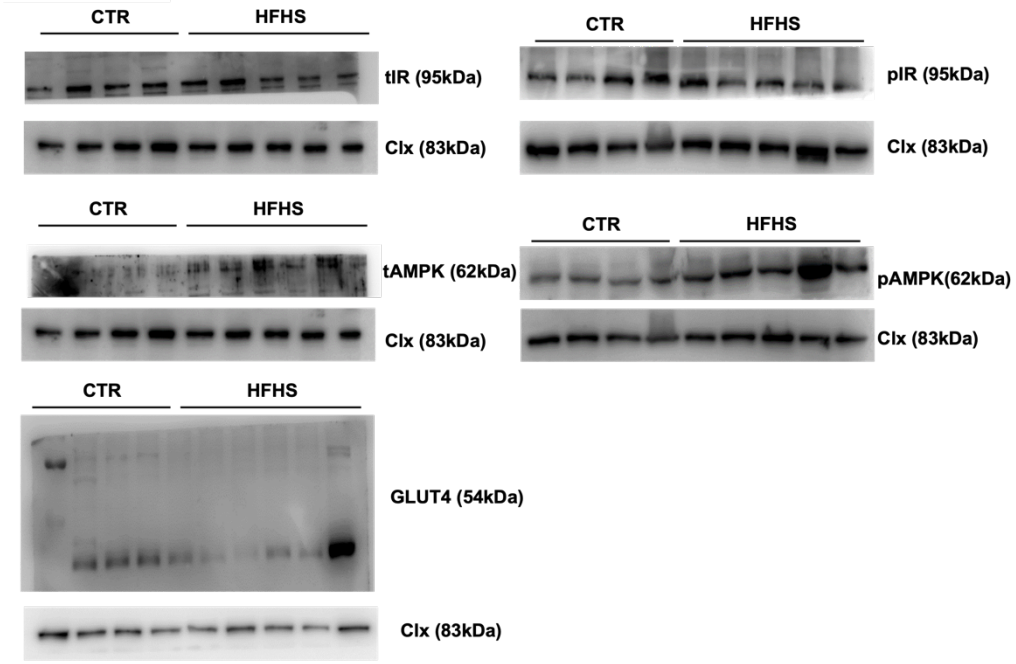
ANEX

MATERNAL DIET-INDUCED OBESITY

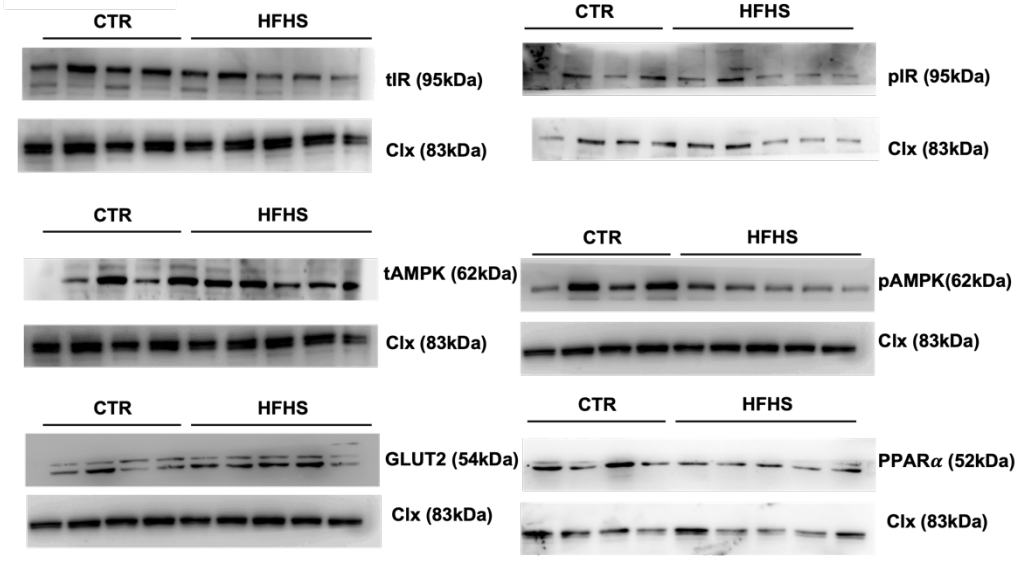




VISCERAL WAT – FEMALES (Figure 10)

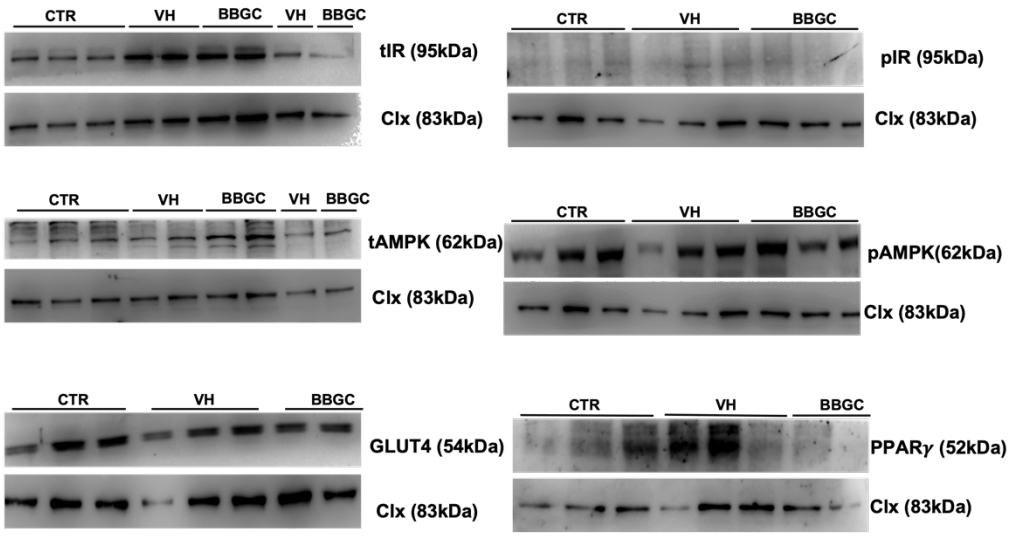


LIVER – FEMALES (Figure 11)

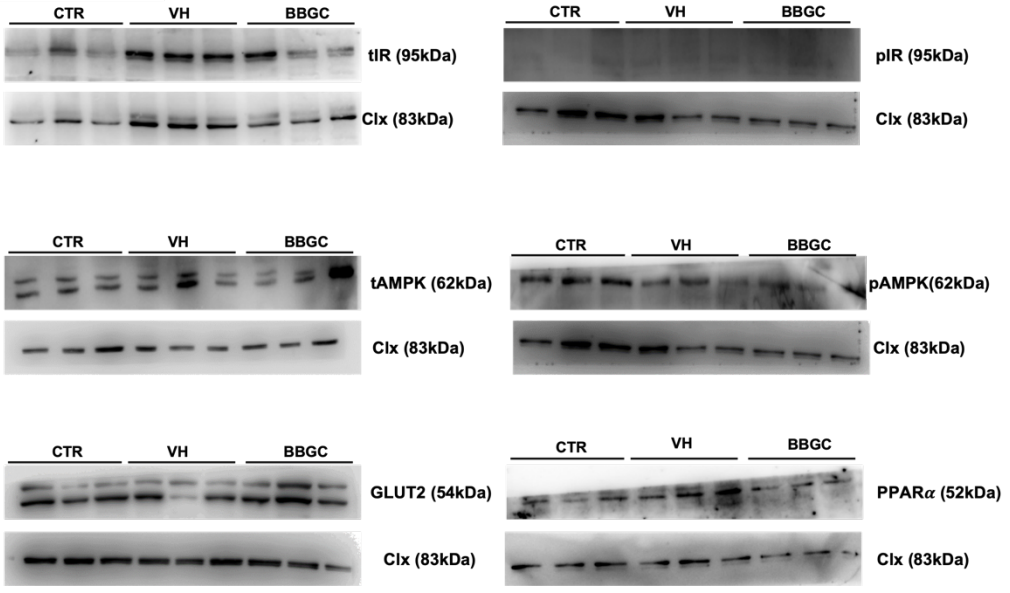


MATERNAL GLYCATION MODEL

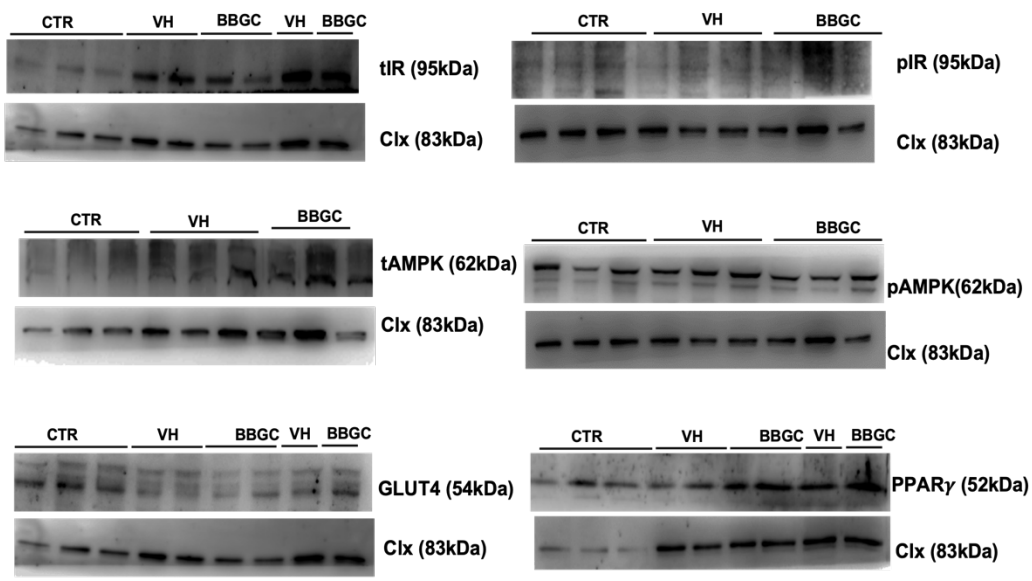
VISCERAL WAT – MALES (Figure 15)



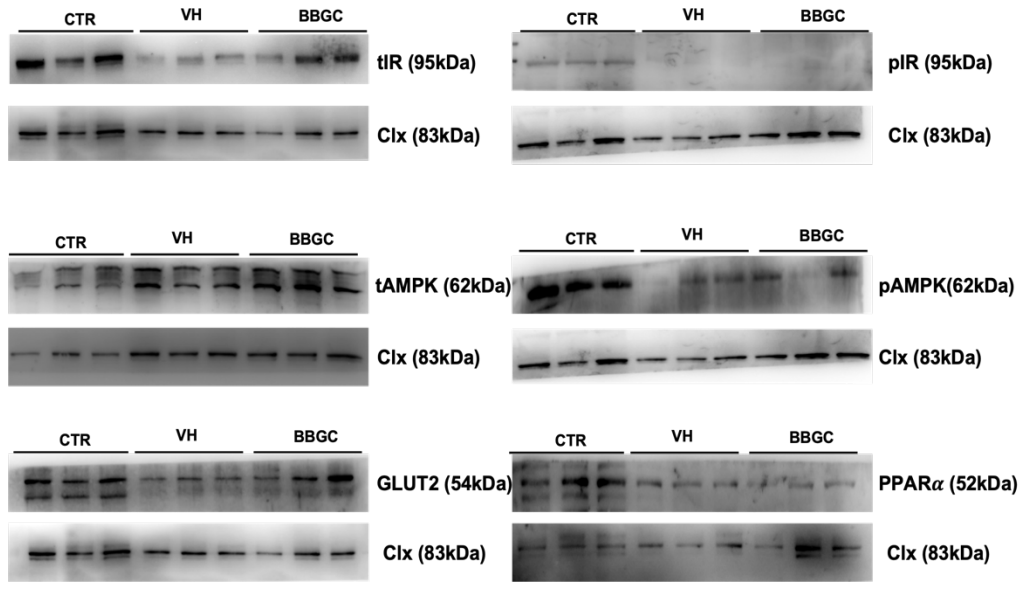
LIVER – MALES (Figure 16)



VISCERAL WAT – FEMALES (Figure 19)



LIVER – FEMALES (Figure 20)



IMPACT OF MATERNAL GLYCATION IN A MODEL OF OVERWEIGTH INDUCED BY NEONATAL HYPERPHAGIA