Universidade de
Aveiro
Ano 2021

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Clínica, realizada sob a orientação científica do Doutor Pedro F. Oliveira, Professor do Departamento de Química da Universidade de Aveiro e da Doutora Ana D. Martins, Investigadora Júnior da Unidade Multidisciplinar de Investigação Biomédica, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto.

o júri

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agradecimentos

Antes de mais, gostaria de agradecer ao professor Pedro Oliveira e à doutora Ana Martins pela oportunidade de participar neste projeto de investigação, por todo o apoio, motivação e por acreditarem nas minhas capacidades.

A toda a equipa do laboratório de Biologia Celular do ICBAS, agradeço todo o apoio e carinho com que me acolheram. Agradeço à Sara Pereira, que me deu a conhecer o laboratório e me ajudou nos primeiros passos que dei neste projeto. Ao Luís Crisóstomo agradeço a sua disponibilidade e ajuda no tratamento de dados deste trabalho. À professora Mariana Monteiro agradeço a sua disponibilidade e ajuda no tratamento dos dados deste trabalho. Deixo também um agradecimento especial à técnica de laboratório Madalena Santos que foi essencial, não só pela sua disponibilidade, apoio no laboratório e por me ter transmitido muito conhecimento sobre imunohistoquímica, mas também pela sua boa disposição e simpatia.

À minha "família bioquímica", Inês Pereira, Manuela Durão e Patrícia Jorge, obrigada por todo o vosso apoio e carinho. Obrigada por serem as minhas melhores *cheerleaders*. Ver-vos lutar pelos vossos sonhos inspira-me a também lutar pelos meus. À minha Sara, que está sempre lá para me aturar e me ouvir em todos os momentos bons e menos bons. Finalmente, mas não menos importante, agradeço a toda a minha família, principalmente aos meus pais. Tudo aquilo que sou, devo-o a vocês.

palavras-chave

Administração de GLP-1, Espermatozoides, Função reprodutora masculina, Genes relacionados à obesidade, Homeostase energética, Restrição calórica, Testículos

resumo

A restrição calórica (CR), pelo seu balanço energético negativo e o GLP-1, por ser uma incretina, constituem dois protocolos de perda de peso, que permanecem pouco investigados, principalmente no que toca ao seu impacto na função reprodutiva masculina, cujo correto funcionamento depende de uma homeostase energética equilibrada. Essa homeostase energética tem sido relacionada a genes ligados ao controlo do metabolismo energético, nomeadamente os genes relacionados à obesidade (ORGs), como gene de obesidade e de massa de gordura associada (*FTO*), recetor de melanocortina-4 (*MC4R*), glucosamina-6-fosfato desaminase 2 (*GNPDA2*) e proteína transmembranar 18 (*TMEM18*). Assim, este projeto teve como objetivo utilizar um modelo animal para estudar o potencial da CR e da administração de GLP-1 na regulação da expressão desses ORGs nos testículos e esperma de ratos Wistar e verificar se há impacto na qualidade espermática. Primeiramente, observamos que a CR promoveu menor ganho de peso e diminuição da resistência à insulina. Por outro lado, a administração de GLP-1, além do esperado aumento nos níveis de GLP-1 ativo, não promoveu qualquer alteração no metabolismo da glucose, no perfil hormonal e consequentemente no peso corporal. Para além disso, a CR promoveu um aumento nos defeitos da cabeça dos espermatozoides, enquanto a administração de GLP-1 melhorou a morfologia espermática. Em relação aos ORGs, observamos a presença de transcritos de *FTO*, *MC4R* e *TMEM18* em testículos de ratos e identificamos esses transcritos, pela primeira vez, em esperma de ratos. Além disso, identificamos, pela primeira vez, a presença de transcritos *GNPDA2* em testículos e esperma de ratos. As proteínas correspondentes também foram identificadas em testículos de ratos, sendo que todas apresentavam localizações celulares distintas. A CR e a administração de GLP-1 promoveram um aumento na expressão dos ORGs nos testículos, porém no esperma a expressão não foi alterada. Também identificamos a presença de transcritos de *NFE2L2* (codifica para um importante fator de transcrição antioxidante) em testículos e esperma de ratos e verificamos que sua abundância é aumentada nos testículos, pela CR e administração de GLP-1. Em seguida, exploramos, individualmente, o papel dos ORGs nos níveis testicular e espermático, nomeadamente na resposta à CR e à administração de GLP-1 e obtivemos algumas pistas relacionadas com o envolvimento potencial de cada um dos ORGs na função reprodutiva masculina. No geral, pudemos perceber que as duas intervenções promoveram uma associação geral de todos os ORGs com uma melhoria no estado oxidativo dos testículos e esperma.

keywords

Caloric Restriction, Energy homeostasis, GLP-1 administration, Male reproductive function, Obesity-related genes, Sperm, Testes

abstract

Caloric restriction (CR), due to its negative energy balance and GLP-1, for being an incretin, constitute two weight loss protocols that remain poorly investigated, particularly its impact on male reproductive function, whose proper functioning depends on a balanced energy homeostasis. That energy homeostasis has been related to genes linked to energy metabolism control, namely the obesity-related genes (ORGs), such as fat mass and obesity-associated (*FTO*), melanocortin-4 receptor (*MC4R*), glucosamine-6-phosphate deaminase 2 (*GNPDA2*) and transmembrane protein 18 (*TMEM18*). Thus, this project aimed to use an animal model to study the potential of CR and GLP-1 administration in the regulation of those ORGs expression in testes and sperm of Wistar rats and verify if there is an impact on sperm quality. Firstly, we observed that CR promoted a lower weight gain and a decrease in insulin resistance. On the other hand, GLP-1 administration, beyond the expected increase in active GLP-1 levels, did not promote any change in glucose metabolism, hormonal profile and consequently in body weight. Furthermore, CR promoted an increase in sperm head defects, while the GLP-1 administration improved sperm morphology. Regarding the ORGs we observed the presence of *FTO*, *MC4R* and *TMEM18* transcripts in rat testes and identified those transcripts, for the first time, in rat sperm. Additionally, we identified, for the first time, the presence of *GNPDA2* transcripts in rat testes and sperm. The corresponding proteins were also identified in rat testes, being that they all presented distinct cellular locations. CR and GLP-1 administration promoted an increase in the expression of the ORGs in testes, however in spermatozoa the expression was not altered. We also identified the presence of *NFE2L2* (encodes for an important antioxidant transcription factor) transcripts in rat testes and sperm and verified that their abundance is increased, in testes, by CR and GLP-1 administration. Then we explored, individually, the role the ORGs at the testicular and sperm levels, namely in the response to CR and GLP-1 administration and we obtained some clues related to the potential involvement of each of the ORGs in the male reproductive function. Overall, we were able to perceive that CR and GLP-1 administration promoted a general association of all ORGs with an improvement in oxidative status both in testes and sperm.

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List of Abbreviations

TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
AL	*Ad libitum*
ATP	Adenosine triphosphate
AC	Adenylyl cyclase
AgRP	Agouti-related peptide
ARC	Arcuate nucleus
ABC	Avidin-biotin complex
BMI	Body mass index
BSA	Bovine serum albumin
CR	Caloric restriction
CCK	Cholecystokinin
CART	Cocaine- and amphetamine-related transcript
cDNA	Complementary DNA
CUX1	CUT-like homeobox 1
cAMP	Cyclic adenosine monophosphate
DEHP	Di-(2-ethylhexyl) phthalate
DPP-4	Dipeptidyl peptidase 4
ELISA	Enzyme-linked immunosorbent assay
FTO	Fat mass and obesity-associated
FRAP	Ferric reducing antioxidant power assay
FSH	Follicle-stimulating hormone
F6P	Fructose-6-phosphate
GABA	Gamma-aminobutyric acid
GWAS	Genome-wide association studies
GLP-1	Glucagon-like peptide 1
GlcN6P	Glucosamine-6-phosphate
GNPDA2	Glucosamine-6-phosphate deaminase 2
G6P	Glucose-6-phosphate

GFAT	Glutamine:fructose-6-aminotransferase
GnRH	Gonadotropin-releasing hormone
GHSR	Growth hormone secretagogue receptor
HBP	Hexosamine biosynthetic pathway
HBSS	Hank's balanced salt solution
HOMA-IR	Homeostatic model assessment for insulin resistance
HPG	Hypothalamic-pituitary-gonadal
IHC	Immunohistochemistry
IR	Insulin receptor
IRX	Iroquois-class homeobox protein
LepR	Leptin receptor
LC	Leydig cell
LED	Low-energy diets
LH	Luteinizing hormone
MC4R	Melanocortin-4 receptor
MTII	Melanotan II
MER	Mild-energy restriction
MYT1	Myelin transcription factor 1
MC	Myoid cell
m^6A	N^6-methyladenosine
NPY	Neuropeptide Y
Y1R	Neuropeptide Y receptor
NFE2L2	Nuclear factor erythroid 2 like 2
Nrf2	Nuclear factor erythroid 2-related factor 2
ORG	Obesity-related gene
PVN	Paraventricular hypothalamic nucleus
PYY	Peptide YY
PPARG	Peroxisome proliferator-activated receptor gamma
PCR	Polymerase chain reaction
PS	Primary spermatocyte
POMC	Proopiomelanocortin

PKA	Protein kinase A
ROS	Reactive oxygen species
REE	Resting energy expenditure
RPGRI1L	Retinitis pigmentosa GTPase regulator-interacting protein 1-like
Runx1t1	Runt-related transcription factor 1
SC	Sertoli cell
SHBG	Sex hormone-binding globulin
SNP	Single nucleotide polymorphisms
Sim1	Single-minded homolog 1
SPT	Spermatid
SPC	Spermatocyte
SPG	Spermatogonia
Srsf2	Splicing regulatory protein serine and arginine rich splicing factor 2
STAT5	Transducer and activator of transcription 5
TMEM18	Transmembrane protein 18
T2D	Type 2 diabetes
UDP-GlcNAc	UDP-N-acetylglucosamine
OGT	UDP–Nacetylglucosaminyl transferase
VLED	Very-low-energy diets
α-MSH	α-melanocyte stimulating hormone
B2M	β2-microglobulin

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1. Introduction

1.1. Influence of energy homeostasis on male fertility

Reproduction is an essential physiological process to ensure continuity and propagation of all the species, and, therefore, it is under the control of sophisticated regulatory networks. However, it is well-known that fertility rates are decreasing worldwide. Human infertility is considered as a major health care problem of different communities and the high prevalence of this situation reinforces its importance.[1] Among humans, infertility is defined as the failure to achieve pregnancy after 12 months of regular unprotected sexual intercourse and is estimated that this problem affect 8–12% of couples in the reproductive age group.[2] Male infertility is the only cause of infertility in 20–30% of cases and a factor contributing to infertility in 50% of all infertility cases.[3] Indeed, the average man's sperm quality, a predictor of male fertility, is declining worldwide. A recent evidence-based meta-analysis has shown that there has been an overall 32.5% decline in sperm concentration in European men over the past 50 years, suggesting a significant decline in male reproductive health, which has serious implications beyond fertility concerns.[4]

In most cases, the infertility is idiopathic, with no clear explanation for impaired spermatogenesis. Although the evidence linking environmental factors and impaired male fertility is weak, there are still some evidence suggesting that semen quality may be influenced by environmental factors and lifestyle. Environmental or occupational exposure to toxic chemicals[5] and various lifestyle factors such as smoking,[6] alcohol consumption,[7] caffeine,[8] advanced paternal age,[9] psychological stress,[10] exposure to radiation emitted by electronic devices,[11] diet choices and nutritional status,[12] influence male reproductive potential and the capacity to achieve fecundation.[13] These findings might have implications on male fertility status and highlight the need for further studies addressing lifestyle consequences.

Decreased male fertility has been widely associated with a diet patterns change, sedentary lifestyle and other modifiable factors.[14,15] Men who have a healthy dietary patterns (health conscious, prudent and Mediterranean dietary pattern) present significantly higher sperm concentration when compared with individuals that have unhealthy dietary patterns (Western dietary pattern, that is a pro-inflammatory hypercaloric diet with low nutritional density). Furthermore, men who adopt healthy diets are also more likely to have a healthier lifestyle, lower body mass index (BMI), and higher physical activity. It was noted that total sperm count, progressive and total motility were also positively associated with a strong adherence to the healthy dietary pattern, especially in men with poor sperm quality.[16,17] Thus, eating a healthy and varied diet is fundamental to maintain a good overall health and consequently a good reproductive health.

Reproductive development and function are sensitive to the metabolic state of the organism and the magnitude of body fuel reserves. Hence, conditions ranging from energy insufficiency to morbid obesity impact the timing of puberty and are frequently linked to fertility problems. This is the result of the close interplay between a diversity of nutritional cues and metabolic signals, including hormones from both the classical (insulin, thyroid hormones, and glucocorticoids) and nonclassical (ghrelin and leptin) endocrine tissues, with different impacts of the hypothalamic-pituitary-gonadal (HPG) axis. The core of this neurohormonal reproductive axis is formed by the hypothalamic decapeptide, gonadotropin releasing hormone (GnRH), pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and gonadal hormones. These major elements are connected by regulatory loops that are essential for the proper control of reproductive hormone secretion and gonadal function.[18] Under normal conditions, GnRH is produced and released from the hypothalamus, which stimulates the production and release of FSH and LH from the anterior pituitary. FSH and LH act on the testes to stimulate spermatogenesis and steroidogenesis, respectively.

Reproduction is an energy-demanding function that is dispensable at the individual level. Thus, under conditions that perturb the organism energy homeostasis, in which indispensable body functions must be preserved,

often result in reproductive impairment. While this phenomenon is especially relevant in the female, because of the substantial energy investment that needs during pregnancy and lactation,[19] such tight association is detectable in both sexes, so that conditions of metabolic stress can perturb puberty onset and gonadal function also in males.[20] Disturbed energy homeostasis caused by dietary problems, such as severe malnutrition or obesity and/or metabolic syndrome, is associated with reproductive dysfunctions, namely in males.[21] Both low and high BMIs (body mass index; less than 19 kg/m^2 or more than 30 kg/m^2 respectively) have been associated with reduced testicular volume and reduced semen quality suggesting impairment of spermatogenesis.[22] Being overweight or underweight causes problems with ovulation in women, and maternal obesity can increase the rate of miscarriage, gestational diabetes, high blood pressure during pregnancy, and birth defects. However, the effect of male weight on fertility and birth outcomes has only recently been understood and seems that paternal weight can play a part just as serious as maternal weight in fertility rates and fetal development.

Not only has overweight and obesity been associated with diabetes mellitus, cardiovascular disease, cancer, and an increased risk of all-cause mortality, but also with male infertility. Increased BMI was found to be significantly linked to a fall in sperm quality, serum testosterone, sex hormone-binding globulin (SHBG) and an increase in serum estradiol. The observed poor sperm quality results from alterations in semen parameters, especially sperm concentration, total sperm count, total motile sperm count, total progressively motile sperm count, sperm morphology, and DNA fragmentation. In addition to impaired semen quality, fertility among obese men may be affected by sexual dysfunction, endocrinopathy, aromatization activity, psychological and thermal effects, sleep apnea, accumulation of lipophilic toxins, and possibly the inflammatory and obstructive elements of epididymitis pathology.[23] Hence, obesity may be contributing to male infertility, as it negatively impacts reproductive function, through numerous and multiple mechanisms. Hormonal abnormalities related to increased adiposity may also be major contributors. They blunt the HPG axis leading to decreased intratesticular testosterone levels, which is vital for spermatogenesis.[24] Elevated scrotal temperatures because of body habitus and inactivity can also impair semen parameters.[25] Increased systemic inflammation in obesity can lead to reactive oxygen species (ROS) and sperm DNA fragmentation, which is associated with reduced pregnancy rates.[26]

In fact, the effect of obesity on male fertility has attracted more attention with a large number of related studies have gradually emerging in the last years. However, despite not being the target of so much study, man with lower BMI may also have problems associated with fertility. It was already been shown that low BMI decrease semen parameters such as total sperm count, semen volume rather than sperm concentration and motility (overall or progressive motility), suggesting that low BMI is also a major risk for semen quality.[27] Few studies evaluated low body weight impacts male reproductive capacity and for these reason the corresponding underlying mechanisms remains unclear. However, was speculated that, analogously to obese men, hormonal imbalance may be involved in reduced semen quality in men with low BMI. Additionally, men with low BMI may have unhealthy lifestyles, as well as slight malnutrition or subclinical adverse conditions, which could affect their reproductive function.[28]

With growing evidence that weight adversely affects male fertility, efforts have focused on possible interventions including weight loss, namely by lifestyle modifications, to achieve an optimization of waist circumference and BMI that eventually restore the reproductive potential. It's been suggested that weight loss should be implemented in obese men for seeking fertility treatment.[29] Hakonsen et al. studied the effects of a short-term diet and exercise on hormones and some semen parameters in obese men. Those authors shown that participants with the largest weight loss had a significant increase in sperm concentration, morphology, serum testosterone, SHBG and testosterone : estradiol ratio.[30] However, as mentioned, low BMI is a pernicious factor for male fertility, which rise the question that whether excessive weight loss affects semen quality in the opposite direction, and whether weight loss treatment should be formulated a degree of restriction.[27]

Thus, despite the fact that the notion that energy homeostasis impacts sperm is still farther away from male collective consciousness than desirable, there is no doubt that body energy balance and consequently the weight

constitute a modifiable factor that strongly impacts male fertility and a balanced diet should be taken into account to improve semen quality and fecundability rates.[31,32]

1.2. Hypothalamic control of body weight

Several brain areas are involved in the regulation of food intake and consequently the body weight, including the brainstem and the hypothalamus.[33,34] These two systems collect information about the nutrient status and respond accordingly, i.e. if the nutrient status shows hunger the action is to eat and preserve energy and, on the other hand, if the nutrient status shows satiety, the action is stop eating and spend energy.[34] Appetite is a complex process that results from the integration of multiple signals at the hypothalamus **(Figure 1)**.

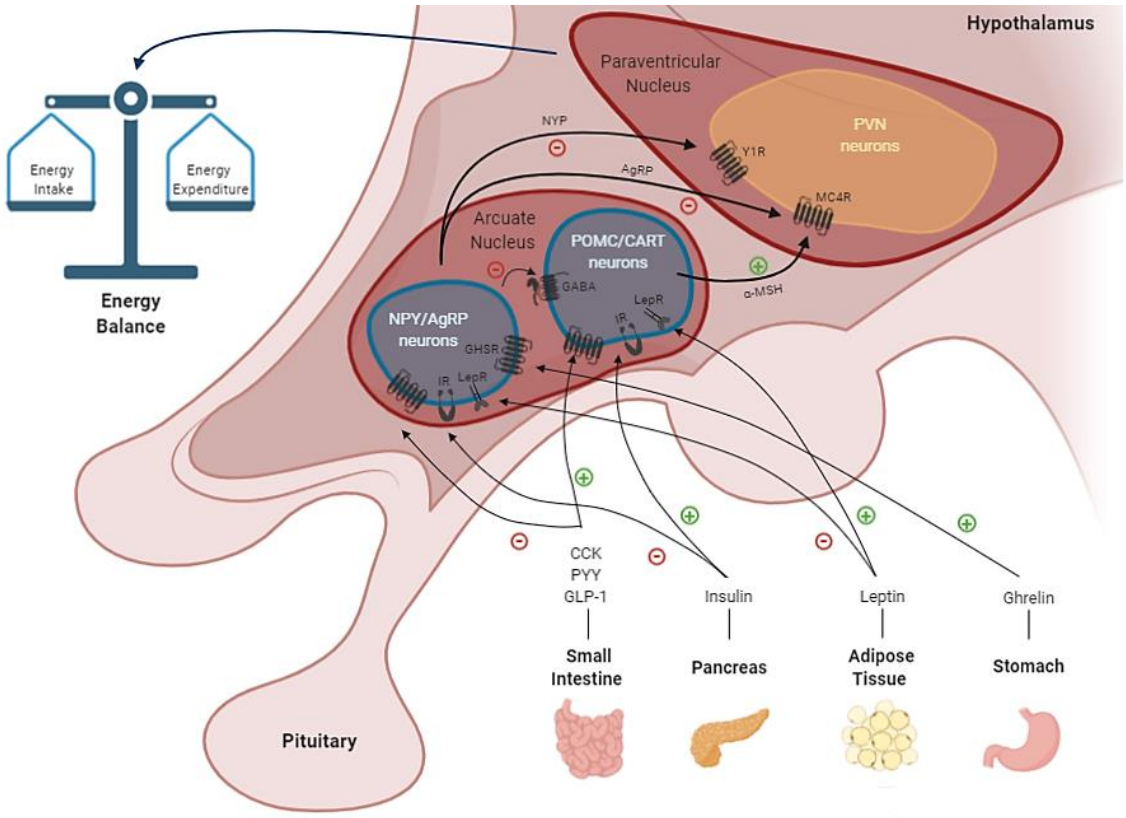


Figure 1 – Hypothalamic control of energy homeostasis. Hormonal circuits from the gastrointestinal system (small intestine, pancreas and stomach) and fat (adipose tissue) that impact the feeding behavior and consequently energy balance through hypothalamic neuroendocrine pathways. (**Abbreviations**: α-MSH, α-melanocyte stimulating hormone; AgRP, agouti-related peptide; CART, cocaine- and amphetamine-related transcript; CCK, cholecystokinin; GABA, gamma-aminobutyric acid; GHSR, Growth hormone secretagogue receptor; GLP-1, glucagon-like peptide 1; IR, insulin receptor; LepR, leptin receptor; MC4R, melanocortin-4 receptor; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular hypothalamic nucleus; PYY, peptide YY; Y1R, neuropeptide Y receptor 1; **Adapted from**: Lizarbe et al. (2013)[35])

The hypothalamic nuclei involved in food intake is composed by second-order neurons such as paraventricular nucleus (PVN) that control sympathetic outflow to peripheral organs and secrete regulatory neuropeptides, being crucial in the feeding behavior regulation.[36] The second-order neurons process the information and project to neurocircuits outside the hypothalamus an integrated response related to the energy intake and expenditure.[37,38] The arcuate nucleus (ARC) controls these second-order neurons by integrating the hormonal and nutritional metabolic signals from the peripheral circulation as well as peripheral and central neuronal inputs.[39,40] There are two distinct and functionally antagonistic types of neurons in ARC: the orexigenic (appetite-stimulating)

neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons (NPY/AgRP neurons) and the anorexigenic (appetite-suppressing) proopiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART) neurons (POMC/CART neurons).[37]

Energy balance is controlled through hypothalamus integration of signals related to long-term storage from adipose tissue and short-term meal-related signals. When this signaling pathway fails, energy balance is deregulated.[38,41,42] Short-term regulation of feeding is based on phasic oscillations in energetic state, mediating the acute indices of hunger and satiety.[43,44] Upon food ingestion, POMC is cleaved to α-melanocyte stimulating hormone (α-MSH) which is released from POMC/CART axons to activate melanocortin 4 receptor (MC4R) on PVN neurons.[45] This leads to reduced food intake while increases energy expenditure.[46-48] Fasting, on the other hand, induces the activation of NPY/AgRP neurons that co-release NPY and AgRP.[49] NPY stimulates food intake via activation of NPY receptor 1 (Y1P) on PVN neurons.[50] AgRP also acts as an inverse agonist of MC4R, preventing the anorexigenic effect of α-MSH.[51] Furthermore, NPY/AgRP neurons directly inhibit POMC neurons via inhibitory gamma-aminobutyric acid (GABA) action at the ARC level.[52] This increase food intake while decrease energy expenditure.[37,43,53]

Gastrointestinal hormones are also important regulators of energy control. Ghrelin is a gastrointestinal hormone that has an orexigenic effect and is considered as a short-term signal of energy insufficiency. It's plasmatic levels increases during fasting and peaks before meals, preceding the hunger sensation.[54] Ghrelin promotes feeding by NPY/AgRP neurons activation, leading to increased food intake, weight and adiposity gain.[55] Upon food ingestion, ghrelin is no longer produced and other hormones are released from the intestine, including glucagon-like peptide (GLP-1), peptide YY (PYY) and cholecystokinin (CCK), exerting anorexigenic effects by POMC/CART neurons activation.[37,54]

Long-term regulation is associated with the signaling of hormones positively correlated with adiposity, i.e. the amount of body fat. Low body fat content encourages feeding and energy preservation, while high body fat suppresses appetite and promotes energy expenditure.[41] Two peripheral hormones, leptin and insulin, act synergistically with gastrointestinal hormones in the regulation of short-term food intake.[44,56] Leptin and insulin receptors (LepR and IR, respectively) are expressed on both POMC/CART and NPY/AgRP neurons.[57] The amount of plasmatic leptin is directly proportional to the body fat content.[56,58,59] Leptin acts on POMC/CART and NPY/AgRP neurons and its pathway is interconnected with insulin pathway. That interconnection triggers the energetic homeostasis gene regulation, mainly leading to over-transcription of anorexigenic neuropeptides and down-transcription of orexigenic neuropeptides, as well as favoring energy expenditure genes. This suppresses appetite and promotes energy expenditure.[54,60,61]

There is a coordinated adjustment to food intake or energy expenditure in response to food restriction and overfeeding to defend a body weight set point. Dysregulation of appetite or loss of caloric sensing can have a significant impact on health.[62] When appetite suppression is attenuated, obesity and associated comorbidities ensue.[63] Alternatively, in conditions like cancer, sepsis and ageing, appetite is suppressed, which exacerbates the associated comorbidities.[64,65]

1.2.1. Glucagon-like peptide 1

GLP-1 is one of the gastro-intestinal hormones involved in the complex control of the energy balance, having a prominent role in glucose homeostasis, gastrointestinal motility and appetite regulation. It is a polypeptide made up of 31 amino acids obtained from proglucagon gene, mainly secreted by endocrine L-cells that predominate in the distal small intestine.[66] GLP-1 is released in response to the presence of nutrients (predominantly sugars and fats) in the stomach and proximal intestine or through direct contact between nutrients and L-cells. GLP-1 release is a process likely to be regulated by a neuroendocrine loop involving both afferent and efferent fibers of the vagus nerve,

that is the major anatomical link between the gastro-intestinal tract and the CNS, since it highly expresses the gastro-intestinal hormones receptors at a variable rate according to the energy status.[67] The secretion of GLP-1 in response to feeding amounts to two phases: the rapid initial phase occurs between 10 to 15 minutes postprandial, and the second and longer phase that takes between 30 and 60 minutes. Once released, GLP-1 diffuses through the capillaries and lymph. Herein, it mediates the activation of the GLP-1 receptor, present throughout the periphery (pancreas, stomach, adipose tissue, heart, and others)[68] and the CNS.[69] In fact, GLP-1 receptors are found on the vagal afferent fibers, that convey signals from the periphery to the brainstem. The rapid rise of circulating GLP-1, in the first phase, suggests a neuronal regulated mechanism.[70]

As GLP-1 goes from the gastrointestinal tract to various peripheral organs, it is subject to degradation by dipeptidyl peptidase 4 (DPP-4). Only 25% of the secreted hormone - active GLP-1 - makes its way to the liver. Further degradation occurs as the hormone continues to peripheral organs. In the end, only 10-15% of the originally secreted GLP-1 reaches those organs. The half-life of native GLP-1 is within 1 to 2 minutes, due to the rapid inactivation by the ubiquitous enzyme DPP-4, combined with the renal clearance. Taking this into account, the highest levels of the active GLP-1 are found immediately within the hepato-portal circulation due to the rapid degradation by DPP-4.[71]

GLP-1 influences energy balance basically by exerting effects on appetite regulation and glucose metabolism. Its essential role is involved with the response to increased levels glycemic after meals, acting directly on the pancreatic islets, specifically in beta cells, where it promotes greater insulin release and in alpha cells, in antagonism to glucagon. In such a way, this incretin is able to reduce blood glucose and increase liver and muscle insulin sensitivity.[72] The effects on weight reduction may also be mediated by its action at the gastrointestinal level since GLP-1 is responsible for delayed gastric emptying, stomach smooth muscle distension and decreased stomach acid secretion, promoting a feeling of satiety and reduced energy consumption capacity. Meanwhile, the absorption and digestion of nutrients are also optimized. Moreover, at the level of the CNS, it seem to promote satiety. [73,74]

Native GLP-1 is known to have a short half-life and the latest methods have been developed to improve its half-life. Thus, based on GLP-1 proprieties and physiological effects, longer acting GLP-1 analogs have been developed as pharmacological approaches used for type 2 diabetes (T2D) and obesity treatment, a condition characterized by hyperglycemia and insulin resistance.[75,76] Among the main approved drugs, albiglutide, dulaglutide, exenatide, liraglutide and lixisenatide are the ones that stand out. These GLP-1 analogues show homologies 50% to 97% with human GLP-1. GLP-1 is physiologically and rapidly degraded by the enzyme DPP-4, resulting in a plasma half-life of about 2 minutes. In order to became GLP-1 resistant to this degradation and consequently increase its half-life, some strategies were used in the synthesis of GLP-1 analogues, which resulted in a division into two large groups, based on its pharmacokinetic profile: short-acting and long-acting.[77] The longer the half-life of GLP-1 analogues, the longer the administration interval and the smaller the fluctuations in their plasma levels, resulting in a continuous activation of GLP-1 receptors. In general, all GLP-1 analogues are effective in weight reduction, being more effective than other therapeutic methods both in monotherapy and in combination with one or more oral antidiabetics or insulin. Still, direct comparisons between GLP-1 analogues showed that liraglutide is superior to albiglutide, dulaglutide, exenatide and similar to lixisenatide in weight reduction.[78,79]

A 56-week study investigated the efficacy and safety of liraglutide 3.0 and 1.8 mg once-daily doses in obese or overweight adults with T2D treated with medication or diet and exercise. The individuals were divided in three groups: use of 1.8 mg of liraglutide, 3.0 mg of liraglutide and placebo. The weight loss in the group that received 3.0 mg had an average weight loss of 6.0%, the one that used 1.8 mg had an average reduction of 4.7% and the placebo group of 2.0%, with the majority who used liraglutide lost 5 to 10% of their initial weight. In addition, there was better glycemic control and reduced systolic blood pressure and C-reactive protein levels. The group that used the 3.0 mg dose also showed improvement in cholesterol and triglyceride levels and improved mental health.[80] Other four randomized controlled trials showed the drugs' effectiveness in promoting weight loss. Three of them related to liraglutide in doses ranging from 1.2 to 3.0 mg daily, which approach or exceed the limit of 1.8 mg used for diabetes management, and one study in that patients used 20 mg distributed in two daily doses had similar results that

demonstrated substantial weight loss when compared to placebo.[81,82] Furthermore, another study analyzed the effectiveness of liraglutide in gastric emptying, weight loss and satiety. It was concluded that the group that used liraglutide at a dose of 0.6 to 3.0 mg/day for 16 weeks, compared to the group that received placebo, achieved a slower gastric emptying for solids, decrease in the maximum tolerated volume in the stomach and greater weight loss, which is a direct consequence of delayed gastric emptying. It was also noticed that there was a relationship between weight loss and time of exposure to the drug and that in T2D patients, the weight reduction was smaller compared to normoglycemic or pre-diabetic individuals.[83] Despite that, the existent information shows that GLP-1 analogues are potentially promising drugs for weight loss.

1.3. Balanced diet versus Unbalanced diet

Energy is essential for the body to survive, grow, keep warm and move around. Thus, the supply and maintenance of energy levels is essential for life, being required to fuel physiological processes.[84] Energy is obtained from the diet and, by definition, a dietary pattern involves the overall diet regarding to quantities, proportion, variety and frequency of consumption.[85]

A balanced diet is an crucial part of a healthy lifestyle and should be sufficient to ensure energy needs, complete to ensure nutritional requirements, harmonious so that the different nutrients are correctly related to each other and appropriate to the individual's biological situation.[86] Excluding breast milk, no other food contains all the essential nutrients.[87] Thus, a balanced diet should provide foods from all five groups (vegetables, fruits, grains, protein and dairy) to fulfil the nutritional needs of an individual.[88] Energy needs and nutrient requirements highly vary with the lifestyle. For example, if physical exercise is often practiced or if there is an elevated activity level, more calories are required.[89] Furthermore, energy requirements of patients suffering from certain diseases or of those going through particular phases of life such as pregnancy and lactation,[90,91] infancy or childhood[92] differ considerably, so those individuals need an adapted diet.[86]

A balanced diet depends on an energy equilibrium. Energy is originated from chemical energy bound in food and its macronutrients that serve as substrates in catabolic pathways. After food ingestion, chemical energy is released and converted into thermic, mechanical, among other forms of energy.[93] Energy balance is defined by the difference between the dietary energy intake and the total energy expenditure, plus the extra energy cost of some life phases/conditions.[94] The energy is measured in calories, being that the relation between the amount of calories ingested and the amount of calories expended is closely related to overall weight. In energy equilibrium, the metabolizable energy consumption matches the amount of energy spent and an individual is considered to be in a steady state. Long-term unbalancing of that equilibrium result in body weight modifications, with a positive (intake > expenditure) or negative (intake < expenditure) energy balance leading to weight gain or loss, respectively.[94,95]

Unfortunately, there is still a devaluation of the importance of having a well-balanced diet. Generally, people take bad food choices, eat too fast,[96] alone and less frequently or even resort to supplementation to save time.[97] This usually leads to nutritional imbalances, resulting in deleterious impacts on health, causing deficiency diseases such as blindness,[98] anemia,[99] scurvy,[100] health threatening conditions like obesity[101] and chronic diseases such as cardiovascular disease,[102] diabetes[103] and osteoporosis.[104] When there is an imbalance between the nutrients needed and the nutrients obtained, there is a malnutrition state. Malnutrition is divided in overnutrition, where exists an excess calorie consumption, and undernutrition where the opposite occurs.[105-107]

Overnutrition is associated with a high prevalence of obesity and it's comorbidities.[86] The excessive intake of foods with a large number of calories, containing processed carbohydrates and fats, and less fiber and nutrients, observed in industrialized countries, is a contributing factor for the development of this pathology, which is aggravated by the lower consumption of fruits, vegetables and sedentary lifestyles.[108] In underdeveloped countries, the main nutritional problem is that most of the population cannot afford enough food to meet all their nutritional requirements, which leads to undernutrition. Thus, protein-energy undernutrition often occurs,[109] being responsible for many deaths

by increasing the risk of developing life-threatening infections.[110] In developed countries, undernutrition is far less common than overnutrition, however certain conditions increase that risk, such as economic problems and disorders or drugs that interfere with intake, metabolism or absorption of nutrients.[86,106]

Within certain limits, there is an adaptation to transient changes in energy intake through physiological and behavioral responses. Energy balance is maintained, and a new steady state is achieved.[95] However, adjustments to low or high energy intakes lead to biological and behavioral disadvantages.[95,111] These adjustments provided by the body are important and may even increase the chances of survival in times of energy imbalance, both excess and scarcity.[112] Both undernutrition and overnutrition cause the loss of energetic homeostasis that trigger cellular stress and coping response mechanisms to resolve the stress.[113] Stress response mechanisms initially promote adaptive strategies to recover homeostasis in the short-term but can become pathogenic due to long-term modification of cellular functions.[85,114] So, although acute fluctuations in the levels of energy intake being tolerated, chronic destabilization is deleterious.[115]

1.3.1. Caloric restriction

Diet and dietary habits have a major impact on quality of life, health, and longevity.[116] They are typically influenced by geographical, religious, ethics, and cultural choices. People often decide or are advised to modify and adapt their diet. There are several types of diets, in which a certain macronutrient (carbohydrates, fats, proteins) is increased or decreased or in which the amount of calories consumed *per day* is increased or decreased. The basic diets (general, light, liquid, reinforced liquid, ovolactovegetarian, vegetarian and vegan) are distinguished by the type of cooking, composition and texture, and can be associated with one or more variants: low microbial content, creamy, gluten free, reinforced in energy, restricted in energy, restricted in dietary fiber, restricted in lactose, restricted in potassium and restricted in salt.[117]

Several reasons lead people to adopt the energy restricted diets, usually designated by caloric restriction (CR), that is based in a reduction in the typical caloric intake, aiming to promote “undernutrition without malnutrition”. In other words, CR is a periodic feeding/fasting diet based on a lowering of the typical calorie consumption by a determinate percentage, while maintaining the necessary nutrients and vitamins to support a healthy life.[118,119] In animal experiments, CR is measured as a percentage of the feed given to an experimental group compared to the amount of food eaten by a control group feed without any restriction, i.e., *ad libitum* (AL), being provided as a single meal once *per day*, usually, at the same time of the day.[120,121]

It was first recognized in 1935 that dietary restricted rats exhibit increased lifespan.[122] Indeed, one of the reasons that lead to the popularity of CR diet is the fact that restricting food intake is widely considered beneficial for quality of life and lifespan, being that aging process is undoubtedly the single most significant contributor to disease and death.[123] Obesity or excessive ingestion of calories are linked to increased incidents of age-related pathologies such as cardiovascular disease, stroke, T2D and cancer.[124,125] It was suggested that CR treatment aiming weight loss and glycemic control can reverse some cases of T2D.[126,127] A study in which non-obese humans were subjected to CR also produced favorable alterations in physiological and behavioral outcomes related to aging. These results raise the possibility that, both obese and non-obese people, can adopt CR in an effort to increase lifespan and quality of life.[128] Another reason why people adopt a CR is associated with an intentional weight loss to promote improvements in some clinical complications, such as obesity.[129,130] Weight loss can also be looked for obese and non-obese people to increase self-esteem and improve health.[131]

Although there are no standard definitions, CR diets are essentially classified as: very-low-energy diets (VLED) in which are ingested less than 800 kilocalories (kcal) *per day*, mild-energy restriction (MER) which involves periods of VLED interchanged by periods of AL, i.e. a slight diet intervention method which provides energy less than AL energy intake but more than 1600 kcal *per day,* and there are low-energy diets (LED) in which are ingested 800–

1600 kcal *per day*.[132] Both VLED and LED are commonly used in clinical practice for the treatment of obesity and T2D but remains controversial if there is a weight maintenance advantage or disadvantage after using VLED compared to MER and LED.[133] Short-term VLED is superior to reduce of body weight, blood glucose and triglycerides levels when compared to LED and MER. However, after long-term follow-up, there is no obvious difference in weight loss between VLED and LED, but glycemic control is still more effective in VLED.[134] The rapid weight loss by VLED is inevitably followed by weight regain.[135] Despite that, the short-term weight loss has long-lasting benefits on glycemic control,[136] maybe due to improved insulin sensitivity remaining from weight loss.[137] The rapid weight loss also increases patient's confidence and the hunger after VLED intervention is attenuated.[138] One concern regarding to the weight loss methods are the possible adverse events. Despite VLED has been consistently shown to be safe for weight loss, a higher caloric intake, such LED, that resulted in similar long-term weight loss would provide a still higher degree of safety.[139]

Although CR have positive effects in health and lifespan, negative effects have also been reported. Severely restricting calories can slow down the metabolism and cause loss of muscle mass. This makes it more difficult to maintain your weight loss in the long term.[140,141] Despite being controversial, CR, when severe and with low amounts of carbs, can also lead to fatigue.[142] Furthermore, reduced fertility, especially in women, has been associated with CR. In fact, studies show that reproductive function is suppressed in women who eat 22–42% fewer calories than are needed to maintain their weight.[143] Researchers believe that severe CR may also affect men's reproductive function, but few studies related to this topic. Thus, it is noticeable that both positive and negative CR outcomes result from the impact of that dietary pattern on the individual body composition and hormonal profile and on other physiological aspects.[144]

1.4. Nutritional genomics

The completion of the Human Genome Project in 1990 has moved a wide step forward on the knowledge about the gene sequences. This was revolutionary since it allowed us, namely, to understand more about nutrition and how individuals differ in their response to nutrients. Nutritional genomics, which refers to the evolving study of gene-nutrient relationships, is not a single term that can be solely defined or explained, in fact it is combination of two broad areas - nutrigenomics and nutrigenetics (**Figure 2**).[145] Nutrigenomics, aims to understand how food components influence gene expression profile or transcriptome and how individual genetic variation affects the way an individual responds to nutrients present in food. It will identify the genes involved in physiological responses to diet and the genes in which small changes (e.g., the polymorphisms), may have significant nutritional consequences; and the influence of environmental factors, such as nutrition, on gene expression.[146] This facilitates the greater understanding of how nutrition affects metabolic pathways and determines the association between diet and diet-related diseases (such as obesity), allowing the understanding of the etiologic aspects of those diseases.[147] Nutrigenetics, the other nutritional genomics area, is based on the understanding of the gene-based differences in response to dietary components. It focuses on the interaction among genes and environmental factors, specifically bioactive components in food and how a diet interacts with the individual's genotype to influence the balance between health and disease. These individual differences may be at the level of single nucleotide polymorphisms (SNPs) rather than at the gene level. Thus, nutrients and genome interact at two levels: 1) nutrients can induce or repress gene expression thereby altering individual transcriptome and 2) SNPs can alter the bioactivity of important metabolic pathways and mediators and influence the ability of nutrients to interact with them. These two levels are tightly linked and have a vital role in the understanding of the effects of diet on an individual and eventually nutrigenomics will lead to evidence-based dietary intervention strategies for restoring health and for preventing diet-related disease.[148]

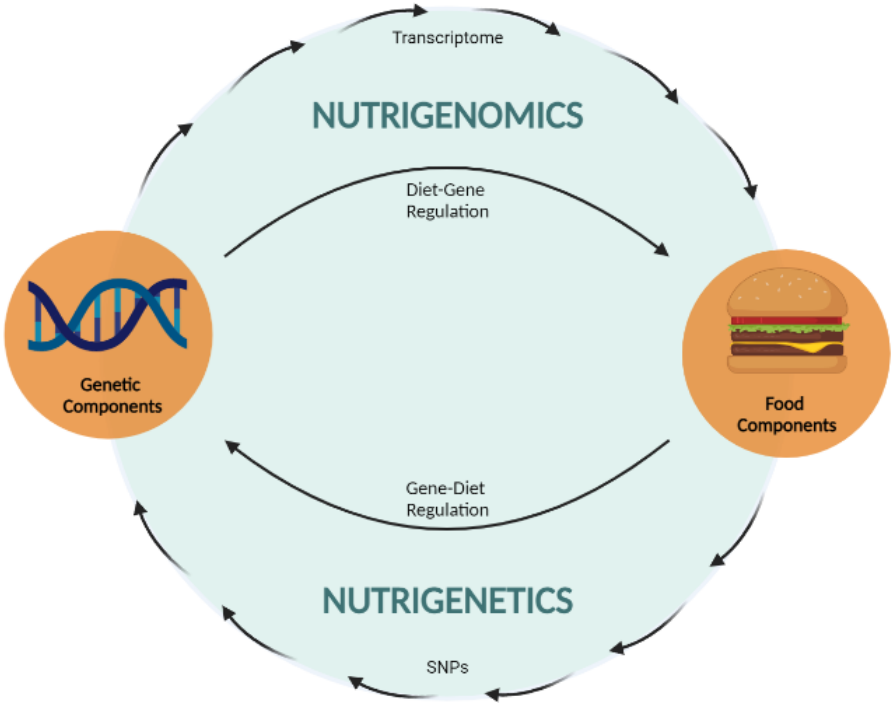


Figure 2 - Nutrigenetics and nutrigenomics. The study of how genetic variations such as SNPs among individuals affect their response to specific food components is defined as nutrigenetics. Nutrigenomics is based on the investigation of how food components modulate changes in gene expression profile or transcriptome. (**Abbreviations**: SNP, single nucleotide polymorphism)

The goal of the HapMap Project was to compare the genetic sequences of different individuals to identify chromosomal regions where genetic variants are shared, being that these genetic differences are known as SNPs, that could serve for the predication of an individual's response to certain factors such as drugs and susceptibility to diet components and dietary patterns. SNPs do not cause a disease, but they can help determine the likelihood that someone will develop a particular condition, for example obesity. Those SNPs, in or near to genes associated with targets of nutrient action such as receptors, enzymes or transporters could alter molecular pathways, might determine the properties and function of the proteins coded by those genes and consequently affect, through different ways, the energy homeostasis. Thus, being the weight regulated by neural mechanisms that modulate appetite and energy expenditure, the disruption of these pathways, due to some SNPs, can possibly lead to obesity.

Obesity arises when there is an imbalance between energy intake and energy expenditure, causing a positive energy balance. Family, twin and adoption studies suggested that the heritability of body weight ranges between 40% and 70% and given that estimated heritability. Hence, genetic approaches can be a useful tool to investigate the mechanisms involved in weight regulation and how those mechanisms are disrupted to contribute to obesity.[149] For instance, monogenic obesity results from a single defective gene on the autosomes that accounts for variations in energy requirements, fuel utilization, tissue metabolic activity, and taste preferences.[150] Nutrigenomics, to date, allowed the description of about 20 single gene disruptions that result in an autosomal form of obesity. Usually, these mutations are in or near to genes somehow associated with the leptin/melanocortin pathway in the CNS that is critical in the regulation of whole-body energy homeostasis, and obesity in these cases appears to be the result of increased appetite and diminished satiety.[151] These genes are classified as obesity-related genes (ORG) and subjects carrying specific SNPs in or near those ORG show a higher obesity susceptibility.[152] Recent technological advances and increases in scale and statistical rigor of genome-wide association studies (GWAS) allowed the identification of those common genetic variants, using body mass index (BMI) as a measure of adiposity.[153] The first wave of GWAS identified the fat mass and obesity-associated (*FTO*) gene, which until nowadays have one of the largest effect size. A second wave of GWAS confirmed the *FTO* and identified the melanocortin-4 receptor (*MC4R*) gene which

possesses a more moderate effect size. A third wave of GWAS identified several novel obesity susceptibility genes, among which there were transmembrane protein 18 (*TMEM18*) gene and glucosamine-6-phosphate deaminase 2 (*GNPDA2*) gene, which also have moderate effects sizes.[154] Because those genes are majority expressed in hypothalamus, liver and adipose tissue, it has been suggested that they have an important role in energetic homeostasis and body weight regulation.[155]

The response or adaptation to a dietary component or pattern depends in part upon the genotype of the individual.[156] The initial step in most obesity treatments is usually the establishment of an adequate CR to create a negative energy balance favoring weight loss.[157] It is evident that improved body weight through CR leads to a better quality of life, improving values related to lipid and glucose metabolism as well as oxidative status.[158,159] Genetic variations related to energy metabolism, physical activity, appetite control, and the utilization of dietary components play an important role in the response to nutritional interventions. In addition to the significant associations between specific polymorphisms and outcomes of CR – nutrigenetics - there is increasing evidence that energy intake influences gene (mRNA levels) expression – nutrigenomics.[160] In fact, CR has been shown to improve longevity and metabolism,[161] and one can hypothesize that these changes are mediated in part by a differential gene expression, namely those associated with energy homeostasis.

1.4.1. Fat mass and obesity-associated gene

Recent studies have revealed a strong association between common variants in the first intron of *FTO* and obesity in both children and adults, with nearly 16% of studied populations homozygous for the risk alleles.[162-164] As adults, these individuals weigh approximately 3 kg more than those homozygous for the low risk alleles as a result of a specific increase in fat mass.[163] The *FTO* gene was first cloned after identification of a fused-toe mutant mouse whose phenotype results from a 1.6-Mb deletion of six genes, including *FTO*.[165] *FTO* gene is located on the human chromosome 16q12.2[166] and is widely expressed in human tissues.[163] However, the highest levels are found in brain, mainly in hypothalamus, an area that plays a key role in the control of energy homeostasis.[167] *FTO* encodes to an alpha-ketoglutarate-dependent dioxygenase and belongs to the non-heme Fe(II)/α-ketoglutarate-dependent dioxygenase AlkB family that includes proteins involved in the oxidative demethylation of N-methylated DNA and RNA bases.

Due to the *FTO* function as a demethylase, it was suggested that it may play a role in metabolism by regulating the expression of some genes through modification of their methylation–demethylation state, therefore affecting the stability and function of targeted RNAs.[167,168] That occurs because increased *FTO* expression and/or activity causes a reduction of N^6-methyladenosine (m^6A) levels in that genes mRNA.[169] Several RNA modifications have been identified, among which the m^6A is the most prevalent.[170] The m^6A modification has become an aspect of interest due to the recognition of the prevalence and functional significance of internal mRNA modifications. It is also the first known modification of RNA that can be regulated by several effectors: “writers” that perform methylation, “readers” that recognize the methylation and “erasers”, like *FTO*, that remove methylation.[171,172] It was therefore proposed that *FTO* plays a role in the regulation of metabolism, possibly by altering gene expression in metabolically active tissues, namely in the liver.

There are at least three potential mechanisms by which *FTO* favor body mass gain: (i) modification of FTO protein expression; (ii) modification of its enzymatic activity; and (iii) abnormal control of the expression of other proteins that in turn modify energy homeostasis parameters.

It was suggested that metabolic signals, such as nutrients and hormones, regulate the expression of hepatic FTO, and altered FTO hepatic levels, in turn, affect glucose and lipid metabolism, probably through its demethylase activity.[163,173] It was demonstrated that *FTO* knockout improves glucose tolerance and insulin sensitivity in mice, suggesting its role in the glucose homeostasis regulation. Since the liver plays a major role in the regulation of

glucose and lipid metabolism, it was hypothesized that hepatic FTO participates in cell metabolism.[168] FTO regulates hepatic lipid metabolism once it seem to have the capacity to alter the methylation state of genes involved namely in fatty acid oxidation, lipolysis and *de novo* lipogenesis. Increased FTO activity reduces the lipolytic genes expression, resulting in reduced fatty acid oxidation and lipolysis. It also stimulates the lipogenic genes expression, increasing *de novo* lipogenesis.[168] This effects consequently increase lipid accumulation. Reduced FTO activity causes the opposite effect (**Figure 3 (1)**).[174-176] Relatively to glucose metabolism, increased hepatic FTO levels are associated with increased availability of the substrate for gluconeogenesis, increased fasting glucose and insulin levels, and impaired glucose tolerance in mice.[177] Lack of FTO reverses hyperglycemia, improves glucose tolerance and insulin sensitivity in normal and obese/diabetic mice.[178] These data suggest that hepatic FTO also participates in the regulation of whole body glucose homeostasis, at least partly, through the regulation of hepatic gluconeogenic gene expression. FTO participates in the regulation of gluconeogenic gene expression by altering the activity of and interacting with transcription factors that regulate glucose-6-phosphatase and phosphoenolpyruvate carboxykinase 1 gene expression, which encode for gluconeogenic enzymes. Thus, increased blood glucose levels and glucose-induced insulin secretion that reduce hepatic FTO expression, leads to reduced gluconeogenesis. Low levels of blood glucose and insulin that stimulate hepatic FTO expression cause the opposite effect (**Figure 3, (1)**).[168] All these findings support the idea that obesity-associated FTO variants may be associated with increased FTO expression and/or activity in the liver.

It was proposed that hepatic FTO is involved in the regulation of blood glucose levels as part of a negative feedback loop. Thus, increased blood glucose levels and glucose-induced insulin secretion may cause a reduction of hepatic FTO expression, leading to reduced gluconeogenic gene expression. Conversely, low levels of blood glucose and insulin, observed during fasting, stimulate hepatic FTO expression, leading to the increased expression of gluconeogenic genes. Impairments in these regulatory mechanisms may contribute to the pathogenesis of metabolic diseases (such as T2D) due to stimulation of *de novo* lipogenesis and inhibition of lipolysis and fatty acid oxidation, leading to an abnormal increase in hepatic glucose production and triglyceride deposition (**Figure 3, (2)**).

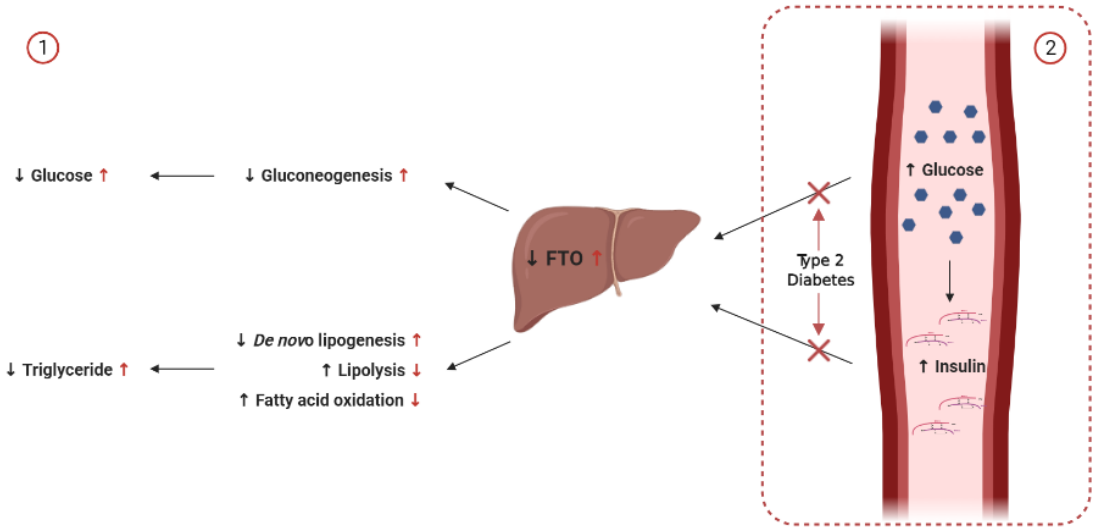


Figure 3 – Hepatic FTO regulation of lipid and glucose metabolism. **(1)** Role of FTO in the nutritional and hormonal regulation of hepatic glucose and lipid metabolism under decreased (black arrows of which those pointing upwards mean increase and those pointing downwards mean decrease) and increased (red arrows of which those pointing upwards mean increase and those pointing downwards mean decrease) hepatic FTO activity. **(2)** Impairments in glucose and insulin regulation of FTO expression may be responsible for an increase in hepatic FTO expression. Increased hepatic FTO expression stimulates gluconeogenesis and *de novo* lipogenesis and inhibits lipolysis and fatty acid oxidation, leading to abnormally increased hepatic glucose production and triglyceride deposition. (**Abbreviations:** FTO, fat mass and obesity-associated; **Adapted from:** Mizuno et al. (2018)[168])

Evidence for a link between altered FTO enzymatic activity and obesity was also described. There are some mRNAs upregulated via FTO-mediated m^{6}A demethylation. For example, individuals homozygous for the rs9939609 SNP risk allele seem to present dysregulated circulating levels of the orexigenic hormone acyl-ghrelin, attenuated postprandial appetite reduction and increased appeal to high-calorie food images. In cell models, overexpression of *FTO* induced the production of ghrelin, that was accompanied by reduced ghrelin pre-mRNA m^{6}A methylation. Furthermore, in peripheral blood cells of homozygous individuals for the risk allele, the expression of ghrelin was increased and the m^{6}A methylation of ghrelin mRNA was reduced.[179] Another example of the link between altered FTO enzymatic activity and obesity comes from the evidence that FTO regulates alternative splicing patterns of several genes, including two splice variants of the adipogenesis-related gene runt-related transcription factor 1 (Runx1t1). The removal of m^{6}A of the Runx1t1 mRNA by FTO, minimizes the recruitment of the splicing regulatory protein serine and arginine rich splicing factor 2 (Srsf2). In its absence there is a skipping of Runx1t1 mRNA exon 6, resulting in an increase of the short product variant, that induces adipogenesis, with a nearly complete elimination of the long product variant. Conversely, m^{6}A-containing transcripts, enhanced by FTO knockdown, are more likely to recruit Srsf2 and induce exon 6 inclusion, thereby inhibiting pre-adipocyte differentiation. (**Figure 4**).[176,180,181]

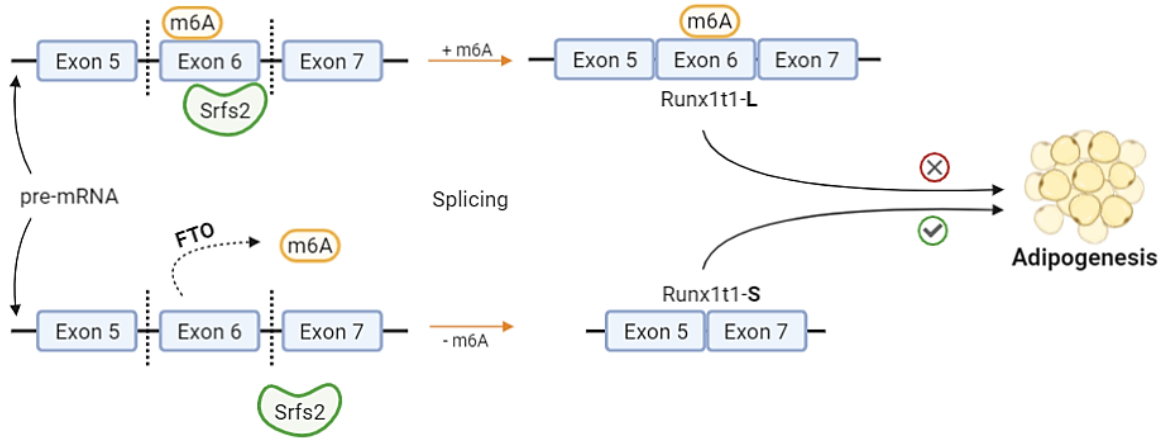


Figure 4 - Alternative splicing of Runx1t1 mediated by FTO-dependent m^{6}A demethylation. The removal of m^{6}A by FTO demethylation leads to a prevalence of the short product of Runx1t1, which enhances (green tick) pre-adipocyte differentiation. Conversely, if no FTO exists, this results in prevalence of the long product of Runx1t1, which inhibits (red cross) pre-adipocyte differentiation. (**Abbreviations**: FTO, fat mass and obesity-associated; m^{6}A, N^6-methyladenosine; Runx1t1-L, long product of runt-related transcription; Runx1t1-S, short product of runt-related transcription factor; **Adapted from**: Yang et al. (2017)[181])

Another potential mechanism by which FTO risk alleles favor body mass gain is through abnormal control of the expression of other genes that encode proteins that in turn modify energetic homeostasis parameters. The first evidence pointed for the product of the FTO neighbor gene retinitis pigmentosa GTPase regulator-interacting protein 1-like (RPGRI1L), that was a transcriptional start site is near to the 5' end of FTO gene. In the presence of some FTO variants (rs17817449 and rs8050136) the site for the transcription of the transcription factor CUT-like homeobox 1 (CUX1) present in FTO intron 1, is blocked leading to a reduced expression of both FTO and RPGRI1L. This transcriptional abnormality leads to reduced trafficking of leptin receptors and decreased hypothalamic response to leptin, that consequently promote an increased food intake by stimulating the orexigenic AgRP/NPY neurons (**Figure 5 (1)**).[182,183] Another gene-expression regulatory mechanism links *FTO* to *iroquois-class homeobox protein (IRX) 3*, although the mechanism is different from that described previously for other genes. IRX3 is expressed in the hypothalamus and is known to regulate whole body energy expenditure through sympathetic innervation of the brown adipose tissue.[184] The noncoding regions of the *FTO* gene are functionally connected with the promoters of *IRX3* as a long-range regulatory element and risk alleles within the regulatory region can affect *IRX3* expression.[184] Some FTO SNPs, namely rs1421085, interact with the promoters of *IRX3* gene, increasing its expression in pre-

adipocytes.[185] This leads to a reduction in mitochondrial thermogenesis that results in body energy imbalance, lipid accumulation and subsequent obesity without a change in physical activity or appetite. There is also an alteration in the adipocytes function, which shift from energy-dissipating beige adipocytes to energy-storing white adipocytes. (**Figure 5 (2)**).[181,186,187]

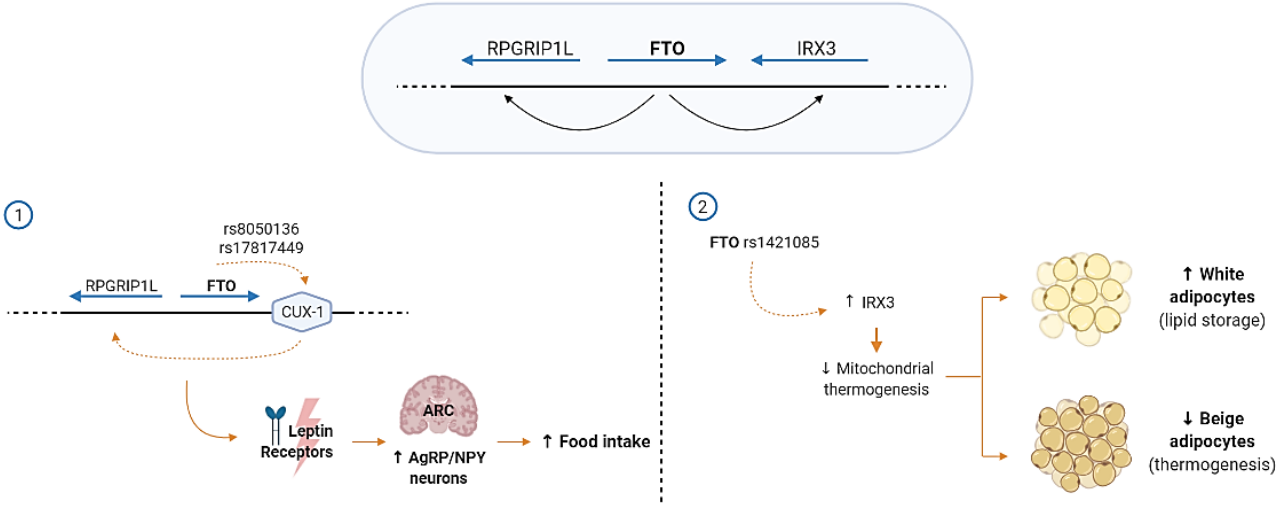


Figure 5 – FTO gene regulation through interactions with neighboring genes. (1) The binding site for the transcription factor CUX1 located in FTO intron 1 is blocked by some FTO variants which downregulate both FTO and RPGRI1L genes, affecting leptin receptors and consequently, food intake. **(2)** FTO rs1421085 increase IRX3 and IRX5 genes expression, reducing the mitochondrial heat production and increasing white adipocytes mass. Arrows pointing upwards mean increase and arrows pointing downwards mean decrease. (**Abbreviations**: AgRP, agouti-related peptide; ARC, arcuate nucleus; CUX-1, CUT-like homeobox 1; FTO, fat mass and obesity-associated; IRX3, Iroquois Homeobox 3; IRX5, Iroquois Homeobox 5; NPY, neuropeptide Y; RPGRIP1L, retinitis pigmentosa GTPase regulator-interacting protein 1-like; **Adapted from**: Yang et al. (2017)[181])

The transformation of the spermatids during spermiogenesis comprises a condensation of nuclear DNA. This complicated process involves a wave of transcription that generates mRNAs needed during later stages when transcription has been largely shut down due to inaccessible DNA. This distinct mRNA processing is vital for the accurate progression of the spermiogenesis.[188] Recently were found two missense mutations (Ser256Asn and Cys326Ser) in the *FTO* gene with a potentially detrimental effect on the functionality of FTO methylation activity, as well as a genetic variant (rs62033438) that is associated with reduced semen quality, which suggests that aberrant demethylation of mRNA by FTO is a factor involved in reduced male fertility.[189] In fact, some studies have revealed that m^6A RNA modification is crucial for male germline development, especially mammalian spermatogenesis.[190,191] However, there is scarce knowledge about the role of m^6A modification in testicular injury or in oxidative stress injury induced by weight loss interventions.

1.4.2. Melanocortin-4 receptor gene

The melanocortin system consists of several agonists, two antagonists, and five receptors. The agonists, including α-MSH, β-MSH, γ-MSH, ACTH, γ3-MSH and desacetyl-α-MSH, are derived from tissue-specific post-translational processing of a pre-prohormone, POMC.[192] Five melanocortin receptors (MC1R-MC5R), a family of five G-protein-coupled receptors, mediate the actions of melanocortins.[193] In the melanocortin system, MC4R, G-protein-coupled seven transmembrane receptor, plays an essential role in controlling both food intake and energy expenditure and consequently regulating body weight.[115] That receptor is encoded by *MC4R* gene**,** located on the human chromosome 18q21.3.[193]

MC4R is implicated in the regulation of body weight, being responsible for controlling the balance between α-MSH and AgRP, secreted by anorexigenic and orexigenic neurons, respectively (**Figure 6**). MC4R is activated by α-MSH, leading to a negative balance that promotes satiety, energy expenditure and consequently weight loss.[115] MC4R responds to stimulation of α-MSH via activation of the stimulatory G protein.[194] When MC4R is activated, a couple of reactions lead to activation of the adenylyl cyclase (AC) that convert adenosine triphosphate (ATP) into cyclic adenosin monophosphate (cAMP) and subsequently activate protein kinase A (PKA). Activated PKA, in turn, leads to phosphorylation of cellular proteins that cause several cellular responses, both in PVN neurons and outside them, related to food intake and energy expenditure, control of lipid and glucose metabolism, linear growth and cardiovascular function.[193,195] On the other hand, AgRP acts as an MC4R antagonist, inducing a positive balance, that leads to increased food intake, energy conservation and weight gain.[115] The melanocortin system is downstream modulated by signals from adipose tissue (leptin), gastrointestinal tract (ghrelin, CCK, PYY and GLP-1) and pancreas (insulin). So, this pathway may be implicated in leptin, insulin and GLP-1 (since those hormones stimulate POMC/CART and inhibits NPY/AgRP neurons) and in ghrelin effects (since NPY/AgRP neurons are stimulated by it).[195,196] By being important in hypothalamic body weight regulation, MC4R is a potential target to combat obesity and to induce weight loss in non-obese people.[195]

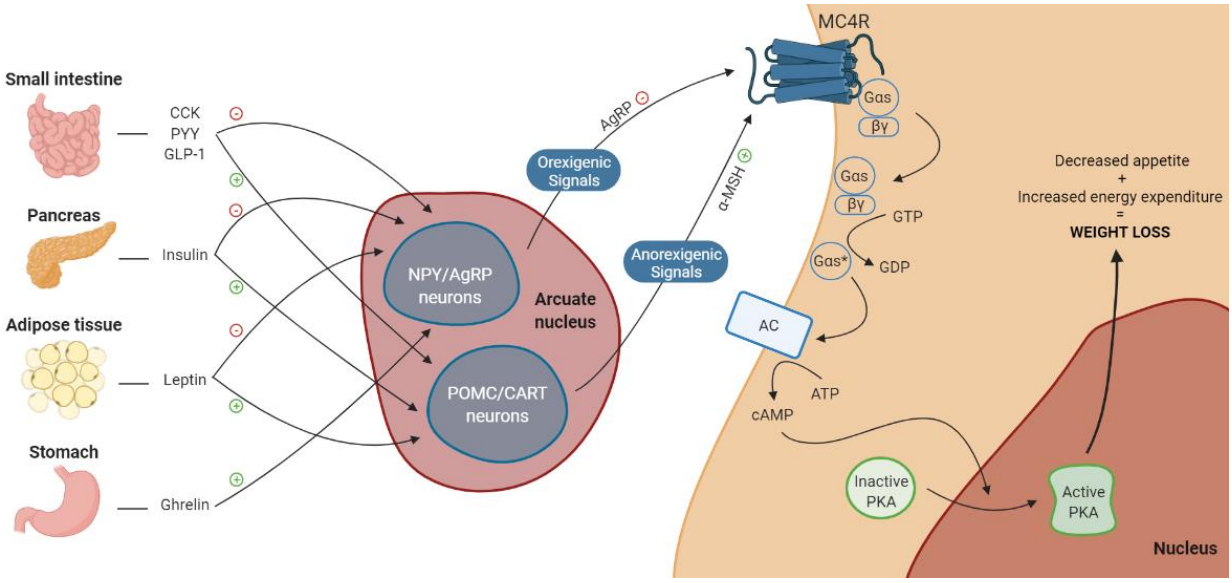


Figure 6 – MC4R involvement in feeding behavior and body weight regulation. Downstream signals from adipose tissue (leptin) or gastrointestinal tract (ghrelin, CCK, PYY and GLP-1) and pancreas (insulin) modulate ("green -" means inhibition; "red +" means stimulation) MC4R, a G-protein coupled receptor that stimulate a cAMP signaling pathway. This leads to triggered responses associated with decreased appetite and increased energy expenditure that ultimately result in weight loss. (**Abbreviations**: ATP, adenosine triphosphate; AC, adenylate cyclase; AgRP, agouti-related peptide; α-MSH, alpha-melanocyte-stimulating hormone; CCK, cholecystokinin; CART, cocaine- and amphetamine-regulated transcript; cAMP, cyclic adenosine monophosphate; Gas, G protein alpha subunit; βγ, G protein beta and gamma subunit; GLP-1, glucagon-like peptide-1; GDP, guanosine diphosphate; GTP, guanosine triphosphate; MC4R, melanocortin 4 receptor; NPY, neuropeptide Y; PPY, peptide YY; POMC, proopiomelanocortin; PKA, protein kinase A; **Adapted from**: Yang et al. (2017)[197])

The first evidence that melanocortinergic pathway is involved in feeding and body weight regulation came from studies in rodents in which the intracerebroventricular administration of α-MSH led to suppression of food intake, revealing the importance of the MC4R in the energetic homeostasis regulation.[198,199] In addition, was shown that intracerebroventricular administration of melanotan II (MTII), an α-MSH analogue, inhibits the hyperphagia and that effect was blocked by coadministration of SHU9119, an MC4R antagonist.[200] Administration of these compounds directly into PVN achieved more potent alterations in food intake, suggesting that neurons in the PVN, that express high levels of MC4R, are primary sites of MC4R action in regulation of feeding behavior.[201] Studies with *MC4R*

knockout mice provided the definitive evidence that MC4R is critical to regulate energetic homeostasis. Homozygous *MC4R* knockout mice display obesity with hyperphagia, hyperglycemia, hyperinsulinemia, increased linear growth, decreased systolic and diastolic blood pressure and decreased energy expenditure.[202-204] *MC4R* knockout mice also do not respond to the stimulation of the MC4R agonist MTII effects.[205] After the description of the phenotype of *MC4R* knockout mice, functionally relevant heterozygous MC4R frameshift mutations were reported in obese humans and since then more than 200 mutations were identified so far.[195] Indeed, MC4R deficiency is the commonest monogenic form of obesity.[206]

Van der Ploeg et al. shown that the *MC4R* is expressed in penile tissues.[207] They also demonstrated that MC4R is involved in male reproductive function, most likely through neuronal circuitry in spinal cord erectile centers and somatosensory afferent nerve terminals of the penis. *MC4R* knockout mice are reported to have reduce sexual motivation, reduced ejaculation efficiency and erectile dysfunction.[207] Another study found that a MC4R agonist, bremelanotide, increased satisfaction levels in men who were taking Viagra.[208] Furthermore, the α-MSH analogue, MTII, induce erection in men and SHU9119 blocks this response.[209] Recently was found that the expression of MC4R on single-minded homolog 1 (Sim1) neurons appears to be essential in the regulation of male sexual behavior, once mice with blocked MC4R expression manifest an inability to reach ejaculation. These sexual defects were reversed only due to the presence of MC4R in Sim1 neurons, suggesting that MC4R expression in those neurons are part of the neural circuit underlying male sexual function.[210]

1.4.3. Glucosamine-6-phosphate deaminase 2 gene

The *glucosamine-6-phosphate deaminase 2* (*GNPDA2*) gene is located on the human chromosome 4p12 and is highly expressed in hypothalamus, alluding an influence on energy balance.[211,212] *GNPDA2* encodes an allosteric enzyme that catalysis the deamination of the glucosamine-6-phosphate (GlcN6P) into fructose-6-phosphate (F6P) in the hexosamine signaling pathway (HSP), one of the main nutrient-sensing pathways.[211,213] GNPDA2 is a counter-enzyme for glutamine:fructose-6-aminotransferase (GFAT), being that the generation of GlcN6P from Fru6P, catalyzed by GFAT, is the first reaction of the HSP (**Figure 7**).[214]

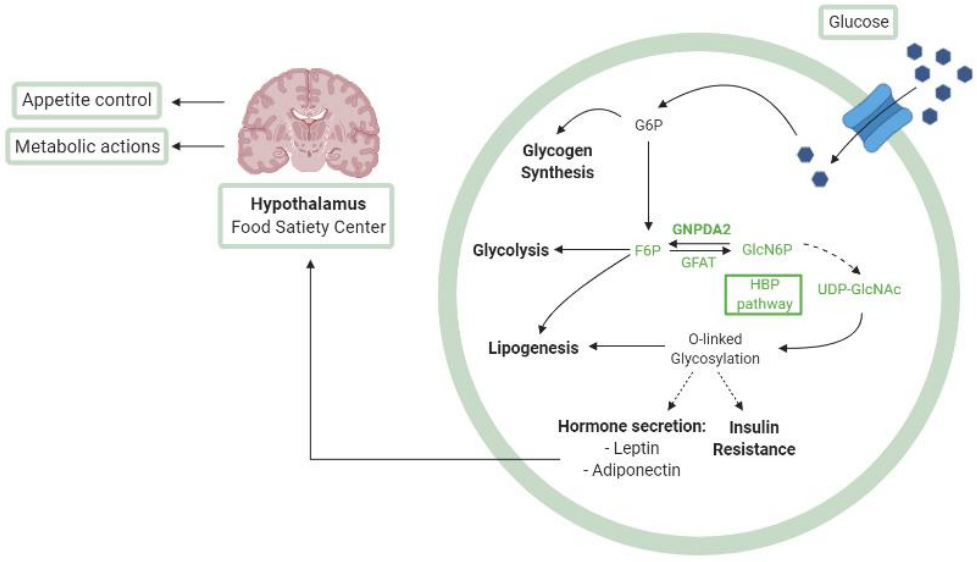


Figure 7 – GNPDA2 role in hexosamine biosynthesis pathway. GNPDA2 participates in hexosamine biosynthesis pathway promoting the deamination of GlcN6P into Fru6P. This pathway is a glucose sensor coupled to a transductional cascade that directly regulates intracellular fuel metabolism, controls glucose metabolism by modulating insulin signaling, and facilitates the release of leptin and adiponectin from adipose tissue. In the central nervous system, leptin acts to influence appetite and modulate neuroendocrine secretion, thereby altering nutrient utilization in various peripheral tissues. (**Abbreviations:** F6P, fructose-6-phosphate; GlcN6P, glucosamine-6-

phosphate; GNPDA2, glucosamine-6-phosphate deaminase 2; G6P, glucose-6-phosphate; GFAT, glutamine:fructose-6-aminotransferase; UDP-GlcNAc, UDP-N-acetylglucosamine; **Adapted from**: Marshall et al. (2006))

Hyperglycemic conditions promote the flux of excess glucose through the hexosamine biosynthetic pathway (HBP).[215,216] Enhanced flux into the HBP begins with glucose uptake and the rapid phosphorylation of glucose to glucose-6-phosphate (G6P). G6P can then be routed to the glycogen biosynthesis pathway or converted to F6P and subsequently catabolized through the glycolytic pathway. Although these pathways represent the major routes for most of the glucose, a small percentage of incoming glucose can be converted to GlcN6P by GFAT, the first and rate-limiting enzyme of the HBP. Once formed, GlcN6P undergoes a series of rapid conversions to UDP-N-acetylglucosamine (UDP-GlcNAc).[217] UDP-GlcNAc is a high-energy substrate used to covalently modify various proteins through the addition of a single monosaccharide (GlcNAc) onto serine or threonine residues. The enzyme catalyzing O-linked glycosylation is UDP–Nacetylglucosaminyl transferase (OGT). These O-GlcNAc modifications has the ability to change the functions many cytoplasmic, mitochondrial and nuclear proteins, including transcription factors.[218] Since the HBP utilizes major macromolecules such as nucleotides, amino acids, carbohydrates, and lipids to generate UDP-GlcNAc, cells may use it as a "sensor" of energy availability coupled to a biological transduction system that desensitizes the glucose transport system as the rate of glucose uptake exceeds the capacity of the major glucose-using pathways.[219] However, the metabolic flux is not always entirely in this direction, since the effects of GFAT are balanced by GNPDA2 that catalysis the conversion of G6P into F6P. However, GNPDA2 can also catalyze the conversion of F6P into G6P, being that the direction of the reaction catalyzed by GNPDA2 could be controlled by the content of its substrates. Thus, GNPDA2 can both produce UDP-GlcNAc or reduce its content.[220]

The elevated UDP-GlcNAc levels enhances O-glycosylation of transcription factors that regulate the expression of genes encoding proteins involved in the insulin-responsive glucose transport system control, being that O-linked protein glycosylation is linked to the induction of insulin resistance.[217,221] Regulation of lipid metabolism appears to constitute another downstream action of the HBP. Enhanced flux through the HSP increases triglyceride synthesis through up-regulation of lipogenic enzymes.[211,222] Furthermore, enhanced hexosamine flux increases the rate of glycogen biosynthesis that is mediated by the intracellular accumulation of G6P and by the glycogen synthase activation.[215] This coordinated regulatory response under hyperglycemic conditions makes sense, since overall glucose uptake would be reduced due to development of insulin resistance, and the excess incoming glucose would be stored through lipogenesis and glycogenesis.[215] In addition, HBP convey nutrient status of adipose tissue to brain, due to regulation of leptin and adiponectin secretion. Leptin exerts multiple behavioral, hormonal, and metabolic actions mainly through its interaction with specific hypothalamic receptors, playing a key role in controlling body weight by acting on food satiety centers in the hypothalamus. Many of leptin's effects attempt to limit excessive storage of energy into lipid stores. This is accomplished through induction of satiety, increased energy expenditure, and long-term control of body weight.[221,223] In addition to affecting the CNS, the combined actions of leptin and adiponectin also affect peripheral tissues, such as liver and muscle, promoting the increased fatty acid oxidation and insulin sensitivity while decreasing hepatic glucose and triglyceride synthesis and the gluconeogenesis.[224] So, under hyperglycemic conditions, there is a reduced nutrient intake, due to decreased appetite at the CNS level, and increased whole-body fuel use and storage at the peripheral tissue level. Thus, GNPDA2 is involved in a pathway highly associated with energy homeostasis (**Figure 7**).[221,223,225] Indeed, it was described that high fat diet-induced obesity is associated with down-regulated expression of the hypothalamic *GNPDA2*.[216]

Wu et al. shown that after overexpression of GNPDA2 enhanced accumulation of lipid droplets, i.e. adipogenesis. Meanwhile, knocking down the gene suppressed adipogenesis. It was also demonstrated that the deficiency of GNPDA2 increased the concentration of IL-1β, IL-8, resistin, MCP-1, and TNF-α, and decreased the concentration of leptin and adiponectin. The opposite was observed with GNPDA2 overexpression. It suggests those inflammatory factors and adipocytokines may mediate the effect of GNPDA2 on adipogenesis.[213] In addition,

GNPDA2 overexpression also upregulated the mRNA expression level of marker genes of adipocyte, such as peroxisome proliferator-activated receptor gamma (PPARG) and signal transducer and activator of transcription 5 (STAT5), that play a central role in the regulation of adipocyte differentiation and metabolism, being highly expressed in adipose tissue. GNPDA2 knockdown downregulated the expression of those genes.[213,226] Furthermore, some genes affected by GNPDA2 have functions in fatty acid metabolic process, regulation of lipid metabolic process, lipid modification and localization, adipocyte differentiation, regulation of lipid storage, carbohydrate homeostasis and response to insulin, reinforcing that GNPDA2 may be critical for lipid and glucose metabolism.[213]

It was also suggested that the risk allele of the rs10938397 SNP may abolish binding sites for transcription factors myelin transcription factor 1 (MYT1),[227] that is expressed in brain and in endocrine cells of pancreas.[228] Pancreatic *MYT1* knockout mice showed abnormal endocrine differentiation, glucose intolerance, and attenuated glucose-induced insulin secretion, suggesting that GNPDA2 may regulate the differentiation and function of the endocrine pancreas through MYT1.[228,229]

To date few studies have focused on the impact of *GNPDA2* in male reproductive function. Despite that the gene and the respective protein were already identified in human Sertoli cells (SCs). Furthermore, in that study, was observed that *GNPDA2* expression in SCs increase after exposure to leptin concentrations similar to those found as circulating levels in individuals with morbid obesity. Considering the function of GNPDA2, that increase is likely to be responsible for promoting the entrance of F6P into glycolysis and pyruvate synthesis. In male subjects with obesity, presenting high circulating leptin levels, this can be a compensatory phenomenon elicited by decreased LDH activity and acetate production.[230]

It is also known that two GNPDA2 SNPs (rs4343755 and rs4695097) were significantly associated with the development of oligozoospermia (low sperm concentration in semen) and azoospermia (no measurable sperm concentration in semen) in males with idiopathic infertility.[231]

1.4.4. Transmembrane protein 18 gene

The *transmembrane protein 18* (*TMEM18*) gene is located on an intergenic region of the human chromosome 2p25.3[232] and is widely expressed but its expression levels vary between different tissues.[233] This gene is abundant in the hypothalamus and brainstem, although also expressed at high levels in other tissues such as skeletal muscle, liver, heart, kidney, reproductive organs and adipose tissue.[155,233,234] *TMEM18* has three transcripts of which the longest one is translated into a 140 amino acid-long protein that has three transmembrane α-helices. TMEM18 has the N-terminal and second loop probably face the inside of the nucleus, whereas the first loop and the C-terminal, that contain a coiled-coil domain, are oriented towards the cytoplasm. A coiled-coil domain is common among DNA or RNA binding proteins, such a transcription factors.[233]

The functional properties of TMEM18 still remain highly obscure, however it was proposed that it exerts it's effects in manifestation of obesity through transcriptional regulation of targets related either to appetite and energy balance at its central sites of action (hypothalamus and other parts of the brain critical for metabolism) or via an impact on adipogenesis in the periphery,[235] due to its capacity for DNA binding, through the terminal positive charged C-terminus domain.[236] That is in line with the demonstration of its expression in the brain, namely hypothalamus and brainstem, which are regions that play a crucial role in the regulation of the food intake or energy expenditure.[155,233] In Gutierrez-Aguilar et al. study, performed in rats, it was demonstrated that rats under high fat diet had downregulated transcripts of *TMEM18* in hypothalamus.[216] Larder et al. demonstrated that *TMEM18* knockout male mice, on normal diet, had increased body weight due to an increased fat and lean mass when compared to wild-type. This phenotype is more pronounced on an HFD, where weight gain is driven by hyperphagia. So, loss of *TMEM18* expression increases BMI because of increased food intake. In contrast, *TMEM18* overexpressed mice was observed a reduced weight gain due to a significant reduction in food intake and an increased energy expenditure.[232] These

results suggest a potential role of *TMEM18* in the regulation of energy balance. However, the central regulation of energy intake by *TMEM18* is still controversial. Landgraf et al. did not see an association of *TMEM18* obesity risk variants with the behavioral traits of hunger, disinhibition, or food addiction in children or adults. Furthermore, in zebrafish, *TMEM18* deficiency did not affect food intake.[237] Another previous study from a Greek childhood cohort indicate that the *TMEM18* obesity risk genotype is not associated with energy intake.[238] This lack of change in reported gene expression might be because *TMEM18* is so widely expressed that *TMEM18* levels in subpopulations of neurons are significantly altered, yet overall expression remains unchanged.[233]

A further possibility is that given the distribution of *TMEM18* among peripheral tissues, the relation of this gene to obesity is not only via a centrally mediated action, being recently proposed *TMEM18* potential role at the hub of a network in adipose tissue related to adipogenesis. Supporting this alternative view, gene expression of *TMEM18* in mice was reduced in adipose tissue during high-fat feeding, but brain levels were unaffected.[239] In a study from Bernhard et al. TMEM18 protein knockdown significantly reduced adipogenesis, which was concordant with the reduced expression of the gene itself.[234] Using patients stratified into non-obese and obese groups, it was observed that *TMEM18* expression was decreased in the adipose tissue of obese patients, and negatively correlated with anthropometric variables of body fat mass, subcutaneous adipocyte size, variables of insulin and lipid metabolism. Landgraf et al. observed that *TMEM18* expression was reduced in adipocytes of children with obesity compared with lean children to a similar extent as shown for adults by Bernhard et al..[237] All those observations indicated a role for that gene in adipogenesis, probably as a transcriptional regulator by acting as a DNA binding-protein for other downstream genes, being a plausible mediator of genetic variation on obesity and T2D.[234]

The differential expression of TMEM18 between obese and lean adipose tissue indicate a role of this gene in adipocyte formation and expression of adipogenic marker genes **(Figure 8)**.[234,237] It was proposed that *TMEM18* might be involved in *PPARG* activation by acting as a transcription factor.[236] *PPARG* is a transcription factor known as a master regulator of adipogenesis,[240] driving expression of critical genes necessary for adipocyte differentiation and function. *PPARG* is activated with the start of adipogenesis and it is increased in the adipose tissue of obese humans.[241] Landgraf et al. shown that *TMEM18* overexpression doubled the *PPARG1* promoter activity. *TMEM18* knockdown promoted the opposite effects, with a reduction *PPARG1* expression and a downregulation of their target genes, that are involved in glucose and lipid metabolism. There is, therefore, a positive correlation between *TMEM18* and *PPARG1* expression, being hypothesized that TMEM18 is an upstream regulator of PPARG signaling, due to its positive charged C-terminal domain that binds DNA and suppresses transcription.[236,237] Mutated mice *TMEM18* lacking the C-terminal domain did not bind to either single or double-stranded DNA, showing that the C terminus is required for *TMEM18* bind to DNA.[236] It was also detected a correlation of both lower *TMEM18* and *PPARG1* expression with increased adipocyte size, lower adiponectin levels and insulin sensitivity. The downregulation of *PPARG1* activity, related to obesity-associated adipocyte hypertrophy, may be driven by a pro-inflammatory stimulus, since adipocyte decreased *TMEM18* expression is accompanied by an increase in macrophage number.[237,242] The coherent downregulation of *TMEM18* and *PPARG1* in response to inflammatory stimuli indicates that TMEM18 is a mediator of inflammation-induced dysregulation of *PPARG1* associated with adipocyte tissue dysfunction and metabolic alterations in the obese state.[237]

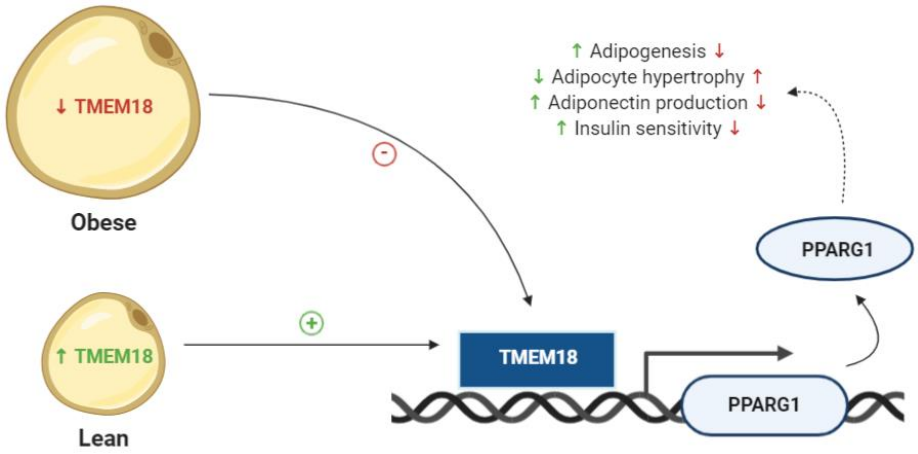


Figure 8 – TMEM18 role in adipogenesis and its involvement in regulation of PPARG1 expression. TMEM18 activates PPARG by upregulating PPARG1 promoter activity and that interaction is dysregulated with obesity and adipose tissue dysfunction. This link affects adipogenesis once PPARG1 is well-characterized regulator of adipocyte development. (**Abbreviations:** PPARG1, peroxisome proliferator-activated receptor gamma; TMEM18, transmembrane protein 18; **Adapted from**: Landgraf et al. (2020)[237])

Given its widespread distribution, *TMEM18* has been implicated in several other disease states aside from obesity, notably in relation to cancer. Was suggested that *TMEM18* act as an important sequence-specific transcription factor involved in the modulation of neural stem cell migration capacity.[243] NCS have an intrinsic tropism for sites of brain injuries, including gliomas, acting as glioma cell tracking.[244] Overexpression of *TMEM18* was found to provide NSCs and neural precursors an increased migration capacity toward glioblastoma cells, due to the increasement of the sensitivity of NSCs to appropriate signals that stimulate cell migration. Functional inactivation of the *TMEM18* resulted in almost complete loss of their migration activity. Thus, *TMEM18* is a novel cell migration modulator, being that the overexpression of this protein could be favorably used in NSC-based glioma therapy.[243]

Relatively to male reproductive function, Pereira et al. found that *TMEM18* and the respective protein were expressed in human SCs, mainly in the cytoplasm.[230] Furthermore, *PPARG* expression was found to be expressed in human testes.[245] This together with the observations that *PPARG* knockout leads to lipid metabolism-associated genes downregulation,[246] raised the hypothesis that TMEM18 is likely to have a regulatory role in fatty-acids metabolism in human SCs.[230] Furthermore, *TMEM18* expression increased after exposure to ghrelin. Thus, *TMEM18* can be a mediator of ghrelin effects on human SCs mitochondrial activity, by promoting fatty-acid metabolism modulation in these cells.[230]

2. Objectives

Caloric restriction, due to its negative energy balance, is a dietary intervention that has been used as a mean to achieve weight loss, both by obese people, as part of the treatment, and by non-obese individuals to improve self-esteem and their own health. GLP-1 is an endogenous hormone incretin, whose action is related to glucose metabolism. It reduces secretion of glucagon by pancreatic alpha cells and, consequently, hepatic gluconeogenesis, in addition to stimulating insulin excretion by beta cells, contributing to the blood glucose control. Concomitantly, it delays gastric emptying and decreases the appetite. Despite the increased popularity of those interventions, they remain poorly investigated, particularly its impact on male reproductive function. The male reproductive function depends on a balanced energy homeostasis, namely for processes such as spermatogenesis. That energy homeostasis has been related to some genes linked to energy metabolism control, namely the obesity-related genes, such as *FTO*, *MC4R*, *GNPDA2* and *TMEM18* that are associated with a predisposition to conditions like obesity. Alterations in energy needs, body weight as well as the rewarding value of food, promoted by nutritional approaches, are known to affect expression of genes involved in the regulation of the energy homeostasis and consequently to the body weight. Thus, the main objective of this project is to study, using an animal model, the potential of CR and GLP-1 administration in the regulation of the expression of the afore mentioned genes in the testes and sperm of Wistar rats and determine if there is an impact on male reproductive function. For that, three different experimental groups will be established: 1) rats fed *ad libitum* diet; 2) rats subjected to chronic GLP-1 intraperitoneal administration; and 3) rats fed under caloric restriction (30%). The following specific objectives were established: (i) identification of the transcripts presence of the four selected genes (*FTO*, *MC4R*, *GNPDA2* and *TMEM18*) in Wistar rats' testes and sperm; (ii) evaluation of the transcript level of the four selected genes in Wistar rats testes and sperm subjected to two nutritional interventions: caloric restriction and GLP-1 administration; and (iii) establishment of correlations between the transcript level of the four selected genes and sperm quality, hormonal and physiological parameters of the Wistar rats subjected to the two nutritional interventions: caloric restriction and GLP-1 administration.

3. Material and Methods

3.1. Animal model and experimental designs

The animal model and experimental designs were performed as previously described by Morais et al.[247] Briefly, sixteen adult male Wistar rats with 6-week-old (150-200 g) (Charles River Laboratories, Barcelona, Spain) were randomly selected from our colony. The rats were housed in our accredited animal colony and maintained with food and water *AL* (Diet Standard, Mucedola, Milan, Italy) in a constant room temperature (20 ± 2 ºC) on a 12 h cycle of artificial lightning. All animal experiments were performed according to the Animal Research: Reporting of In Vivo Experiments guidelines, and the study was licensed by the Portuguese Veterinarian and Food Department (0421/000/000/2016). After 1 week of acclimatization, body weight-matched rats were randomly divided into three groups: 5 were used as controls (CTR), 6 were subjected to caloric restriction (CR) and 5 were subjected to a peritoneal implantation of a mini-pump for chronic delivery of 3.5 pmol/min/kg GLP-1 (GLP-1). Rats from the CTR and GLP-1 groups were fed *AL* with a standard chow diet (4RF21 certificate, Mucedola, Italy), whereas the rats in CR group received 30% less chow diet than the former for 28 days. Food consumption and the animal's weight were monitored in a daily basis during the 28 days of the study duration. After the experimental period, animals were euthanized with carbon dioxide and killed by exsanguination after cardiac puncture. Blood was collected into chilled EDTA tubes and immediately centrifuged, and plasma was stored at - 20 ºC until further assays. Testes were removed and processed for histochemistry, protein, DNA, RNA and metabolite extraction and stored at –80 ºC until further use. Epididymis were isolated and placed in prewarmed (37 ºC) Hank's Balanced solution (pH 7.4), crushed with a stiletto blade, and incubated for 5 min (37 ºC) to allow sperm to disperse into the medium, and the suspension was then collected and stored at -80 ºC until further use.

3.2. Blood glucose and hormone measurements

Blood glucose and hormone levels were measured as previously described by Morais et al.[247] In short, blood glucose level was determined in whole blood by the glucose oxidase method using a One Touch Ultra glucometer (Lifescan, Milpitas, CA). Plasma concentrations of total and active GLP-1 (EZGLP1T-36K and EGLP-35K; Merck Millipore, Darmstadt, Germany), insulin (EZRMI-13K; Merck Millipore, Darmstadt, Germany), total ghrelin (EZRGRT-91K; Merck Millipore, Darmstadt, Germany) and leptin (EZRL-83K; Merck Millipore, Darmstadt, Germany) were determined by enzyme-linked immunosorbent assay (ELISA) using specific commercial kits according to the manufacturer instructions. Insulin resistance was calculated using the following homeostatic model assessment (HOMA) formula:

$$\text{HOMA-IR} = (\text{Glucose}\,[\text{mg/dL}] * \text{Insulin}\,[\text{mU/L}]) / 405.$$

3.3. Neuropeptides expression

The RNA expression of *NPY*, *AgRP*, *POMC*, and *CART* in the hypothalamus were studied by using TaqMan real-time polymerase chain reaction (PCR) in a Step One Plus system (Applied Biosystems, Foster City, CA) using specific primers and probes obtained from inventoried TaqMan Gene Expression Assays (Applied Biosystems), as previously described by Morais et al..[247] All reactions were carried out using the following cycling parameters: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 1 minute. For data

analysis, the RNA level of the gene of interest was normalized for both tissues using β-actin values according with the $2^{-\Delta\Delta Ct}$ method.

3.4. Microscopy sperm parameters evaluation

The sperm parameters of the animals under study, including concentration, viability and morphology were performed as described by Martins et al.[248] being that the procedures are briefly presented below.

3.4.1. Concentration

For the evaluation of the concentration, a preliminary slide observation was performed, allowing the calculation of the most appropriate dilution to be used. The dilution of 1:40 (50 µL of sperm with 2 mL of Hank's balanced salt solution (HBSS)) was used. Then, 10 µL of the diluted sample was placed in a Neubauer chamber, and 15 minutes were allowed to allow the sperm to settle. Using a microscope, spermatozoa were counted in four quadrants. Each quadrant has an area of 1 mm^2 and a layer of solution is 0.1 mm thick, which makes a volume of 0.1 mm^3 per square, thus being able to calculate the concentration of spermatozoa/mL, taking into account the factor dilution with the following formula:

$$Concentration = average\ number\ of\ spermatozoa\ per\ quadrant\ x\ 10^6\ x\ dilution\ factor.$$

3.4.2. Viability

Staining with eosin-nigrosin was used to assess spermatozoa viability by examining sperm smears from the epididymis, where was mixed 5 µL of sperm and 10 µL of the dye. Dead sperm were stained pink due to impaired membrane integrity, while viable sperm appeared white. Among 333 isolated and non-overlapping sperm count, viable and non-viable spermatozoa were counted, finally calculating the percentage of viability using the following formula:

$$\%\ Viability = (nº\ of\ viable\ spermatozoa\ /\ nº\ of\ spermatozoa\ counted)\ x\ 100$$

3.4.3. Morphology

For the evaluation of morphology, Diff-Quick staining was used to stain intracellular structures. A drop of solution was mixed with sperm (5 µL) and another of HBSSf (10 µL) at one end of the slide and a smear was performed. After drying, the slide was subjected to staining solutions in the following order: clear fixative solution for 1 minute; pink solution for 2 minutes and finally blue solution for 2 minutes. Having removed the excess dye, the slide was immersed in H_20 and the sperm defects / anomalies were evaluated. Only one type of sperm defect was counted, from the most severe (head) to the least (tail), with a count of a minimum of 333 spermatozoa that were classified as: normal, head defects, tail defects and neck defects. Finally, the percentage of each defect was calculated according to the formula:

$$\%\ Defect = (nº\ of\ spermatozoa\ with\ the\ same\ defect\ /\ nº\ of\ spermatozoa\ counted)\ x\ 100$$

3.5. RNA isolation from rat testes and sperm

To extract total RNA from the rats' testes and sperm the NYZ Total RNA Isolation kit (MB13402; NZYTech, Lisboa, Portugal) was used according to the manufacturer instructions. Briefly, the disrupted tissues (around 20 mg) were lysed and then the samples were vortexed for 10 s. The mixture was transferred to a column provided with the kit. After washing the column with the washing buffers provided, testes and sperm RNA was eluted from the column with 50 µL and 40 µL of elution buffer, respectively. RNA concentration and purity were measured using the microplate reader SYNERGY H1 (BioTek Instruments, VT, USA) at 260/280 nm, and the samples with ratios of absorbance between 1.8 and 2.1 were analyzed to be of acceptable quality. The RNA samples were stored at −80°C until use.

3.6. Complementary DNA (cDNA) synthesis

Reverse transcription of the total RNA was performed to synthesize cDNA using the NYZ M-MuLV First-Strand cDNA Synthesis Kit (MB17201; NZYTech, Lisboa, Portugal) according to the manufacturer instructions, in a final volume of 20 µL. Briefly, 10 µL of a mix, containing random hexamers, oligo$(dT)_{18}$, deoxynucleotides, $MgCl_2$ and an optimized reverse transcription buffer, and 2 µL of another mix, containing the M-MuLV Reverse Transcriptase (RNase H minus) and a ribonuclease inhibitor, were added to each sample that contained 1 µg of RNA. The volume was adjusted to 17 µL with RNase-free water. The samples were then incubated at 25ºC for 10 min, for reagent activation, 50 min at 37°C, for conversion, and 85ºC for 10 min to inactivate the reaction. Then was added 1 µL of RNase H (*E. coli*), followed by an incubation at 37ºC for 20 min.

3.7. Conventional polymerase chain reaction (PCR)

To identify the ORG transcripts in rat testes and sperm we performed conventional PCR assays according to standard methods. In brief, exon–exon spanning primers were designed for the *rattus norvegicus* genes *FTO*, *MC4R*, *GNPDA2* and *TMEM18*, that are described in **Table 1**. To amplify the selected genes, it was used the NZYTaq II 2× Green Master Mix (MB358; NZYTech, Lisboa, Portugal), which is a premixed ready to use a solution containing a Taq-derived DNA polymerase, dNTPs, reaction buffer and other additives to optimize the reaction. cDNA was amplified in a final volume of 12.5 µL. To each sample, it was added 2 µL of cDNA, 0.1 µL of primer forward (50 µM), 0.1 µL of primer reverse (50 µM), and 6.5 µL of Green Master Mix 2x. The volume was adjusted to 12.5 µL with RNase-free water. The conditions of PCR cycles were adjusted to better fit optimal temperatures of enzyme activation, DNA denaturation, annealing, and dissociation. The protocol for PCR, performed in T100™ Thermal Cycler (BioRad, Hemel Hempstead, UK), was the following: 5 min at 95°C for enzyme activation, 30 s at 95°C for DNA denaturation, 1 min at 72°C for extension and, 7 min at 72°C for final denaturation. The annealing temperatures were adjusted to each pair of primers and can be consulted in **Table 1**. The samples were run in a 2% agarose (MB20702, NZYTech, Lisboa, Portugal) gel, stained with GreenSafe (MB13201, NZYTech, Lisboa, Portugal). The results were revealed in the ChemiDoc™ Imaging System (BioRad, Hemel Hempstead, UK).

3.8. Real-Time Polymerase chain reaction (qPCR)

To perform the qPCR assays, we used qPCR Green Master Mix (2×) (NZYTech, Lisboa, Portugal), which, already contains a DNA polymerase enzyme, buffer and SYRBR green nucleic acid stain. cDNA was amplified in a final volume of 16 µL. To each sample containing 0.8 µL of cDNA, was added 0.64 µL of forward primer (10 µM), 0.64 µL of reverse primer (10 µM), and 8.0 µL of Green Master Mix. The volume was adjusted to 16 µL with RNase-

free water. The conditions of qPCR cycles were adjusted to better fit optimal temperatures of enzyme activation, DNA denaturation, and primer annealing and dissociation conditions. The protocol for qPCR was the following: 10 min at 95°C for polymerase activation, 15 s at 95°C for DNA denaturation and, 1 min at 72°C for extension. The annealing temperatures and the number of cycles required for the exponential phase of amplification were optimized to each set of primers. Along with the primer sequences, the annealing temperatures and the number of cycles can be consulted in **Table 1**. Melting curve was determined by an additional step: 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C. qPCR reactions were carried in duplicate, and the optical density was assessed by a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hemel Hempstead, UK). Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to β2-microglobulin (*B2M*).

Table 1 - Primer sequences and PCR conditions used to assess gene expression and mRNA abundance in rats' testes and sperm. β-2 microglobulin (B2M) was used as a housekeeping control.

Gene Name	GenBank Accession Number	Primer sequence (5´-3´)	Amplicon Length	Annealing Temperature	Cycles
B2M	NM_012512.1	Forward: CCGTGATCTTTCTGGTGCTTGTC Reverse: CTATCTGAGGTGGGTGGAACTGAG	150 bp	58.0 ºC	35
MC4R	NM_013099.3	Forward: ACAAGAACCTGCACTCACCC Reverse: ATGCGAGCAAGGAGCTACAG	192 bp	62.0 ºC	35
FTO	NM_001039713.1	Forward: CAGAGATCCCGATACGTGGC Reverse: CTGTGAGCCAGCCAAAACAC	151 bp	57.6 ºC	35
GNPDA2	NM_001106005.1	Forward: ACCATCCCGAAAGCTACCAT Reverse: GGACCTATTCCTCCAACAAAAAGAT	181 bp	58.4 ºC	35
TMEM18	NM_001007748.1	Forward: AAGCATGGTGAATGGGGACC Reverse: CACACTCAAACCTGCGTGAC	127 bp	56.4 ºC	35
NFE2L2	NM_031789.2	Forward: CAATGACTCTGACTCCGGCA Reverse: AGGGGCACTGTCTAGCTCTT	139 bp	56.6 ºc	35

3.9. Immunohistochemistry (IHC)

For FTO, MC4R, GNPDA2 and TMEM identification and localization on rats' testes we performed IHC, where were used specific antibodies: anti-FTO, anti-MC4R, anti-GNPDA2 and anti-TMEM18, respectively. Briefly, 3 µm formalin-fixed paraffin embedded tissue sections mounted on adhesive microscope slides (Superfrost Plus, Thermo Scientific, Rockford, IL) were deparaffinized, rehydrated in two changes of xylene (10 min each) and 5 min in graded alcohols (100%, 96% and 70% ethanol). Then, tissue sections were subjected, during 8 min, to antigen retrieval through heating by microwaving at 900W in 10 mM citrate buffer (pH 6.0), with 0.1% Tween 20 in cases where permeabilization was required (FTO, GNPDA2 and TMEM18). Once cooled and washed with PBS, the endogenous

peroxidase activity was blocked with a diluted solution of 0.3% hydrogen peroxide in methanol for 15 minutes. After slides washing, they were incubated in a normal rabbit serum (X0902, Dako) in FTO slide and normal swine serum (X0901, Dako) in MC4R, GNPDA2 and TMEM18 slides (1:5 in 10% bovine serum albumin [BSA]) for 20 minutes in a humidity chamber, to block nonspecific binding sites.

The incubation with the primary antibodies was performed overnight at 4°C. The list of antibodies used in IHC, along with the concentrations used, can be consulted in **Table 2**. For a negative control, specimens were processed in the absence of a primary antibody. The slides were washed and then, for reaction detection, FTO slides were incubated for 60 min with secondary biotinylated antibody polyclonal rabbit anti-mouse (1:200, E0354, Dako) and MC4R, GNPDA2 and TMEM18 slides were incubated 60 min with biotinylated secondary antibody polyclonal swine anti-rabbit (1:200, E0353, Dako). Afterwards, slides, after being washed, were incubated with the avidin-biotin complex (ABC) (1:100 dilution in 5% BSA; VectorLaboratories, Peterborough, UK) for 30 minutes and then, after washing the slides, the color of the antibody staining was revealed with the DAB substrate (Liquid DAB + Substrate Chromogen System, K3468, Dako), during 1 min for FTO and TMEM18, and 5 min for MC4R. All the sections were washed in distilled H_2O and counterstained with a 1:2 diluted Harris Hematoxylin. The slides were rinsed in running tap water for 5 min and the sections were dehydrated through a graded series of ethanol (70, 96, and 100%) for 5 min each and cleared in two changes of xylene for 5 min each. Lastly, the coverslips were placed over the sections using mounting solution (Entellan new, 107961, Sigma-Aldrich). The immunohistochemical analysis were imaged with a Microscope Digital Camera (Olympus EP50).

Table 2 - List of antibodies, and respective concentrations, used for identification and localization of the ORG correspondent proteins.

Antibody	Company	Catalog Number	Concentration for IHC
Anti-FTO	Abcam	ab92821	1:300
Anti-MC4R	Abcam	ab24233	1:75
Anti-GNPDA2	Abcam	ab106363	1:150
Anti-TMEM18	Abcam	ab100954	1:150

3.10. Ferric reducing antioxidant power assay (FRAP)

The samples of testes and sperm were collected, washed with PBS and dried by centrifugation (*8000* g, 10 min at 4º C). The pellets were then diluted in the adequate volume (3 µL / mg of tissue or for 1 µL / 1 million of sperm) of 1% SDS Buffer (1% Noridet P40, 0.5% Sodium deoxycholate, 0.1% Sodium dodecyl sulphate 10% in PBS, 0.1% Phenylmethanesulfonyl fluoride 100 mM, 0.1% cocktail mix of protease and phosphatase inhibitors, 0.1% sodium orthovanadate 100 mM) and homogenized with a microtubule piston. The samples were let to rest for 20 min at 4ºC and then centrifuged at *14000* g, 4 ºC for 20 min. The pellet of cellular debris was discarded, and protein concentration of the supernatant was quantified by Pierce Bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Massachusetts, USA), using the microplate reader SYNERGY H1 (BioTek Instruments, VT, USA). Total antioxidant potential of the samples was determined against standards of L-ascorbic acid, by following the absorbance changes at 595 nm due to the reduction of the Fe^{3+}-2,4,6-Tripyridyl-s-Triazine (TPTZ) complex to a colored Fe^{2+}-TPTZ complex. Absorbance results were corrected by using a blank, using H_2O instead of the sample. The changes in absorbance values of tested reaction mixtures were used to calculate the sample's FRAP value (µmol of antioxidant

potential / L of protein) using the equation bellow and taking into account that the FRAP value of the ascorbic acid 1 mM is 2.

$$\frac{ABS\ Amostra^{40\ min} - ABS\ Amostra^{0\ min}}{ABS\ Standard^{40\ min} - ABS\ Standard^{0\ min}} \times FRAP\ value\ of\ standard$$

3.11. Data and statistical analysis

All data presented are expressed as mean ± standard error of the mean (SEM) unless otherwise specified. The Kolmogorov-Smirnov test was used to determine the normality of the groups. Comparison of independent groups was carried out by using either an unpaired t-test or a Mann Whitney U test. For multiple comparation analysis a one-way analysis of variance (ANOVA) test was used, with either ordinary one-way ANOVA or Kruskal-Wallis test depending on the normality of the groups. P-value < 0.05 was considered as statistically significant. All the correlations were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution and a confidence interval of 95%. P-value < 0.05 was considered as statistically significant. Statistical analysis was carried out using GraphPad (Prism, LaJolla, CA; version 8.0.1) for Windows.

4. Results

4.1. Caloric restriction and GLP-1 administration impact on body weight, hormonal profile and neuropeptides expression

4.1.1. Caloric restriction decreases body weight gain

CR and GLP-1 analogues administration are known to influence the body energy balance, being that the body weight changes are a function of that energy balance. Thus, we started by evaluating how the rats' body weight responded to 28 days of CR and GLP-1 administration (**Figure 9**).

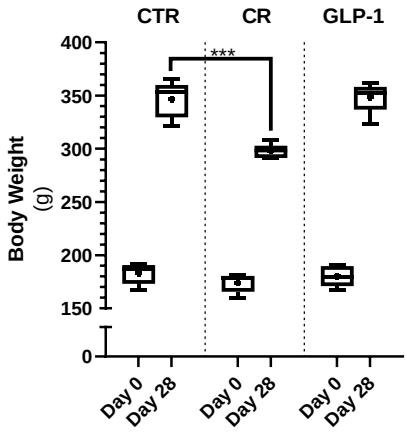


Figure 9 – Body weight response to 28 days of CR and GLP-1 administration. The body weight of the rats from the CTR, CR and GLP-1 groups was measured in day 0 and day 28 of the experiment. Results are expressed as mean ± SEM (*n* = 5 for CTR and GLP-1 condition and *n* = 6 to CR condition). * indicates statistically significant differences as compared to the CTR group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$). (**Abbreviations**: CR, caloric restriction; CTR, control; GLP-1, glucagon like-peptide 1)

Although CR and the administration of GLP-1 analogues are widely known for the consequent loss of body weight, that effect was not observed in this study neither for the CR nor for the GLP-1 group. This because the weight of the rats from the CR, GLP-1 and control groups on the last day of the experiment (298.0 ± 2.6 g, 348.4 ± 6.6 g and 346.5 ± 7.7 g, respectively) was found to be higher compared to the first day (174. ± 3.6 g, 180.2 ± 4.4 g and 182.9 ± 4.5 g, respectively), therefore presenting a body weight gain (**Figure 9**).

All the experimental groups showed an increase in body weight in day 28 relatively to day 0, however the body weight gain seemed to be different between the groups, hence we calculated the body weight gain (**Figure 10**), which consists of the difference between the body weight on the last day (day 28) relative to the first day of the experiment (day 0).

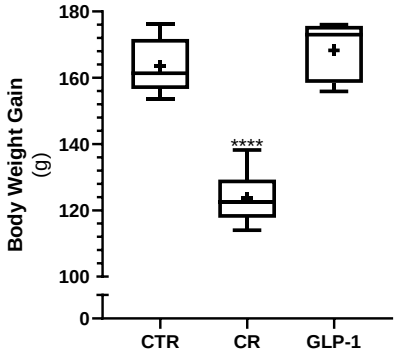


Figure 10 – CR and GLP-1 administration influence on rats' body weight gain. The body weight gain of the rats from the CR, GLP-1 and CTR groups was calculated considering the difference of weight between the day 28 and day 0 of the experiment. Results are expressed as mean ± SEM (*n* = 5 for CTR and GLP-1 condition and *n* = 6 to CR condition). * indicates statistically significant differences as compared to the CTR group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$). (**Abbreviations**: CR, caloric restriction; CTR, control; GLP-1, glucagon like-peptide 1)

We could observe that there was a significantly smaller body weight gain in the group subject to CR compared to the control group (123.8 ± 3.3 g vs 163.6 ± 3.8 g), which did not occur in the GLP-1 group (**Figure 10**).

4.1.2. Caloric restriction alters glucose metabolism and hormone profile

Since we were analyzing how rats body weight responds to CR and GLP-1 administration, we considered important observe if there was a change in fasting glucose levels (**Figure 11, (a)**), that is an important source of energy, and in the fasting levels of hormones related to glucose metabolism such as insulin (**Figure 11, (b)**), that maintains normal blood glucose levels by facilitating cellular glucose uptake, and ghrelin (**Figure 11, (d)**), that is known for its role in appetite initiation.[249] In individuals with insulin resistance, cells don't respond normally to insulin and glucose can't enter the cells as easily. As a result, blood glucose levels rise even if more insulin continues to be produced to try to lower the glucose levels.[250] So, we also calculated fasting HOMA-IR, that is a simple method to assess insulin resistance (**Figure 11, (c)**).

During the fasting period, triglycerides accumulated in adipose tissue are broken down into fatty acids and glycerol to be used as an energy source. The liver uses these fatty acids to produce ketone bodies that will provide the energy needed to maintain tissues, especially the brain, during the unfed state.[225] So, we measured the fasting levels of leptin (**Figure 11, (e)**) that is widely known as an adiposity signal, being closely linked to weight loss. We also calculated the leptin/ghrelin ratio (**Figure 11, (f)**) once it has been suggested to be a better marker and a predictor of energy restriction treatment success or failure.[251]

GLP-1 is a secreted peptide that acts as a key determinant of blood glucose homeostasis by virtue of its actions in slowing gastric emptying, enhancing pancreatic insulin secretion, and suppressing pancreatic glucagon secretion. We measured the total GLP-1 (**Figure 11, (g)**) secreted from intestinal endocrine L-cells, which would provide information about its secretion. Active GLP-1 is a gut hormone derived as a result of proglucagon processing. Once active GLP-1 is secreted, the enzyme DPP-4 quickly cleaves the majority of the hormone and the remaining active GLP-1 enters circulation, being transported to peripheral organs where it stimulates the incretin effect.[74] Therefore, we measured the biologically active form of GLP-1 (**Figure 11, (h)**) that effectively reaches the liver. This GLP-1 form is also relevant, once it is the form that in fact promote the physiological effects on the body such as the incretin effect. Furthermore, as there is an experimental group in which active GLP-1 was administered, we wanted

to verify if active GLP-1 actually had its levels increased. We also considered the active/total GLP-1 ratio (**Figure 11, (i)**) to understand how the active GLP-1 varies relatively to the total GLP-1.

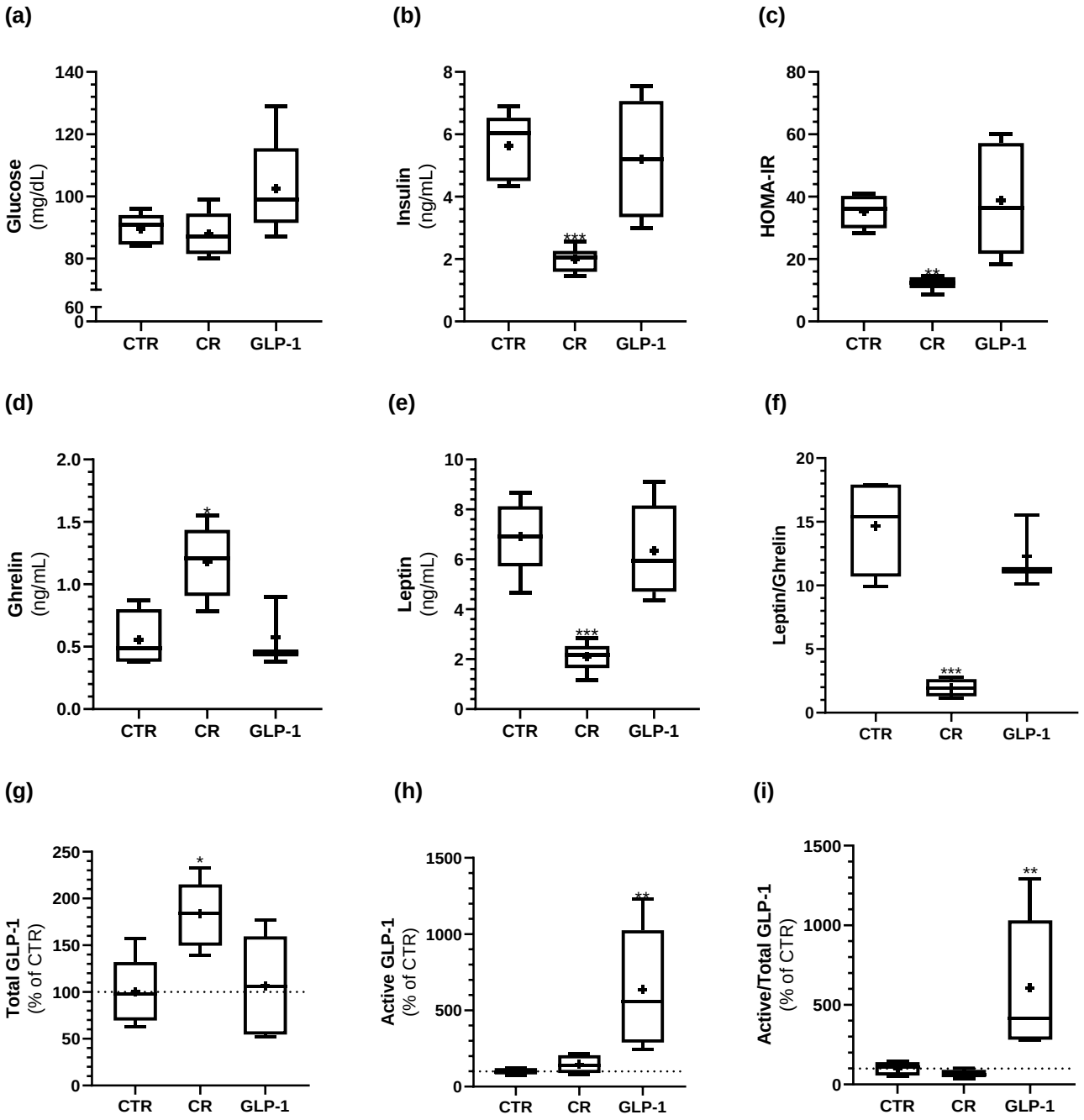


Figure 11 – Fasting blood glucose and hormone levels. Fasting blood glucose **(a)** and some hormone measurements (insulin **(b)**, ghrelin **(d)** and leptin **(e)**) were performed in rats from the CTR, CR and GLP-1 groups. Furthermore, were assessed the plasma levels of total **(f)** and active GLP-1 **(g)**. HOMA-IR **(c)**, Leptin/Ghrelin ratio **(f)** Active/Total GLP-1 ratio **(i)** were also calculated. Results are expressed as mean ± SEM (n = 5 for Control and GLP-1 condition and n = 6 to CR condition). * indicates statistically significant differences as compared to the control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$). (**Abbreviations**: CR, caloric restriction; CTR, control; GLP-1, glucagon like-peptide 1; HOMA-IR, homeostatic model assessment for insulin resistance)

Fasting blood glucose levels (**Figure 11, (a)**) were similar in both experimental groups when compared to control group. Fasting insulin plasma levels (**Figure 11, (b)**) and HOMA-IR (**Figure 11, (c)**) had no significant changes in the GLP-1 group when compared to the control group. On the other hand, these values were significantly lower in CR rats when compared with the control rats (1.98 ± 0.16 ng/mL vs 5.62 ± 0.48 ng/mL and 12.25 ± 0.89 vs 35.3 ± 2.4).

Fasting ghrelin plasma levels (**Figure 11, (d)**) were significantly higher on the CR group when compared with the control group (1.03 ± 0.18 ng/mL vs 0.56 ± 0.12 ng/mL) and there was no significant difference between GLP-1 and control groups. Concerning to leptin (**Figure 11, (e)**) its fasting plasma levels were significantly lower in the rats under CR when compared with the control group (6.92 ± 0.66 ng/mL vs 2.10 ± 0.27 ng/mL) and once again there was no significant difference between GLP-1 and control groups. Leptin/ghrelin ratio (**Figure 11, (f)**) was only significantly decreased in CR group when compared to control group (3.07 ± 1.14 vs 14.67 ± 1.96).

Regarding to the total GLP-1 levels (**Figure 11, (f)**) a statistical difference was observed in the CR group when compared to the control group (183.9 ± 14.6 % vs 100.0 ± 16.1 %, values expressed on % of total GLP-1 in control group) and no statistical difference was observed between GLP-1 and control group. The reverse was observed relatively to the active GLP-1 levels (**Figure 11, (g)**) since was observed statistical differences between the GLP-1 and the control groups (637.3 ± 178.3 % vs 100.0 ± 9.5 %, values expressed on % of active GLP-1 in control group), which was not observed to CR group. Active/total GLP-1 ratio (**Figure 11, (h)**) was increased in the GLP-1 group when compared to the control group (607.9 ± 193.1 % vs 100.0 ± 19.2 %, values expressed on % of active/total GLP-1 in control group).

4.1.3. Caloric restriction and GLP-1 administration did not affect the neuropeptides expression

It is well known that food intake regulation is one of the most important factors involved in body weight control. To regulate food intake, the brain must alter appetite. Appetite is controlled by a series of short-term hormonal and neural signals that derive from the gastrointestinal tract, such as ghrelin and GLP-1. Other hormones such as insulin and leptin, together with circulating nutrients, indicate long-term energy stores, both of which are signals of fat mass. All these signals act at several CNS sites but the pathways converge on the ARC in the hypothalamus. The ARC contains populations of neurons that express orexigenic neuropeptides such as NPY and AgRP, and anorexigenic neuropeptides such as POMC and CART.[37,41] Thus, being these four neuropeptides so closely linked to appetite control and considering that this is an aspect that can be affected by the energy homeostasis perturbation promoted by nutritional interventions, their expression was measured at the hypothalamic level by Morais et al.[247] in this animal model. However, no significant differences in hypothalamic gene expression for *NPY*, *AgRP*, *POMC*, and *CART* were found by them, between the rats from the CR and GLP-1 groups and the control rats.

4.2. Caloric restriction and GLP-1 administration affect sperm morphology

The hormonal dysregulation is responsible for the disruption of several reproductive events. Thus, in conditions such as caloric restriction and in the administration of GLP-1 analogues, where a hormonal dysregulation associated with the disruption of the body energy homeostasis can occur, the male reproductive function can be disturbed. This occurs because the reproduction is a physiological function highly dependent on a correct energy balance, namely for successful spermatogenesis and sperm production.[125,252] Therefore, we performed the measurement of some parameters related to male reproductive function such as the testes weight (**Figure 12, (a)**) and epididymis weight (**Figure 12, (b)**). From the body and testes weight we calculated the gonadotropic index (**Figure 12, (c)**), that is a metric that represents the relative weight of the gonad to the body weight. We also analyzed the sperm quality, namely through the sperm concentration (**Figure 13, (a)**), viability (**Figure 13, (b)**) and morphology being that the subtypes of sperm morphological defects were analyzed, which were divided into: head, neck and tail defects (**Figure 13, (c)**).

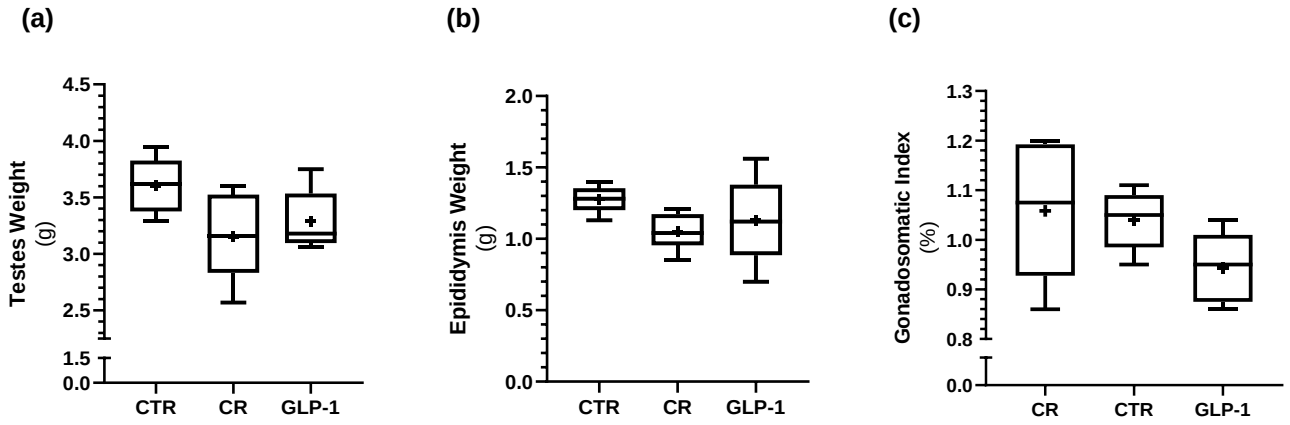


Figure 12 – Impact of CR and GLP-1 administration on testes and epididymis weights and on gonadosomatic index. The response of the of the rats' reproductive structures of the rats from CTR, CR and GLP-1 groups was evaluated by the sum of the weights of the right and left testis **(a)** and epididymis **(b)**. Results are expressed as mean ± SEM (*n* = 5 for CTR and GLP-1 condition and *n* = 6 to CR condition). * indicates statistically significant differences as compared to the CTR group (* *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 and **** *P* < 0.0001). (**Abbreviations**: CR, caloric restriction; CTR, control; GLP-1, glucagon like-peptide 1)

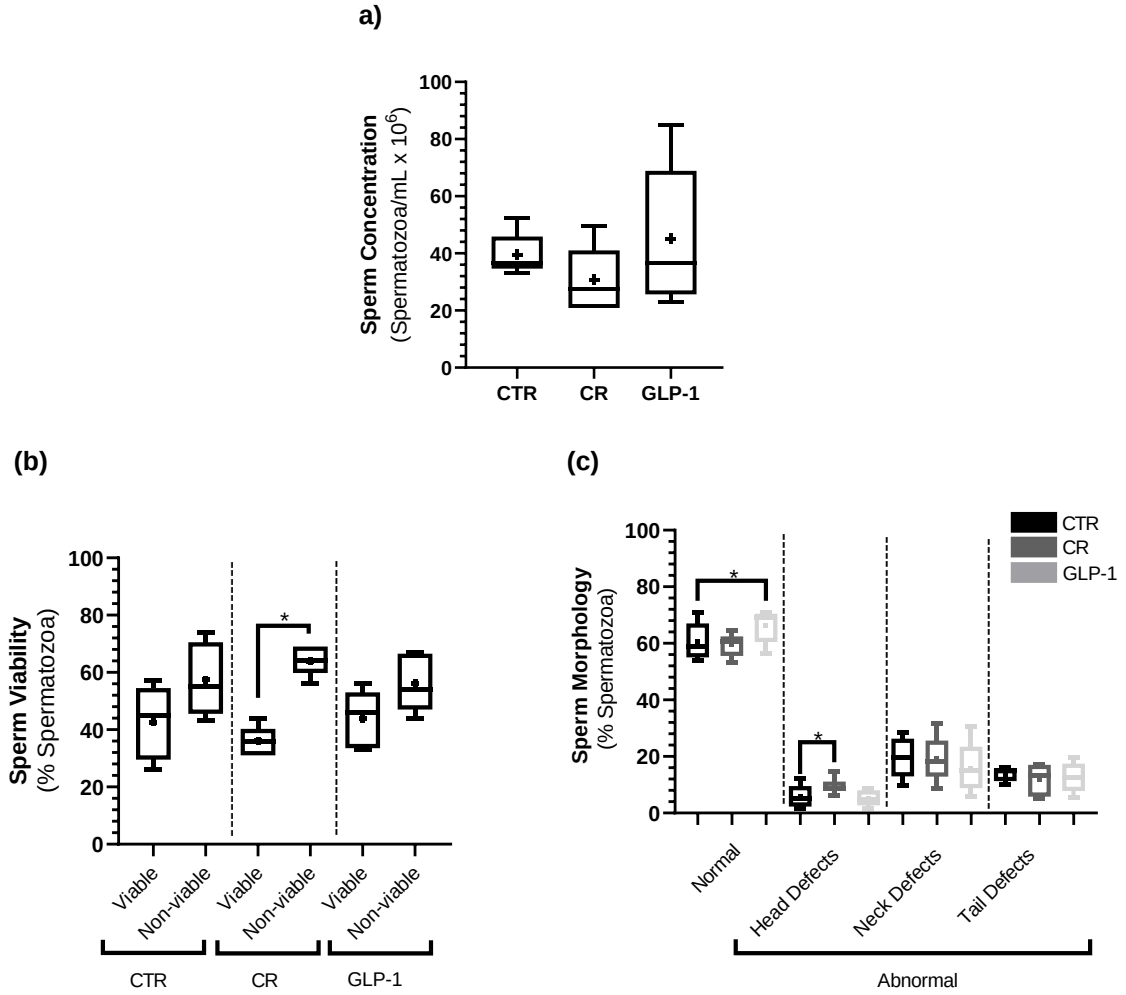


Figure 13 – Impact of CR and GLP-1 administration on sperm parameters. The sperm quality of the rats of CTR, CR and GLP-1 groups was evaluated by the measurement of sperm concentration **(a)**, viability **(b)** and morphology **(c)**. Within the morphology parameter, was also analyzed the sperm defects specifically in the head, neck and tail. Results are expressed as mean ± SEM (*n* = 5 for CTR and GLP-1 condition and *n* = 6 to CR condition). * indicates statistically significant differences as compared to the CTR group (* *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 and **** *P* < 0.0001). (**Abbreviations**: CR, caloric restriction; CTR, control; GLP-1, glucagon like-peptide 1)

Regarding the weight of the rats' testes (**Figure 12, (a)**) and epididymis (**Figure 12, (b)**), and regarding the gonadosomatic index, no significant differences were observed either in the CR or in the GLP-1 groups when compared to the control group. About sperm quality, we observed significant differences regarding sperm morphology (**Figure 13, (c)**) but not in the sperm concentration (**Figure 13, (a)**) and viability (**Figure 13, (b)**). The rats in the CR group showed a significant increase in the percentage of head defects compared to rats in the control group (9.36 ± 0.79 % vs 5.73 ± 1.35 %). The rats in the GLP-1 group had a significant increase in the percentage of sperm with normal morphology. (66.21 ± 2.06 % vs 60.98 ± 2.31 %). Coherently, the rats in the GLP-1 group, despite not being statistically significant, appear to have a lower percentage of all sperm morphology defects when compared to the control rats. Another interesting observation was the fact that, in the control rats, the percentage of viable sperm was higher than the non-viable percentage and that the animals from the other groups, especially in CR group, the percentage of non-viable sperm was increased.

4.3. Identification of *FTO*, *MC4R*, *GNPDA2* and *TMEM18* transcripts in rat testes and sperm and location of the corresponding proteins in rat testes

4.3.1. FTO, MC4R, GNPDA2 and TMEM18 transcripts are present in rat testes and sperm

Caloric restriction and GLP-1 analogues administration are known to promote weight loss due to hormonal dysregulations and consequently affect the energy homeostasis that is an essential aspect to achieve a normal male reproductive function. Most of the ORGs encode for proteins that are somehow involved in the leptin–melanocortin signaling pathway, being that this pathway is important in controlling the energy homeostasis in the hypothalamus by coordinating the appetite, food intake and energy expenditure.[253,254] Thus, we hypothesized that ORGs, namely *FTO*, *MC4R*, *GNPDA2* and *TMEM18*, could be part of the mechanism by which caloric restriction and administration of GLP-1 may affect the male reproductive system. Thus, we started by determining the presence of the four selected ORGs transcripts in both rat testes and sperm by conventional PCR (**Figure 14**).

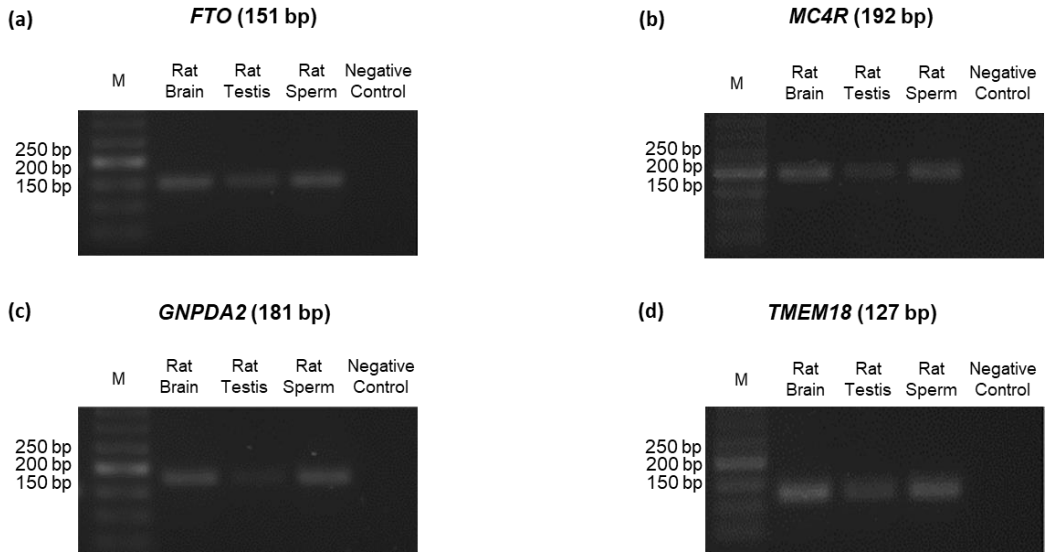


Figure 14 - Identification of ORGs transcripts in rats' testes and rats' sperm by conventional PCR. *FTO* **(a)**, *MC4R* **(b)**, *GNPDA2* **(c)**, and *TMEM18* **(d)** transcripts in both Wistar rat testes and sperm were identified by conventional PCR. A molecular weight marker was used for determining the approximate size of the nucleic acid fragment. A cDNA-free sample was used as a negative control and a rat brain sample was used as a positive control. (**Abbreviations**: FTO, fat mass and obesity associated; GNPDA2, glucosamine-6-phosphate deaminase 2; MC4R,

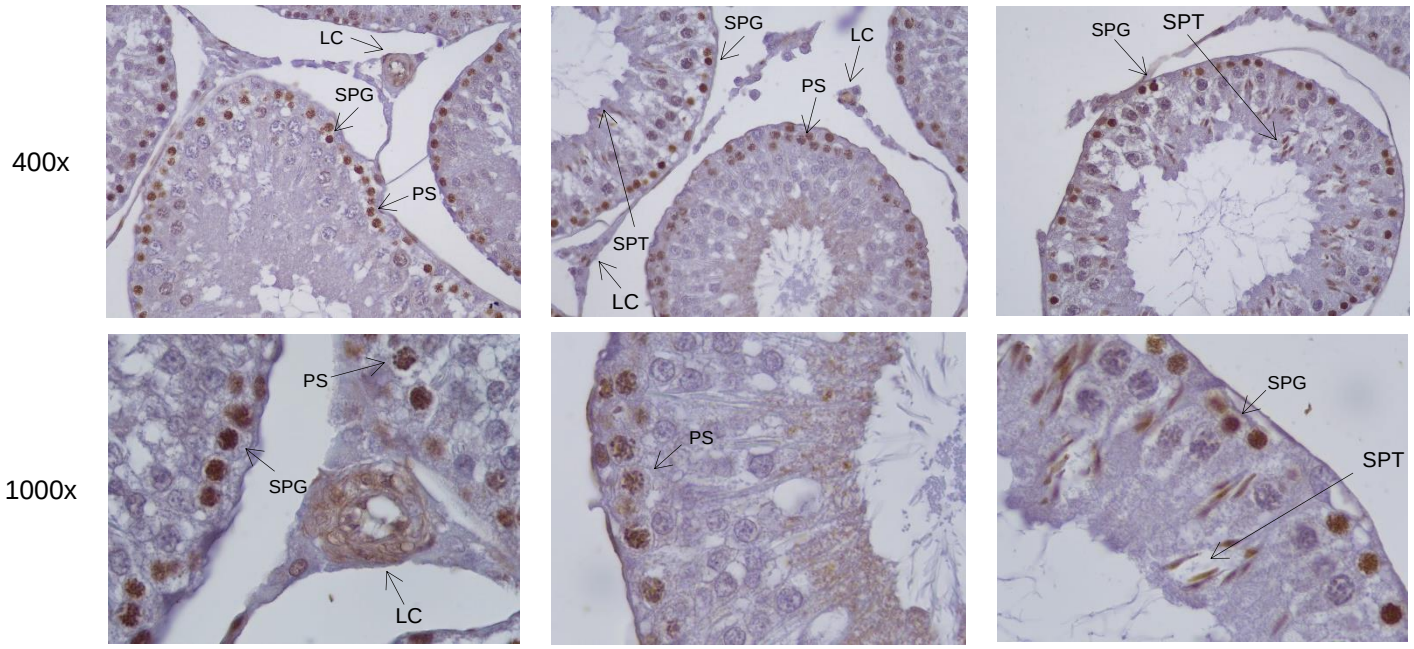
melanocortin 4 receptor; M, molecular weight marker; PCR, polymerase chain reaction; TMEM18, transmembrane protein 18)

We confirmed the presence of *FTO*, *MC4R* and *TMEM18* transcripts in rat testes, has it had been already reported by Zhao et al.[255], Mountjoy et al.[256] and Almén et al.,[233] respectively. We also identified those transcripts, for the first time, in rat sperm. *FTO* transcript was identified by a 151 bp staining band (**Figure 14, (a)**), *MC4R* transcript at 192 bp staining band (**Figure 14, (b)**) and TMEM18 transcript at 127 bp staining band (**Figure 14, (d)**). Additionally, we were able to identify, for the first time, the presence of *GNPDA2* transcripts in both rat testes and sperm, at 181 bp staining band (**Figure 14, (c)**).

4.3.2. FTO, MC4R, GNPDA2 and TMEM18 proteins are present in rat testes in specific locations

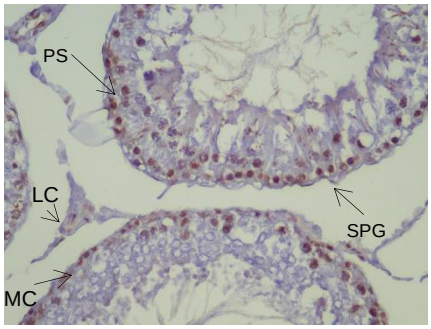
Systematic studies quantifying transcripts and proteins at genomic scales revealed the importance of multiple processes beyond transcript concentration that contribute to establishing the expression level of a protein, such as: translation rates, translation rate modulation, modulation of a protein's half-life, protein synthesis delay and protein transport. Thus, the direct comparison between protein and mRNA abundances from the same location or from the same cell type may not always be appropriate.[257] So, once identified the presence of ORGs transcripts in the rat testes and sperm we further proceed to the investigation of the ORGs protein expression and location on rat testes by immunohistochemistry (**Figure 15**).

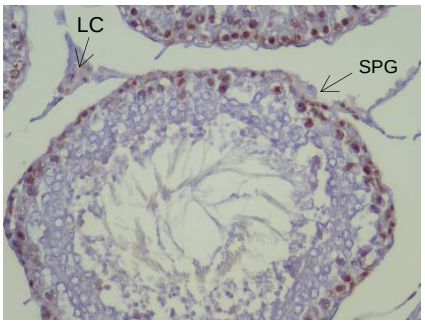
(a) FTO

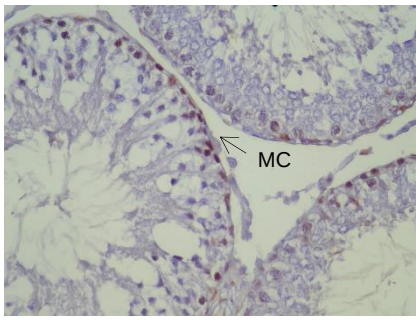


(b) MC4R

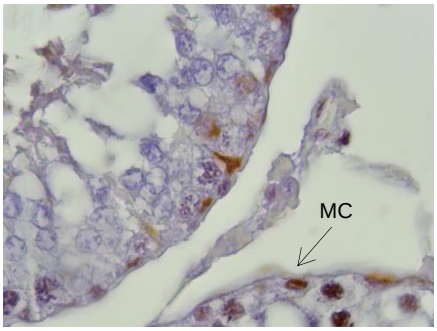
400x

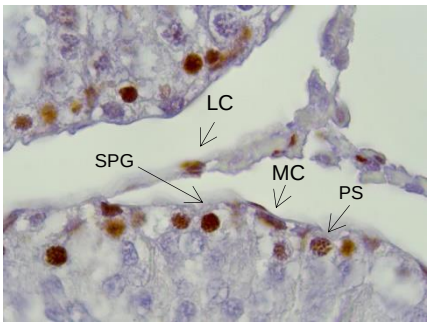


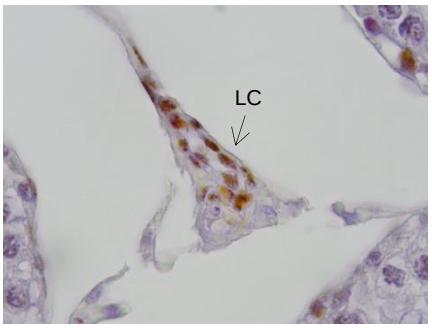




1000x

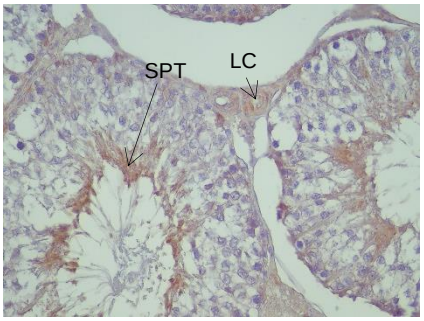


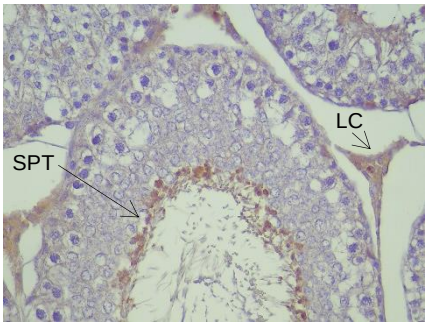


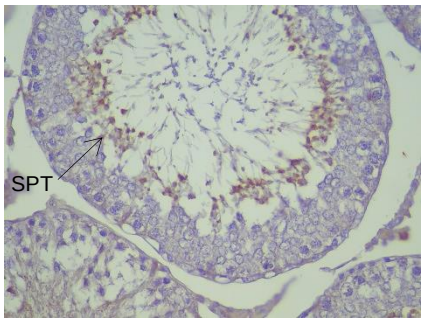


(c) GNPDA2

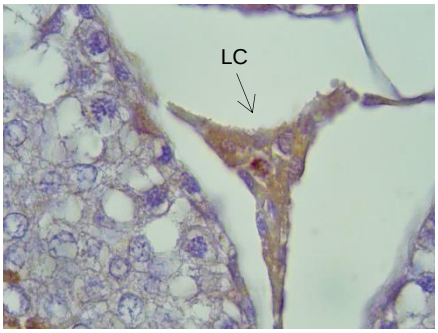
400x

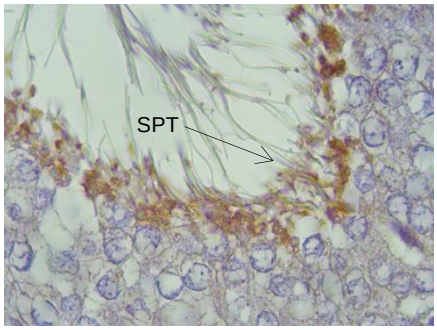


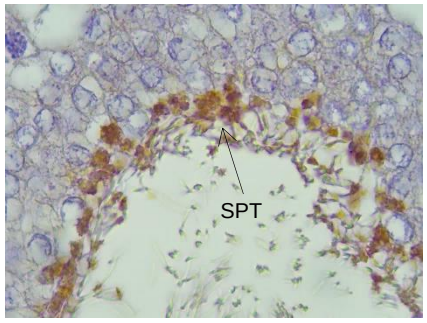




1000x







(d) TMEM18

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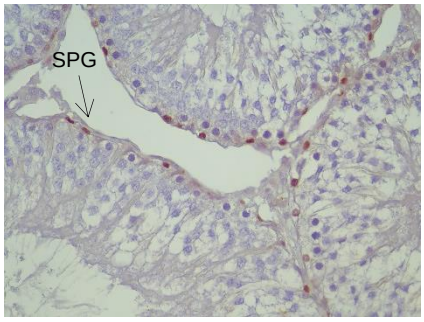

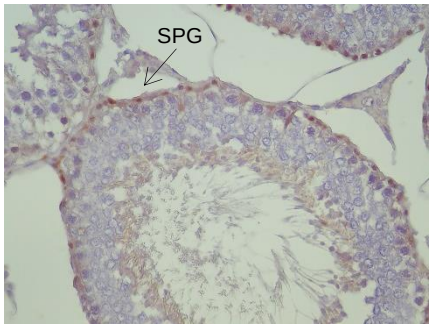

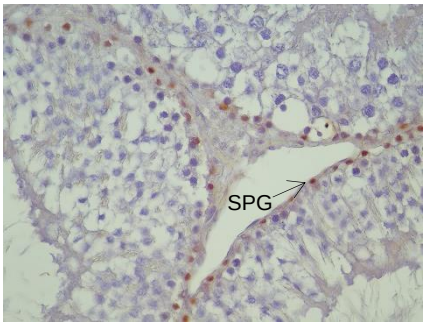

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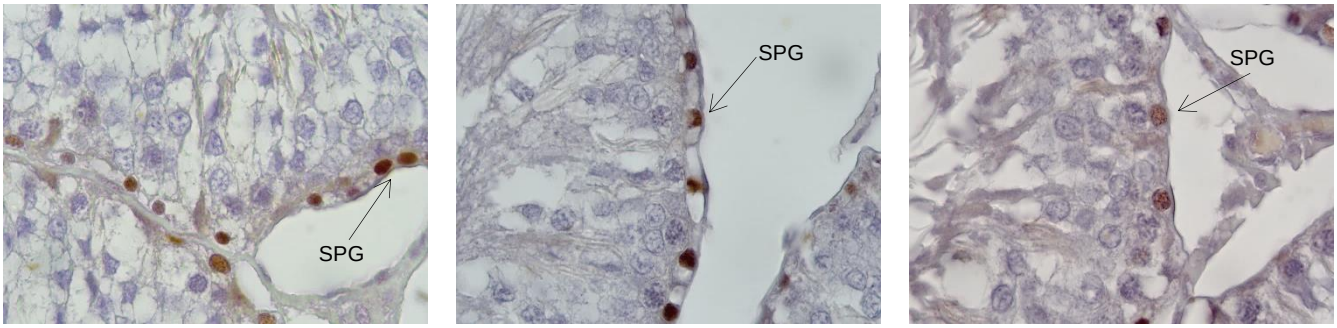


Figure 15 - Immunohistochemistry staining for the studied ORGs in rats' testes. Immunohistochemical staining for the expression of FTO **(a)**, MC4R **(b)**, GNPDA2 **(c)** and TMEM18 **(d)** in rats' testes. The images were obtained in two different magnifications: 10x (eyepiece) * 40x (objective) and 10x (eyepiece) * 100x (objective). (**Abbreviations**: FTO, fat mass and obesity associated; GNPDA2, glucosamine-6-phosphate deaminase 2; LC, Leydig cell; MC4R, melanocortin 4 receptor; MC, myoid cells; PS, primary spermatocyte; SC, Sertoli cell; SPC, spermatocyte; SPG, spermatogonia; SPT, spermatid; TMEM18, transmembrane protein 18)

By IHC, we were able to identify the presence of all the studied ORGs proteins in rat testicular tissue. FTO was present in rat testes (**Figure 15, (a)**), specifically in primary spermatocytes, spermatogonia and Leydig cells. MC4R was highly expressed in the primary spermatocytes, spermatogonia, myoid cells and Leydig cells (**Figure 15, (b)**). GNPDA2 appears to be present in late elongated spermatids and Leydig cells (**Figure 15, (c)**). TMEM18 was expressed in the early undifferentiated spermatogonia (**Figure 15, (d)**).

4.4. Expression of *FTO*, *MC4R*, *GNPDA2* and *TMEM18 transcripts* in testes and sperm of rats subjected to caloric restriction and to GLP-1 administration

Nutrigenomics states that energy intake influences gene (mRNA levels) expression.[258] Nutritional interventions such as caloric restriction and GLP-1 analogues administration have associated a decrease in food intake.[259,260] Thus, we hypothesized that in the highly energy demanding testes and sperm, the expression of the selected ORGs, that are in various ways related to the body energy homeostasis, might respond to those interventions and that this response could be part of the mechanism through these genes may promote potential effects in male reproductive function.

4.4.1. FTO, MC4R, GNPDA2 and TMEM18 expression in rats' testes is increased by caloric restriction and GLP-1 administration

We observed that CR promoted an increase *FTO*, *MC4R*, *GNPDA2* and *TMEM18* expression in rat testes, since the values of their mRNA abundance where higher when compared to the control group (5.30 ± 1.75 vs 1.01 ± 0.06 (**Figure 16, (a)**), 4.77 ± 1.37 vs 1.00 ± 0.04 (**Figure 16, (b)**), 2.89 ± 0.69 vs 1.02 ± 0.09 (**Figure 16, (c)**), 8.87 ± 3.75 vs 1.04 ± 0.16 (**Figure 16, (d)**), respectively). Relatively to GLP-1 administration, *FTO*, *MC4R, GNPDA2* and *TMEM18* also suffered an increase in their expression in testes because, once again, their mRNA abundance where higher when compared to the control group (3.43 ± 0.64 vs 1.01 ± 0.06 (**Figure 16, (a)**), 9.03 ± 0.61 vs 1.00 ± 0.04 (**Figure 16, (b)**), 8.27 ± 0.53 vs 1.02 ± 0.09 (**Figure 16, (c)**), 10.52 ± 3.75 vs 1.04 ± 2.01 (**Figure 16, (d)**), respectively).

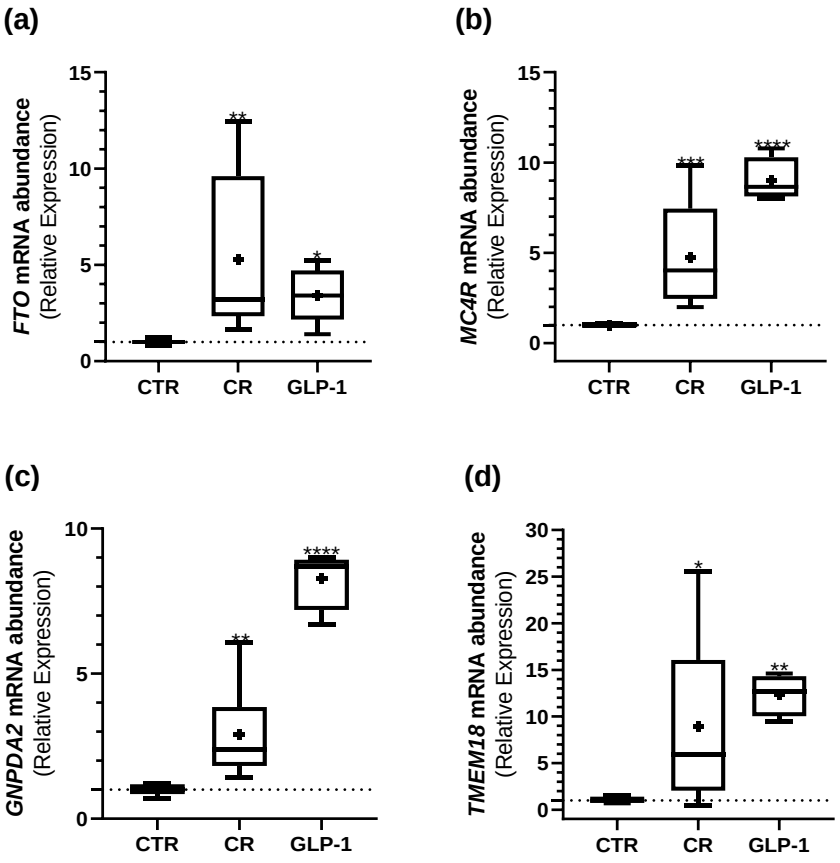


Figure 16 – Effect of CR and GLP-1 administration on the studied ORGs expression in rats' testes. The expression of ORGs (*FTO* **(a)**; *MC4R* **(b)**; *GNPDA2* **(c)**; *TMEM18* **(d)**) in rat testes subjected to CR and GLP-1 administration was accessed by qPCR. Results are expressed as mean ± SEM (*n* = 5 for CTR and GLP-1 condition and *n* = 6 to CR condition). * indicates statistically significant differences as compared to the CTR group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$). (**Abbreviations**: CR, caloric restriction; CTR, control; FTO, fat mass and obesity associated; GLP-1, glucagon-like peptide-1; GNPDA2, glucosamine-6-phosphate deaminase 2; MC4R, melanocortin 4 receptor; qPCR, quantitative polymerase chain reaction; TMEM18, transmembrane protein 18)

4.4.2. FTO, MC4R, GNPDA2 and TMEM18 expression is not changed in rat sperm by caloric restriction and GLP-1 administration

Contrary to what was observed regarding the expression of the ORGs under study in the rat testes, CR and the administration of GLP-1 did not promote any alteration in the expression of these ORGs in the rat sperm, when compared to the rats from the control group (**Figure 17**).

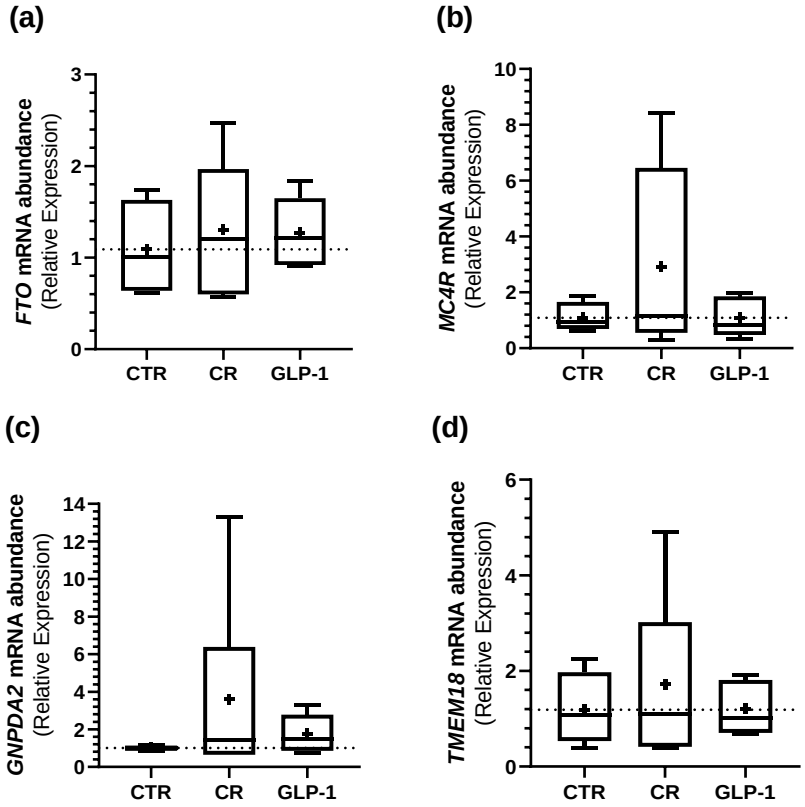


Figure 17 – Effect of CR and GLP-1 administration on the studied ORGs expression in rats' sperm. The expression of ORGs (**(a)** *FTO*; **(b)** *MC4R*; **(c)** *GNPDA2*; **(d)** *TMEM18*) in rats' sperm subjected to CR and GLP-1 administration was accessed by qPCR. Results are expressed as mean ± SEM (*n* = 5 for CTR and GLP-1 condition and *n* = 6 to CR condition). * indicates statistically significant differences as compared to the CTR group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$). (**Abbreviations**: CR, caloric restriction; CTR, control; FTO, fat mass and obesity associated; GLP-1, glucagon-like peptide-1; GNPDA2, glucosamine-6-phosphate deaminase 2; MC4R, melanocortin 4 receptor; qPCR, quantitative polymerase chain reaction; TMEM18, transmembrane protein 18)

4.5. Caloric restriction and GLP-1 administration impact on the rat testes and sperm oxidative status

4.5.1. Total antioxidant capacity in rat testes and sperm is not altered by caloric restriction and GLP-1 administration

Oxidative stress has been identified as one of the many mediators of male infertility by causing sperm dysfunction. Martins et al. found, using this animal model, that CR promote a decreased oxidative stress-induced damage in testes.[248] On the other hand, Zhao et al. observed that Di-(2-ethylhexyl) phthalate (DEHP), a common environmental endocrine disrupting chemical, promote testicular injury due to oxidative stress and that effect was

associated with increased global levels of m^{6}A RNA modification, particularly in nuclear factor erythroid 2-related factor 2 (Nrf2) mRNA, an important antioxidant transcription factor. Additionally, they observed a decreased expression of an important RNA methylation modulator gene - *FTO*. We hypothesized that the observed reduction of the oxidative stress-induced damage in testes and sperm under CR conditions might be associated with the increased *FTO* expression found by us. Oxidative stress can be caused by an increased ROS or due to a decreased antioxidant capacity, so we first analyzed the antioxidant capacity in both rat testes and sperm by FRAP assay to determine if the reduced oxidative stress in the testes of the CR rats observed by Martins et al. was associated with increased antioxidant properties (**Figure 18**).

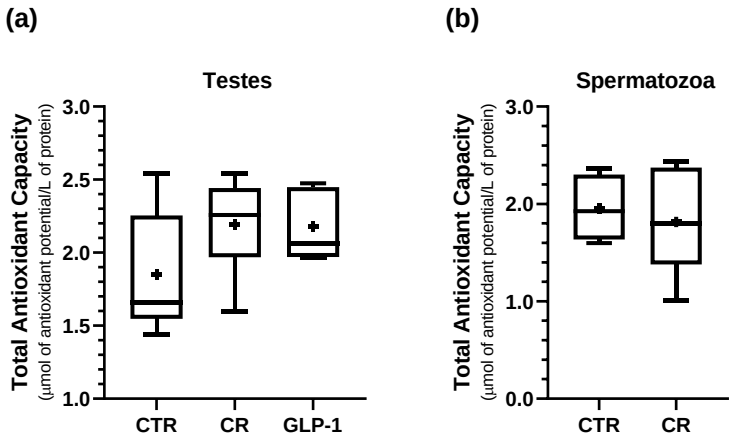


Figure 18 – Effect of caloric restriction and administration of GLP-1 on the total antioxidant capacity. The FRAP was measured in the rat testes **(a)** and sperm **(b)** of the rats subjected to CR and GLP-1 administration. The antioxidant power is expressed by the FRAP value (µmol of antioxidant potential / L of protein). are expressed as mean ± SEM (*n* = 5 for CTR and GLP-1 condition and *n* = 6 to CR condition). (**Abbreviations**: CR, caloric restriction; CTR, control; GLP-1, glucagon like-peptide 1)

We observe that the total antioxidant capacity was not altered in rats' testes neither by CR nor GLP-1 administration (**Figure 18, (a)**) in the rat sperm by CR (**Figure 18, (b)**), when compared to the sperm from the rats of the control group.

4.5.2. NEF2L2 is expressed in rat testes and sperm and its testicular expression is increased by caloric restriction and GLP-1 administration

NRF2, encoded by nuclear factor erythroid 2 like 2 (*NFE2L2*) gene, is a transcription factor that regulates the cellular defense against toxic and oxidative insults through the regulation of the expression of genes involved in oxidative stress response. As mentioned, it was reported by Zhao et al. that oxidative stress injury involves inhibition of the Nrf2 mediated antioxidant pathway due to increased m^{6}A modification of the *Nrf2* mRNA, which was correlated with a decreased *FTO* expression.[255] Thus, through conventional PCR, we intend to observe if the *NFE2L2* gene is expressed in rat testes and sperm (**Figure 19**).

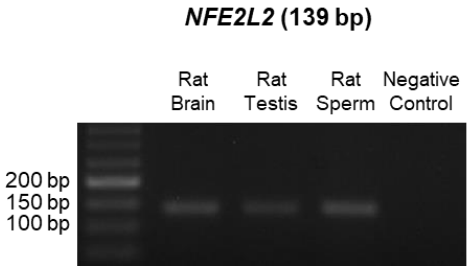


Figure 19 - Identification of *NFE2L2* gene transcripts in rat testes and sperm by conventional PCR. *NFE2L2* transcripts in both Wistar rats' testes and sperm were identified by conventional PCR. A molecular weight marker was used for determining the approximate size of the nucleic acid fragment. A cDNA-free sample was used as a negative control and a rat brain sample was used as a positive control. (**Abbreviations**: PCR, polymerase chain reaction; M, molecular weight marker; NFE2L2, nuclear factor erythroid-derived 2-like 2)

By conventional PCR, we observed the presence of *NFE2L2* transcripts, as a 139 bp staining band, in rat testes and sperm, has it had been already reported by Wadja et al.[261] (**Figure 19**).

Then we proceeded to the quantification of the expression of the *NFE2L2* gene by qPCR, in the testes (**Figure 20, (a)**) and sperm (**Figure 20, (b)**) of rats subjected to both CR and GLP-1 administration.

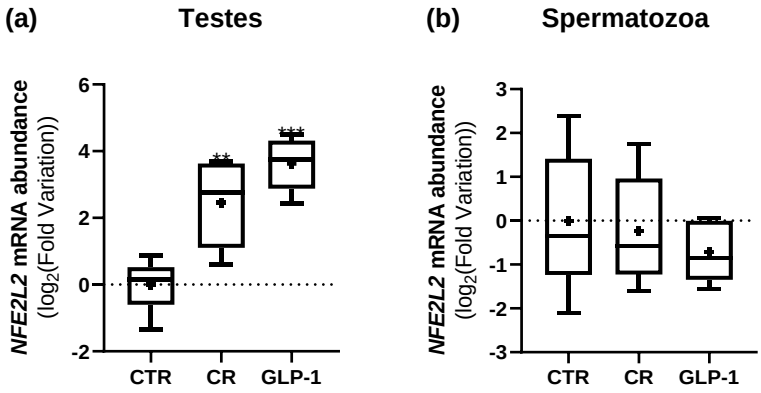


Figure 20 – Effect of CR and GLP-1 administration on *NFE2L2* expression in rat testes and sperm. The expression of *NFE2L2* in rats' testes **(a)** and sperm **(b)** subjected to CR and GLP-1 administration was accessed by qPCR. Results are expressed as mean ± SEM (n = 5 for CTR and GLP-1 condition and n = 6 to CR condition). * indicates statistically significant differences as compared to the CTR group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$). (**Abbreviations**: CR, caloric restriction; CTR, control; GLP-1, glucagon like-peptide 1; qPCR, quantitative real-time polymerase chain reaction; NFE2L2, nuclear factor erythroid-derived 2-like 2)

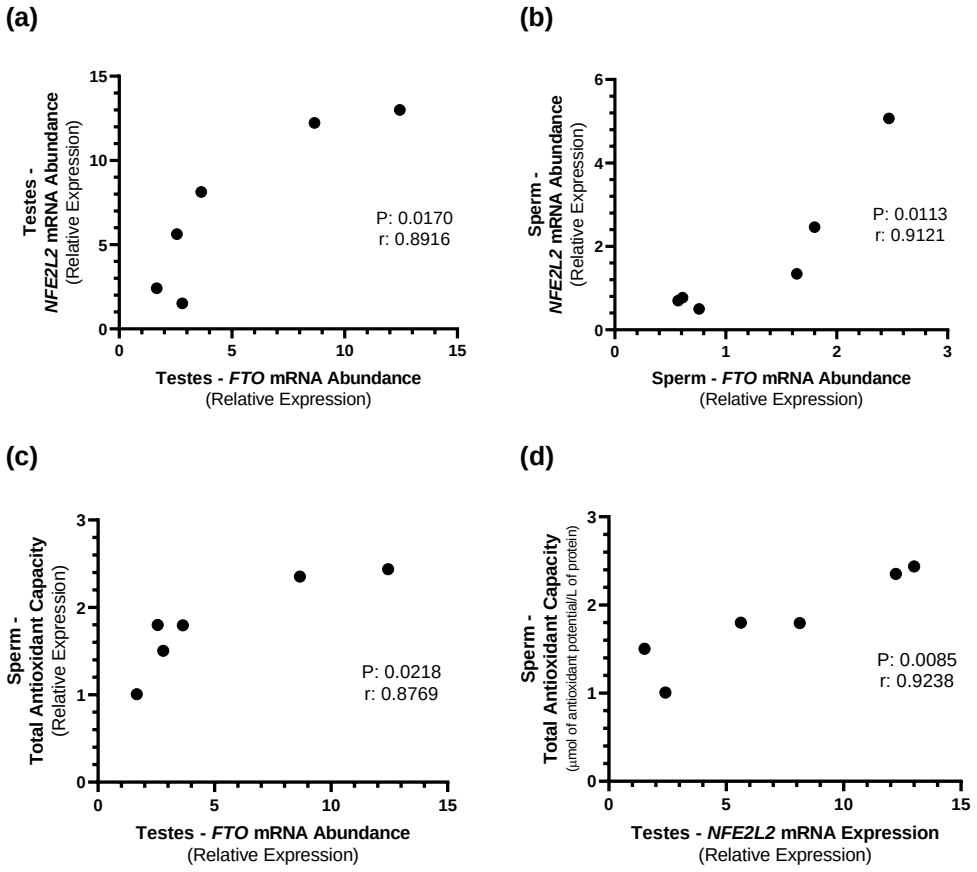
We could observe that *NFE2L2* expression in rat testes was increased in animals from the CR and GLP-1 groups (**Figure 20, (a)**) when compared to those of the control group (7.15 ± 1.98 vs 1.11 ± 0.23 and 13.77 ± 2.96 vs 1.11 ± 0.23, respectively). On the other hand, in rat sperm, there was no alteration in the *NFE2L2* expression in samples from CR and GLP-1 groups when compared to control group (**Figure 20, (b)**).

4.6. Correlations between the ORGs and the sperm quality in rats under caloric restriction and GLP-1 administration

One of our main objectives was to understand whether the ORGs under study were associated with the sperm quality in rats subjected to CR and GLP-1 administration. Thus, based on the functions and pathways in which the ORGs are known to be involved (in tissues other than testes and sperm), we examined the correlations (through Pearson correlations) between the results we obtained that seemed relevant and that could somehow give highlights about the role of these same genes on male fertility, and how they are related to changes in sperm quality caused by CR and GLP-1 administration. It is important to mention that, unless otherwise stated, the correlations were not observed in the control group.

4.6.1. FTO expression association with sperm oxidative status and concentration in rats under CR

It is already known the connection of *FTO* in the testes with their oxidative state.[255] In this follow-up, Pearson correlation between testes *FTO* mRNA abundance and testes' *NFE2L2* mRNA abundance revealed that under CR conditions these two genes become positively correlated (**Figure 21, (a)**; P: 0.0170; r: 0.8916). Pearson correlation also revealed that, in sperm, these two genes also are positively correlated (**Figure 21, (b)**; P: 0.0113; r: 0.9121) in animals under CR. Furthermore, also relatively to those animals, we observed that sperm total antioxidant capacity was positively correlated, through a Pearson correlation, with testes *FTO* mRNA abundance (**Figure 21, (c)**; P: 0.0218; r: 0.8769) and testes *NFE2L2* mRNA abundance (**Figure 21, (d)**; P: 0.0085; r: 0.9238). In addition, one of the parameters indicative of sperm quality, sperm concentration, was correlated with sperm *FTO* mRNA abundance (**Figure 21 (e)**; P: 0.0015; r: 0.9685) and sperm *NFE2L2* mRNA abundance (**Figure 21, (f)**; P: 0.0012; r: 0.9721).



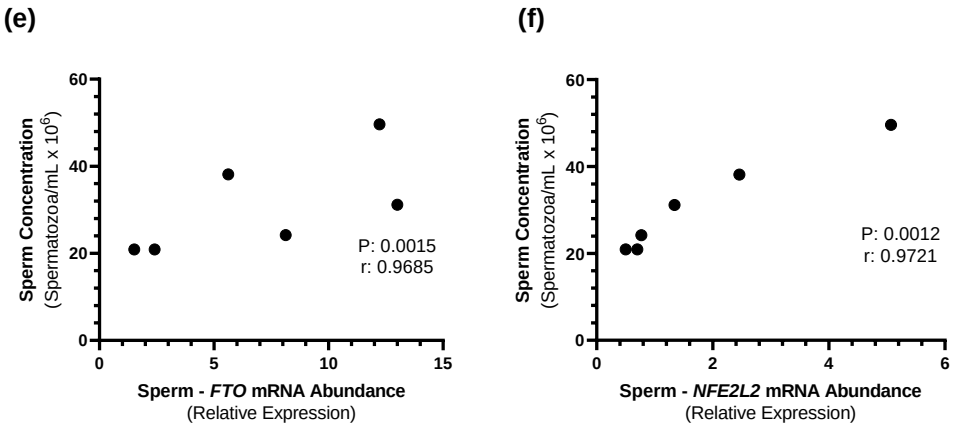


Figure 21 – ***FTO*** **abundance association with the sperm oxidative status and concentration in rats under CR.** Correlations between *FTO* and *NFE2L2* mRNA abundances in both testes **(a)** and sperm **(b)**, between sperm total antioxidant capacity and testes *FTO* **(c)** and *NFE2L2* **(d)** mRNA abundances and lastly between sperm concentration and sperm *FTO* **(e)** and *NFE2L2* **(f)** mRNA abundances. The associations were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: CR, caloric restriction; FTO, fat mass and obesity associated; NFE2L2, nuclear factor erythroid-derived 2-like 2)

Another indicative evidence of the indirect relationship of *FTO* with the sperm oxidative status and consequently sperm quality, under CR conditions comes from the existence of a positive correlation between *NFE2L2* mRNA abundance in the testes of these rats with ghrelin plasmatic levels (**Figure 22, (a)**, P: 0.0341; r: 0.8452). In turn, ghrelin plasmatic levels are positively correlated with sperm viability (**Figure 22, (b)**, P: 0.0234; r: 0.8723) and with sperm total antioxidant capacity (**Figure 22, (c)**, P: 0.0017; r: 0.9663).

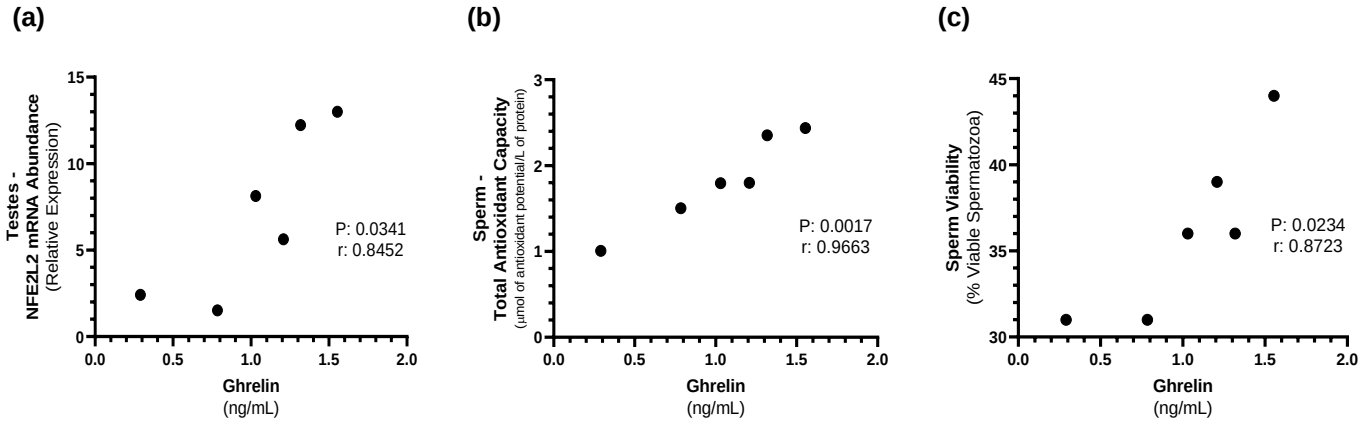


Figure 22 – ***FTO*** **abundance indirect association with the sperm oxidative status and viability in rats under CR.** Correlations between *NFE2L2* mRNA abundance and ghrelin plasmatic levels **(a),** and between ghrelin and sperm viability **(b)** and total antioxidant capacity **(c)**. The associations were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: CR, caloric restriction; NFE2L2, nuclear factor erythroid-derived 2-like 2)

4.6.2. Association between FTO expression and the sperm morphology in rats subjected to GLP-1 administration

Pearson correlation between *FTO* mRNA abundance in sperm and the active GLP-1 plasmatic levels revealed that, in the rats subjected to GLP-1 administration, these two parameters become positively correlated (**Figure 23**; P: 0.0442; r: 0.8881).

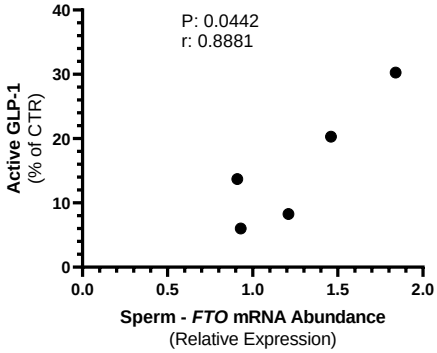


Figure 23 – ***FTO*** **abundance association with the active GLP-1 in rats subjected to GLP-1 administration.** Associations between *FTO* mRNA abundance and active GLP-1 plasma levels were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: FTO, fat mass and obesity associated; GLP-1, glucagon like-peptide 1)

In this experimental group, we also observed that *FTO* transcript abundance is negatively correlated with the insulin plasmatic levels (**Figure 24, (a)**; P: 0.0121; r: -0.9532) and with the HOMA-IR value (**Figure 24, (b)**; P: 0.0103; r: -0.9578). These two mentioned correlations, despite already existing in the control group, their strength intensified in the CR group, since the P value decreased. In turn, the insulin levels are negatively correlated with the percentage of sperm with normal morphology in the rats subject to GLP-1 administration (**Figure 24, (c)**; P: 0.0384; r: -0.8982).

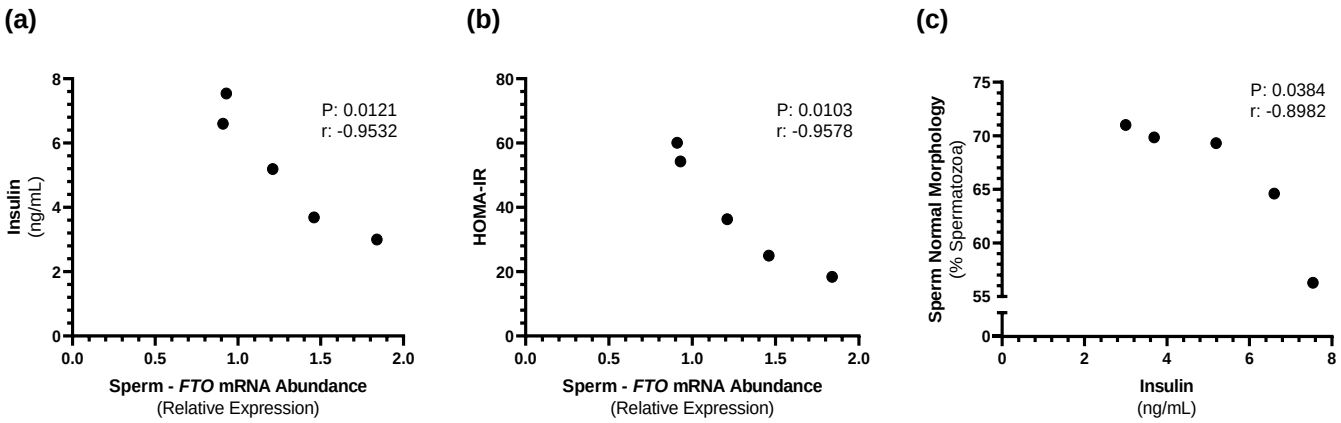


Figure 24 – ***FTO*** **abundance indirect association with the percentage of sperm with normal morphology in rats subjected to GLP-1 administration.** Associations of *FTO* mRNA abundance in sperm with plasmatic insulin levels **(a)** and with HOMA-IR **(b),** and between plasmatic insulin plasmatic levels and sperm with normal morphology **(c).** These associations were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: FTO, fat mass and obesity associated; GLP-1, glucagon like-peptide 1; HOMA-IR, homeostatic model assessment for insulin resistance)

4.6.3. Association between MC4R and neuropeptide NPY expressions in rats under CR

MC4R is involved in the melanocortin pathway, and its activation induce satiety and increase energy utilization, thereby promoting weight loss. Briefly, that receptor is stimulated by POMC/CART neurons and inhibited by AgRP/NPY neurons.[197] In this follow-up, we observed that there is an interesting positively correlation between the *MC4R* transcript abundance in testes of rats under CR and the hypothalamic *NPY* transcript abundance (**Figure 25**, P: 0.0486; r: 0.8807).

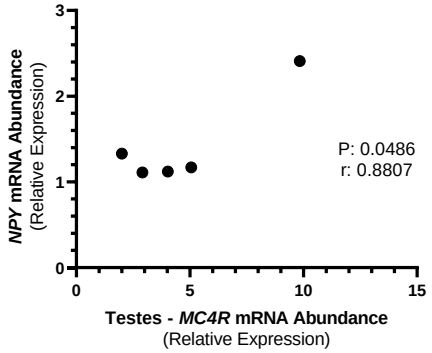


Figure 25 – ***MC4R* abundance association with the neuropeptide *NPY* abundance in rats under CR.** Association of *MC4R* mRNA abundance in testes and the hypothalamic *NPY* mRNA abundance was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: CR, caloric restriction; MC4R, melanocortin 4 receptor; NPY, Neuropeptide Y)

NPY neurons in the arcuate nucleus of the hypothalamus are critical control centers for insulin's central action on control energy homeostasis, as well as glucose homeostasis regulation. Furthermore, NPY activation is also associated with energy expenditure reduction.[262] In this sequence, Pearson correlations between testes *NYP* mRNA abundance and glucose plasmatic levels revealed that under CR conditions these two parameters become positively correlated (**Figure 26, (a)**; P: 0.0125; r: 0.9074). Furthermore, we observed a positive correlation between testes *NYP* mRNA abundance and resting energy expenditure (REE) (**Figure 26, (b)**; P: 0.0188; r: 0.8859).

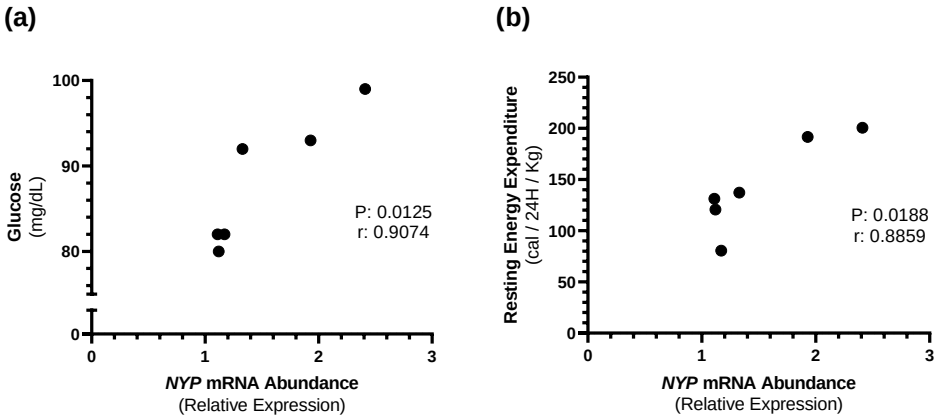


Figure 26 – ***NPY* neuropeptide abundance association with the glucose plasmatic levels and REE in rats under CR.** Associations of the hypothalamic *NPY* mRNA abundance with the glucose plasmatic levels **(a)** and REE **(b)** were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: CR, caloric restriction; NPY, Neuropeptide Y; REE, resting energy expenditure)

4.6.4. Association between MC4R expression and sperm oxidative status in rats under CR

In addition, we realized that *MC4R*, like *FTO*, may be involved in regulating the testes' oxidative status in the rats under CR. We observed strong positive correlations between *FTO* mRNA abundance with testes and sperm *MC4R* mRNA abundance (**Figure 27, (a)**; P: 0.0125; r: 0.9521 and **Figure 27, (b)**; P: 0.0008; r: 0.9761, respectively). The *MC4R* mRNA abundance, in turn, showed to be positively correlated with sperm total antioxidant capacity (**Figure 27, (c)**; P: 0.0462; r: 0.8189) and with *NFE2L2* mRNA abundance in testes (**Figure 27, (d)**; P: 0.0082; r: 0.9251).

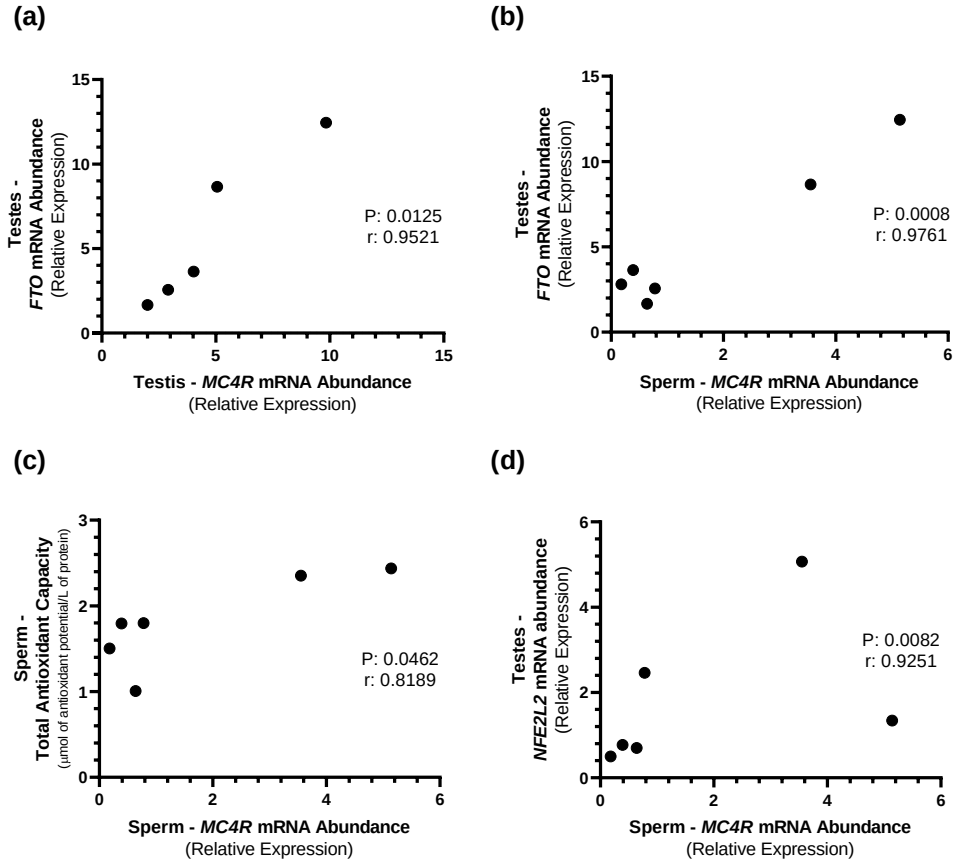


Figure 27 – ***MC4R* abundance direct and indirect association with the sperm oxidative status in rats under CR.** Associations between testes **(a)** and sperm **(b)** *MC4R* mRNA abundance with testes *FTO* mRNA abundance and between sperm *MC4R* mRNA abundance with sperm total antioxidant capacity **(c)** and with *NFE2L2* mRNA abundance **(d)**. These correlations were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: CR, caloric restriction; FTO, fat mass and obesity associated; MC4R, melanocortin 4 receptor; NFE2L2, nuclear factor erythroid-derived 2-like 2)

4.6.5. Association between MC4R expression and the sperm oxidative status in rats subjected to GLP-1 administration

In the GLP-1 experimental group, we discovered that the *MC4R* mRNA abundance is strongly and positively correlated with the *NFE2L2* mRNA abundance in sperm of that rats (**Figure 28**; P: < 0.0001; r: -0.9984), being that this correlation was not observed in the control rats. This might indicate a possible indirect role of *MC4R* in the regulation of the oxidative status in the sperm of rats subjected to GLP-1 administration.

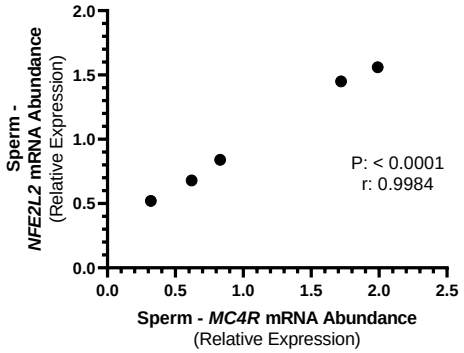


Figure 28 – ***MC4R*** **abundance indirect association with the sperm oxidative status in rats subjected to GLP-1 administration.** Association of *MC4R* mRNA abundance and the *NFE2L2* mRNA abundance in sperm of rats subjected to GLP-1 administration was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: GLP-1, glucagon like-peptide 1; MC4R, melanocortin 4 receptor; NFE2L2, nuclear factor erythroid-derived 2-like 2)

4.6.6. Association between GNPDA2 expression and the head defects in sperm of rats under CR

GNPDA2 is part of the HSP, in which the final product is UDPGlcN6P, the substrate used by OGT for O-GlcNAc glycosylation of proteins. This glycosylation, in turn, is an indispensable process for the formation of morphologically normal sperm. In this follow-up, we observed a positive correlation between the *GNPDA2* transcript abundance in testes of rats under CR and the head defects of their sperm (**Figure 29**; P: 0.0393; r: 0.8334).

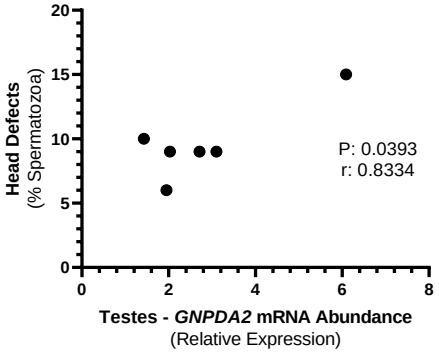


Figure 29 – ***GNPDA2*** **abundance association with the head defects in sperm of rats under CR.** Association of *GNPDA2* mRNA abundance in testes and the head defects of sperm of rats under CR was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: CR, caloric restriction; GNPDA2, glucosamine-6-phosphate deaminase 2)

4.6.7. Association between GNPDA2 expression and sperm oxidative status and concentration in rats subjected to GLP-1 administration

Pearson correlation between sperm *GNPDA2* mRNA abundance and sperm *NFE2L2* mRNA abundance revealed that, in rats subjected to GLP-1 administration, these two genes become positively correlated (**Figure 30, (a)**; P: 0.0088; r: 0.9623). Furthermore, we observed that sperm *GNPDA2* mRNA abundance is positively correlated, through Pearson correlation, with sperm concentration of those rats (**Figure 30, (b)**; P: 0.0270; r: 0.9198).

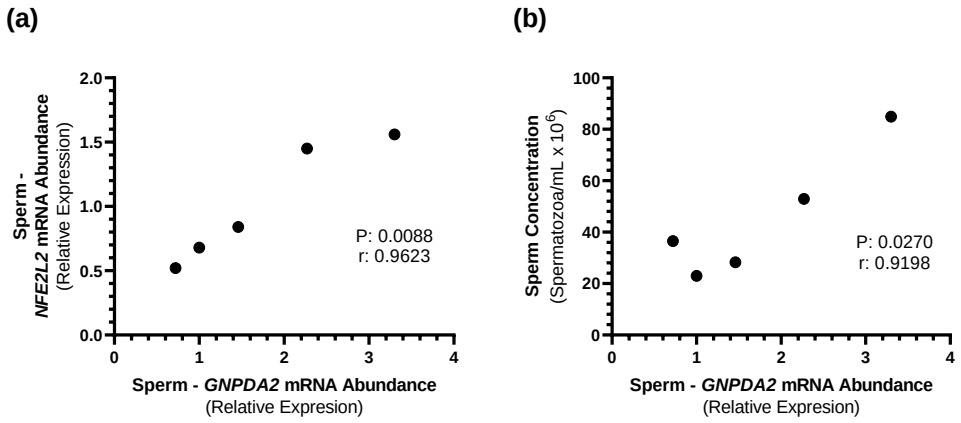


Figure 30 – ***GNPDA2*** **abundance association with the sperm oxidative status and concentration in the rats subjected to GLP-1 administration.** Associations between *NFE2L2* mRNA abundance in sperm **(a)** and sperm concentration **(b)** with *GNPDA2* mRNA abundance also in sperm. The associations were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: GLP-1, glucagon like-peptide 1; GNPDA2, glucosamine-6-phosphate deaminase 2; NFE2L2, nuclear factor erythroid-derived 2-like 2)

4.6.8. Association between TMEM18 expression and sperm concentration and viability in rats under CR

TMEM18 activates PPARG, particularly by upregulating PPARG promoter activity, being that PPARG seem to be related with the improvement of insulin resistance.[263,264] In this follow-up, under CR conditions, Pearson correlation between sperm *TMEM18* mRNA abundance and HOMA-IR value revealed that under CR conditions these two parameters become negatively correlated (**Figure 31, (a)**; P: 0.0044; r: 0.9452). Furthermore, we observed a positive correlation between sperm concentration and TMEM18 mRNA abundance in sperm (**Figure 31, (b)**; P: 0.006; r: 0.9804) and a negative correlation between sperm concentration and HOMA-IR (**Figure 31, (c);** P: 0.0160; r: -0.8950).

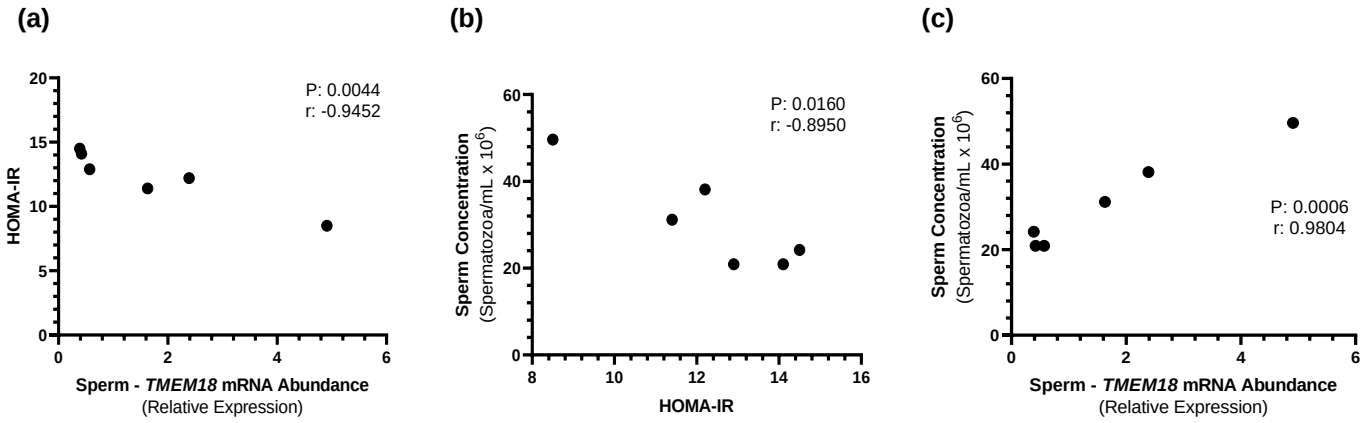


Figure 31 – ***TMEM18*** **abundance association with sperm concentration of the rats under CR.** Association between *TMEM18* mRNA abundance in sperm and HOMA-IR **(a)** and associations of sperm concentration with *TMEM18* mRNA abundance in sperm **(b)** and HOMA-IR **(c)** were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: CR, caloric restriction; HOMA-IR, homeostatic model assessment for insulin resistance; TMEM18, transmembrane protein 18)

In addition to being associated with sperm concentration, TMEM18 also seemed to be associated with sperm viability. This because we observed the existence of a positive correlation between *TMEM18* mRNA abundance and sperm viability (**Figure 32**; P: 0.0258; r: 0.8657) in rats under CR.

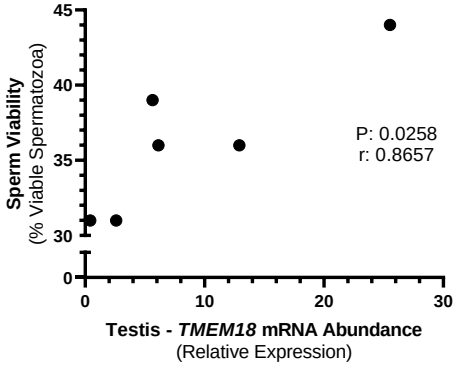
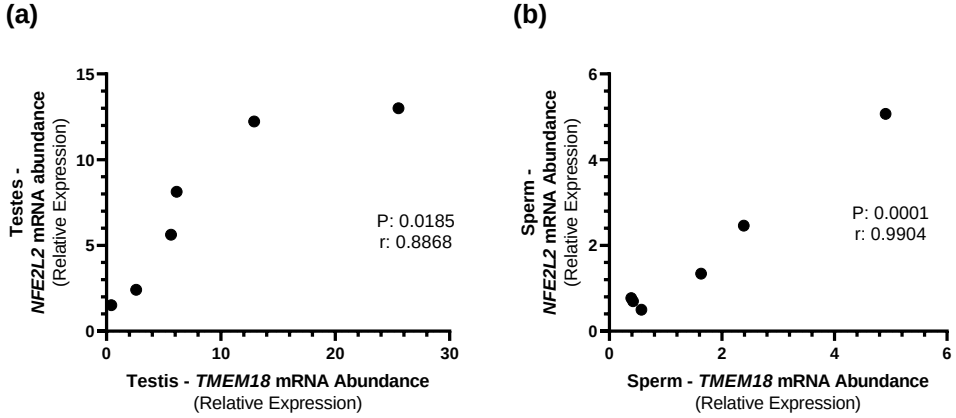


Figure 32 – ***TMEM18*** **abundance association with sperm viability in rats under CR.** Association between *TMEM18* mRNA abundance in sperm and sperm viability was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: GLP-1, glucagon like-peptide 1; TMEM18, transmembrane protein 18)

4.6.9. Association between TMEM18 expression and sperm oxidative status of in rats under CR

Two transcription factors, Nrf2 (encoded by *NFE2L2*) and PPARG, have been shown to play key roles in establishing a cellular antioxidative defense system. Regarding to this, Pearson correlation between testes *TMEM18* mRNA abundance and testes *NFE2L2* mRNA abundance revealed that under CR conditions the abundances of the two transcripts become positively correlated (**Figure 33, (a)**; P: 0.0185; r: 0.8868). Pearson correlation also revealed that, in sperm, the abundances of these two transcripts also become positively correlated (**Figure 33, (b)**; P: 0.0001; r: 0.9904). Another evidence of the association of TMEM18 with the sperm oxidative status in the rats under CR comes from the fact that we observed strong positive correlations between *TMEM18* mRNA abundance with testes and sperm *FTO* mRNA abundance (**Figure 33, (c)**; P: 0.0020; r: 0.9630 and **Figure 33, (d)**; P: 0.0031; r: 0.9539, respectively). This happens because *FTO* abundance also seemed be linked to the decrease in testes oxidative stress. The final indication of the association of *TMEM18* abundance with the sperm oxidative status comes from the positive correlation between the TMEM18 mRNA abundance in sperm with the sperm total antioxidant capacity (**Figure 33, (e)**; P: 0.0412; r: 0.8293).



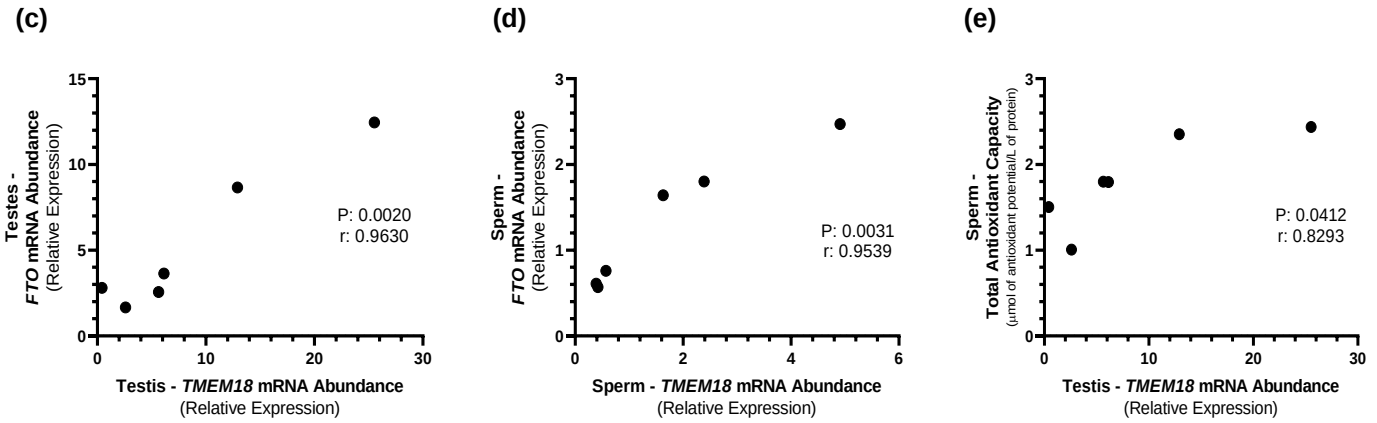


Figure 33 – ***TMEM18*** **abundance direct and indirect association with the sperm oxidative status in rats under CR.** Associations between *TMEM18* mRNA abundance and *NFE2L2* mRNA abundance in both testes **(a)** and sperm **(b)**, between *TMEM18* mRNA abundance and sperm total antioxidant capacity **(c)** and between total *TMEM18* mRNA abundance with *FTO* mRNA abundance in both testes **(d)** and sperm **(e)**. The associations were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: CR, caloric restriction; FTO, fat mass and obesity associated; NFE2L2, nuclear factor erythroid-derived 2-like 2; TMEM18, transmembrane protein 18)

4.6.10. Association between TMEM18 expression and sperm oxidative status in rats subjected to GLP-1 administration

Similarly to what was observed in the CR group, in the GLP-1 experimental group, we observed, through Pearson correlations, that the *TMEM18* mRNA abundance is strongly and positively correlated with the *NFE2L2* mRNA abundance in sperm of those rats (**Figure 34**; P: 0.0075; r: -0.9659).

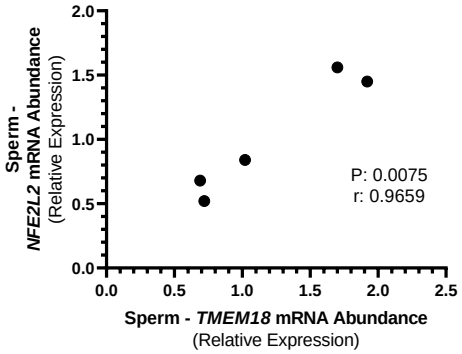


Figure 34 – ***TMEM18*** **abundance indirect association with the sperm oxidative status in rats subjected to GLP-1 administration.** Association between *TMEM18* mRNA abundance and the *NFE2L2* mRNA abundance in sperm was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant. (**Abbreviations**: GLP-1, glucagon like-peptide 1; NFE2L2, nuclear factor erythroid-derived 2-like 2; TMEM18, transmembrane protein 18)

4.6.11. Association between the abundance of the ORGs transcripts in sperm and testes of rats under CR and GLP-1 administration

Through Pearson's correlations, we observed that there are associations between the abundance of the different ORGs transcripts under study, in both sperm and testes of rats subjected to CR (**Table 3**) and to administration of GLP1 (**Table 4**), being that all these associations translate into positive correlations. Most of the presented correlations did not exist in the control group and became highly significant in the animals of the CR and GLP-1 groups. In **Tables 3** and **4** we can observe that essentially in rats subjected to CR, there is a generalized correlation between all ORGs in testes and sperm, indicating that their response to CR is somehow interconnected.

Table 3 - Pearson correlation coefficients between the ORGs abundance in sperm and testes of the rats under CR.

		Testes				**Sperm**			
		FTO	***MC4R***	***GNPDA2***	***TMEM18***	***FTO***	***MC4R***	***GNPDA2***	***TMEM18***
Testes	***FTO***								
	MC4R	P: 0.0125 r: 0.9521							
	GNPDA2	P: 0.0050 r: 0.9420	P: < 0.0001 r: 0.9990						
	TMEM18	P: 0.0020 r: 0.9630	P: 0.0015 r: 0.9885	P: 0.0022 r: 0.9611					
Sperm	***FTO***	---	---	---	---				
	MC4R	P: 0.0008 r: 0.9761	P: 0.0348 r: 0.9047	P: 0.0180 r: 0.8884	P: 0.0025 r: 0.9591	---			
	GNPDA2	P: 0.0094 r: 0.9198	P: 0.0102 r: 0.9582	P: 0.0027 r: 0.9571	P: 0.0021 r: 0.9627	---	P: 0.0082 r: 0.9251		
	TMEM18	---	---	---	---	P: 0.0031 r: 0.9539	---	---	

Table 4 - Pearson correlation coefficients between the ORGs abundance in sperm and testes of the rats subjected to GLP-1 administration.

		Testes				**Sperm**			
		FTO	***MC4R***	***GNPDA2***	***TMEM18***	***FTO***	***MC4R***	***GNPDA2***	***TMEM18***
Testes	***FTO***								
	MC4R	---							
	GNPDA2	---	---						
	TMEM18	P: 0.0276 r: 0.9185	---	---					
Sperm	***FTO***	---	---	---	---				
	MC4R	---	---	---	---	---			
	GNPDA2	---	---	---	---	---	P: 0.0052 r: 0.9733		
	TMEM18	---	---	---	---	---	P: 0.0134 r: 0.9498	---	

(**Abbreviations**: CR, caloric restriction; FTO, fat mass and obesity associated; GLP-1, glucagon-like peptide-1; GNPDA2, glucosamine-6-phosphate deaminase 2; MC4R, melanocortin 4 receptor; ORG, obesity related gene; TMEM18, transmembrane protein 18)

5. Discussion

The male reproductive function depends on a balanced energy homeostasis.[252] GWAS have identified a variety of new SNPs associated with metabolism and maintaining energy balance, being highly associated with obesity. Most of these SNPs are in introns or intergenic regions, suggesting that they affect the regulation of the corresponding gene or nearby genes. Little is known about most of these ORGs, being that the understanding of their regulation may be a key step to provide important clues. Many genes involved in energy homeostasis are regulated in response to feeding and fasting or by dietary components.[239] Thus, the identification of such regulatory patterns would provide information about a potential role in the male reproductive function, namely regarding the influence in the molecular mechanisms responsible for ensuring male reproductive physiological functions.

To understand the regulation of the ORGs and if they are nutritionally regulated, we examined how changes in the energy balance affect their expression. Thus, in this study, we proposed to study whether the four selected ORGs - *FTO*, *MC4R*, *GNPDA2* and *TMEM18* - are expressed in rat testes and sperm and how their expression can be modulated by weight loss interventions such as CR and administration of GLP-1. In addition, we intended to determine if those conditions affect the male reproductive potential, namely through sperm quality analysis. To achieve these purposes, a group of rats was subjected to a 28-day food restriction of 30% less calories and another group was subjected to intraperitoneal implantation of an osmotic mini-pumps for delivery of active GLP-1 during the same 28-day period. Intraperitoneal implantation was meant to ensure that the peptide would be delivered near to the nervous terminals as occurs in normal physiological conditions and to ensure the vagal pathway activation, while also increasing the systemic GLP-1 levels. The GLP-1 dosage selected was chosen based on the presumption that basal GLP-1 secretion would not be affected in a negative feedback loop, as previously reported.[265] An additional control group of rats not subjected to any food restriction or intervention was also included.

As we are dealing with two interventions that affect body energy balance and bearing in mind that the ORGs under study are largely related to the body energy homeostasis, we started by analyzing the response of plasma levels of glucose and GLP-1 and of some hormones related to energy homeostasis (leptin, ghrelin and insulin) to both CR and administration of GLP-1. Indeed, it was reported that the levels of these hormones are severely altered in individuals even after 48 hours of CR,[266] which illustrate the importance of these hormones acting as energy sensors. Furthermore, we analyzed the hypothalamic expression of four important neuropeptides - *NPY*, *AgRP*, *POMC* and *CART* – since they are centrally involved in the neuroendocrine function of controlling the body energy balance which includes the control of energy intake and the energy expenditure, being that their expression was not altered by CR or GLP-1 administration. The energy balance not only encompasses energy consumption but also includes energy expenditure and for this reason, one of its components - REE - was also measured being that this parameter was not affected by CR or GLP-1 administration.

Relatively to the rats subjected to CR, their plasma levels of total GLP-1 increased relatively to the control group, as already been reported in individuals under CR,[267] while active GLP-1 and active/total GLP-1 were not altered. This seems to indicate that CR promoted greater GLP-1 secretion, but the degradation by DPP-4 would also have intensified to the point where the active GLP-1 did not undergo significant changes. Consequently, the proportion of active GLP-1 relative to total GLP-1 remained. We calculated the HOMA-IR value since it is an important aspect when it comes to energy balance because in individuals with insulin resistance, the cells are unable to use insulin effectively, and it is essential for regulating the amount of glucose that circulates in the bloodstream. The HOMA-IR value, which is influenced by variations in insulin and glucose levels, was reduced in these rats when compared to the control rats. CR promoted a decrease in the fasting plasma levels of insulin but did not change the fasting plasma levels of glucose when compared to the control group. Thus, the decrease in the value of HOMA-IR was due exclusively to the effect that CR had on insulin levels. As could be expected, as glucose lowering effects of GLP-1 are dependent on the establishment of hyperglycemia, a decrease of fasting glucose was not expected to

occur. We observed an improvement in insulin resistance that may be explained by the sustained increase in total GLP-1 release, supporting the notion of an inverse correlation between GLP-1 and insulin levels.[268] In fact, we observed a positive correlation between insulin levels and HOMA-IR, in CR subjected rats. Still, despite GLP-1 levels were effectively increased and insulin levels decreased, we did not observe the correlation between those parameters in these animals.

A hallmark of continuous CR is the increased appetite signals and decreased satiety signals. Specifically, CR is known to increase hunger once there is a decrease in the satiety hormone leptin and an increase in the hunger hormone ghrelin,[269] which was observed in this study. It is important to note that the increased biological hunger in the presence of restricted energy intake can lead to a physiological barrier to weight loss maintenance by increasing the likelihood overconsumption of calories. There is evidence that these metabolic hormonal changes can persist for over a year, posing a significant barrier to long-term weight loss maintenance.[269,270] The fact that there is a decreased leptin/ghrelin ratio also suggests an increased appetite in the rats under CR. Previous studies have found that decreased leptin to ghrelin ratio correlated with increased hunger and appetite[251] therefore, we can suppose that with the observed lower leptin to ghrelin ratio, in our CR animals, hunger and appetite should be increased. In fact, this suggests an increased vulnerability to body weight regain. Furthermore, we observed a negative correlation between leptin and insulin. This relation makes sense since there is a bidirectional feedback loop, or adipoinsular axis, between adipose tissue and pancreatic β-cells via leptin and insulin. When adipose stores diminish, falling plasma leptin levels would permit increased insulin production, thereby eliciting in the deposition of additional fat.[56] Although there is a negative correlation between these two hormones, both had their levels decreased when compared to the control group, as already mentioned. This might indicate that there was a decrease in adiposity, leading to a decrease in leptin levels, and that even so, the CR did not allow insulin levels to increase due to the feedback loop, and that inclusive decreased, with a decrease in insulin resistance.

CR is known to promote weight loss[123], which was not observed in our study. Rats subjected to CR did not lose weight relative to the control groups but gained significantly less weight. This could mean that the CR rats' energy balance was equally positive (i.e. energy intake > energy expenditure). However, this energy balance was less positive than that of the control rats, thus resulting in lower body weight gain. The changes in the energy balance occurred due to changes in one side of the energy balance equation - energy intake - for obvious reasons of caloric restriction of 30%, but this should still have continued to surpass the rats' energy expenditure. In individuals subject to CR the energy expenditure seems to decrease over time due to metabolic function, being that this decline can occur through decreases in both resting energy expenditure (e.g., adaptive thermogenesis) and non-resting energy expenditure (e.g., reductions in energy use for daily living).[269] However, regarding the resting energy expenditure, we did not observe significant differences compared to the control group. The hypothalamic expression of the neuropeptides *NPY*, *AgRP*, *POMC*, and *CART*, that are highly influential relatively to the energy balance, was also not altered by CR. The fact that there was a lower energy intake (despite the energy expenditure having remained unchanged) may then have been the responsible factor for the significant lower body weight gain in the rats subjected to CR.

Concerning the animals from the experimental group subjected to administration of active GLP-1, as expected we observed increased levels of active GLP-1 and increased active/total GLP-1 ratio, when compared to control group. The levels of total GLP-1 did not change indicating that there was no induction of GLP-1 secretion at the level of β-cells. So, the active GLP-1 levels increased exclusively due to exogenous administration. Glucose and insulin fasting plasma levels were not altered by the administration of GLP-1 and therefore there was no change in the value of HOMA-IR. Once again, as GLP-1 activity is glucose-dependent, GLP-1 do not stimulate insulin secretion or suppress glucagon secretion when glucose levels are not raised.[271] Thus, considering that glucose levels were normal in these rats, it makes sense that insulin levels were also unchanged by GLP-1 administration. Although GLP-1 is widely associated with achieving satiety, the fasting plasma levels of leptin and ghrelin, the satiety and hunger hormones, respectively, were not changed in this group when compared to the control rats. This is consistent with

the fact that the rats subjected to GLP-1 administration did not show any significant differences regarding to the body weight or body weight gain when compared to the control group. This shows that the confirmed increase in active GLP-1 levels in these rats, by intraperitoneal administration, did not promote any effects regarding to the measured parameters that are related to body energy balance and consequently to body weight. In fact, GLP-1 therapy, more precisely in individuals with T2D or with obesity, are known to reduce food intake, appetite and hunger and promote fullness and satiety with the ultimate result of promoting weight loss. However, GLP-1' pharmacokinetic/pharmacodynamic profile is such that native GLP-1 is not therapeutically useful. Thus, although GLP-1 might be effective when given continuously, it is highly susceptible to enzymatic degradation *in vivo*, namely to cleavage by DPP-4 that occurs quickly and generates a non-insulinotropic metabolite. Strategies to take advantage of the therapeutic potential of GLP-1, based on an understanding of the factors that influence its metabolic stability and pharmacokinetic/pharmacodynamic profile, have therefore been the focus of research. Strategies include DPP-4 resistant GLP-1 analogues and selective enzyme inhibitors to prevent *in vivo* degradation of the peptide. These analogs maintain their affinity for the GLP-1 receptor and are more stable, resulting in greater potency than native GLP-1.[272] This could be a possible explanation for the fact that the administration of GLP-1 did not promote its known effects regarding the inhibition of food intake and consequent impact body weight.

The observed altered hormonal profile, mainly in CR group, associated with the biometric parameters, highlights the impact of nutritional interventions on overall body energetic signaling. It is well known that there are several genes, such as *FTO*, *MC4R*, *GNPDA2* and *TMEM18,* that encode factors that regulate food and energy intake and factors implicated in energy expenditure. Polymorphisms or genetic variants in those genes are highly associated with obesity, and that is why they are known by obesity-related genes.[34] As energy balance is known to modulate spermatogenesis, we hypothesized that CR and GLP-1 administration could impact the male reproductive function altering sperm quality and this effect could be mediated or associated with the expression of the selected ORGs.

FTO is known to be highly expressed in brain tissue and controls appetite and feeding behavior which is consistent with the role of this gene in the regulation of body mass. But the role of *FTO* in other tissues, including testes, is still not clear. Our study confirmed that *FTO* is expressed in rat testes, as it was previously reported by Zhao et al.,[255] and we reported its expression in rat spermatozoa for the first time. We were also able to show that the expression of the correspondent protein in the testes is mainly located in primary spermatocytes, spermatogonia and Leydig cells.

How *FTO* is affected by nutritional status is somewhat controversial, namely in the hypothalamus, which has been the main target tissue of study. The majority of the studies[273-275] found an increased *FTO* gene expression after CR, being in agreement with our results, but for example Poritsano et al.[276] claimed a decreased *FTO* expression after CR. To our knowledge, there are no other studies that have investigated the effect of CR or the effect of GLP-1 administration on the expression of *FTO* in the testes and sperm. In this follow-up, we demonstrated that *FTO* expression in testes was increased in the rats subjected to CR and to GLP-1 administration, but it remained unchanged in sperm.

We have hypothesized that the observed reduction of the oxidative stress-induced damage in testes conditions of CR demonstrated by Martins et al[248], in this same animal model, could be associated with the increased *FTO* expression we observed in the rat testes. In fact, the CR is known to promote a decrease in oxidative damage in several tissues and organs although that effect is dependent on the duration of CR and is tissue specific.[277] The proposed relationship could be mediated by an increase in the *NFE2L2* gene expression that encodes for Nrf2 protein which is, in turn, an important transcription factor of antioxidant defense related genes. We raise this hypothesis taking in consideration that Zhao et al. observed in a study related with the effects of the DEPH. They showed an oxidative stress-induced testicular damage promoted by this compound, having also been observed a decreased *FTO* expression. These two aspects were then related because the decreased *FTO* expression led to an increased global level of m^6A RNA modification, particularly in *Nrf2* mRNA. This occurred because *FTO* is an RNA methylation

modulator gene responsible for removing specific methylations. The altered m^{6}A modification is known to have an important role in male reproductive dysfunction, so in this case, the altered m^{6}A modification in *Nrf2* mRNA lead to reduce Nrf2 protein levels, affecting the Nrf2-mediated antioxidant system and leading to aggravated oxidative stress and testicular injury.[255] In agreement with our hypothesis, we found that the increased *FTO* expression in testes was correlated with the also increased *NFE2L2* expression in testes. That positive correlation was as well observed in sperm, although the expression of *FTO* and *NFE2L2* were not changed in the CR group when compared to the control rats. Interestingly, *FTO* and *NFE2L2* expression in rat testes was shown to be positively correlated with the unchanged sperm total antioxidant capacity. In addition, *FTO* and *NFE2L2* expressions, this time in rats' sperm, were positively correlated with the sperm concentration. This suggests that CR may be indirectly inducing the Nrf2-mediated antioxidant pathway in the rat testes and consequently decreasing the oxidative stress, through the increased *FTO* expression. Perhaps, this might be associated with the sperm quality, namely with the sperm concentration, that despite not having undergone significant changes compared to the control group, shown a positive correlation with two genes that seem to be linked to the control of oxidative stress in the testes. Thus, the *FTO* and *NFE2L2* genes in sperm constitute possible targets for increasing sperm concentration.

Moreover, we observed that CR also promoted the existence of a positive correlation between *NFE2L2* expression in testes and the ghrelin plasma levels, that are both increased in the CR group. The hormone ghrelin, in turn, became positively correlated with sperm viability. In fact, ghrelin has already been associated with improved sperm viability, apparently due to the fact that it is an endogenous antioxidant that attenuates the oxidative stress response.[278] Thus, these results suggest that *FTO* might be indirectly associated with a better sperm quality, and that effect could be attributed to the antioxidative effects of ghrelin on the rat sperm, especially on its plasma membrane, which probably protects the sperm structure against oxidative damage.

Concerning the rats subjected to GLP-1 administration we found an interesting positive correlation between *FTO* expression in sperm of those rats and the active GLP-1 fasting plasma levels. So, the active GLP-1 administration may be associated with the *FTO* expression in rat sperm, despite its expression not being significantly different from that observed in the testes of the animals of the control group. Moreover, despite not showing significant differences compared to control rats, plasma insulin levels and HOMA-IR value in those rats are negatively correlated with sperm *FTO* expression. Plasma insulin levels, in turn, are negatively correlated with the percentage of the morphologically normal sperm, which in this experimental group are statistically higher compared to the control rats. This suggests that an improvement in sperm morphology could be indirectly associated with the expression of *FTO* in the sperm of rats subjected to GLP-1 administration, even though despite showing a slight increase in the expression of this gene, this is not statistically significant. That improvement could be mediated by the impact of *FTO* expression in both insulin plasmatic levels and insulin resistance.

MC4R encodes for a receptor that belongs to the melanocortin receptor family which has a central role in weight being a regulation key regulator of energy homeostasis, food intake and body weight.[193] These roles are quite clear in terms of its expression in the brain, yet their role in the male reproductive system remains unknown. Thus, we started by checking if *MC4R* is expressed in rat testes, as it was previously reported by Mountjoy et al.,[256] and reported its expression in rats' sperm for the first time. We were also able to show that the expression of its protein in the testes is mainly located in primary spermatocytes, spermatogonia, myoid cells and Leydig cells. Still regarding *MC4R*, we demonstrated that its expression in testes was increased in the rats subjected to CR and to GLP-1 administration.

We observed a positive correlation between the increased expression of *MC4R* in the testes of rats subjected to CR, and the hypothalamic expression of the neuropeptide *NPY*, which is known as an inhibitor of the *MC4R*. This correlation is somewhat intriguing, since this neuropeptide is orexigenic, which means that it is an appetite stimulator promoting a positive energy balance, while the *MC4R* is involved in appetite reduction. It was already demonstrated that during negative energy balance, such as food deprivation and reduced circulating glucose levels, NPY/AgRP neurons are activated. Electrophysiological studies have shown that glucose alters excitability of NPY/AgRP neurons,

and a decrease in glucose levels in glucose-inhibited NPY/AgRP neurons leads to Ca^{2+} oscillations and neuronal depolarization, that in turn, promotes food intake and restoration of blood glucose levels.[279] In this way, we might be facing negative feedback. As individuals are subject to CR, the fact that there is a high expression of *MC4R*, which has an anorexigenic role, may have further potentiated a negative energy balance. *MC4R* expression may have reached a certain level at which it became harmful by acutely lowering plasma glucose levels. This may then be promoting an increase in *NPY* expression, but although its expression is slightly higher in CR animals than in the animals from the control group, it does not reach a statistically significant. In fact, we observed a positive correlation between the hypothalamic expression of NPY and the plasma glucose levels. In addition, a positive correlation was observed between the expression of *NPY* and the REE, although both were not significantly different from the control. This somehow makes sense since *NPY* inhibits the receptor that is responsible for a decrease in energy expenditure, an effect that is often referred to as a consequence of CR.[123,262] The possible increased *NPY* expression may be associated with the increased levels of ghrelin, which is one of the enhancers of orexigenic neurons that release *NPY*, and due also to the observed decreased levels of leptin and insulin in these CR rats, which are two inhibitors of these same neurons.

We noticed that the expression of *MC4R* in the testes and in the sperm of rats subjected to CR is positively correlated with the *FTO* expression of in the testes of these rats, and *FTO*, as already observed, seems to be associated with the oxidative status of the rat testes. In this follow-up, we observed that the expression of *MC4R* in the sperm of these rats is positively correlated with the total antioxidant capacity also in the sperm and with the expression of *NFE2L2* in testes, which is a transcription factor also known as *Nrf2*, and regulates the expression of antioxidant proteins that protect against oxidative damage. In the rats subjected to GLP-1 administration we also observed a strong positive correlation between the expression of *MC4R* in sperm and the *NFE2L2* expression in sperm. This leads us think that *MC4R*, in rats subjected to CR and GLP-1 administration, similarly to what was observed to *FTO*, has an active role in protecting against testicular oxidative damage, especially in sperm.

GNPDA2 encodes an allosteric enzyme that catalysis the deamination of the GlcN6P into F6P in the hexosamine signaling pathway, that is one of the main nutrient-sensing pathways.[211,213] Our study reported for the first time that *GNPDA2* is expressed in rat testes and sperm. We were also able to show that the expression of its protein in the testes is mainly located in late spermatids and Leydig cells. Still concerning *GNPDA2*, we demonstrated that its expression in testes was increased in the rats subjected to CR and to GLP-1 administration.

It was already been reported that the overexpression of GFAT enzyme, that is the GNPDA2 counter-enzyme, in skeletal muscle and adipose tissue resulted in insulin resistance.[280] Elevated levels of UDP-GlcNAc, that is the final product of the HSP, enhances O-glycosylation of transcription factors that regulate the expression of genes encoding proteins involved in the insulin-responsive glucose transport system control, existing a link between O-linked protein glycosylation and the induction of insulin resistance. So, excess hexosamine flux seem to cause insulin resistance.[217,221] In this study, we observed that rats under CR present a significant decreased insulin resistance (HOMA-IR) when compared to the control group and this may be associated with the overexpression of the *GNPDA2* that moves this HSP in the opposite direction to the formation of UDP-GlcNAc which is also associated with the induction of insulin resistance. The HSP is also known to convey nutrient status of adipose tissue to brain, due to regulation of leptin and adiponectin secretion. We noticed that the leptin levels were decreased in CR group, when compared to that observed in the animals of the control group. The fact that GNPDA2 - counter enzyme of GFAT that promotes HSP flux - is overexpressed, along with the fact that insulin resistance and leptin levels have been shown to be decreased may indicate that the HSP is not actually occurring towards the formation of the final product - UDPGlcN6P - that it is the substrate used by OGT for O-GlcNAc glycosylation of proteins.

Glycosylation, which is the enzymatic process attaching glycans or carbohydrates to proteins, lipids, or other organic molecules, is a main reaction and indispensable in the process for spermatogenesis and attainment of full competence of sperm. Glycosylation of sperm occurs during spermatogenesis, maturation process during epididymal transit, and capacitation.[281] Relatively to sperm quality we were able to observe a significant increase in head defects

in the sperm from rats of the CR group, compared to those of the control group. Higher levels of sperm head defects are related with lower fertility potential.[282] Indeed, higher number of sperm head defects are present in infertile individuals[282] and are also related with low pregnancy rates.[283] We noticed a positive correlation between these head defects and the *GNPDA2* expression in the testes and sperm from the rats subjected to CR, which lead us to hypothesize that these effects are somehow related to the CR impact on the *GNPDA2* expression, namely due to an associated decreased of UDP-GlcN6P and consequently O-glycosylation. A study recently showed that the sperm from high fertile bulls possessed a higher abundance of O-linked glycans e.g., galactosyl (β-1,3)N-acetylgalactosamine and N-linked glycans like [GlcNAc]1-3, N-acetylglucosamine than the low fertile bull sperm.[284] Thus, the effect of CR on *GNPDA2* seems to reverse the HSP flow in the opposite direction to the formation of UDP-GlcN6P, which on the one hand is beneficial by decreasing insulin resistance but on the other hand seems to be associated with a lower sperm quality, namely by the increase of head defects.

Relatively to the rats subjected to GLP-1 administration we noticed that, despite not having undergone significant changes compared to the control group, the expression of *GNPDA2* in sperm is positively correlated with their expression of *NFE2L2* also in sperm. In addition, there is a positive correlation between *GNPDA2* expression also in sperm and the sperm concentration. Thus, in sperm, *GNPDA2* may play a role in controlling the oxidative state through the Nrf2-mediated antioxidant pathway that eventually affects positively sperm concentration.

TMEM18 gene encodes a three-transmembrane domain protein with a positive charge C-terminus domain and its function are still under debate, being practically null the information that exists about its role on the male reproductive function.[233] Our study confirmed that *TMEM18* is expressed in rat testes, as it was previously reported by Álmen et al.,[233] and reported its expression in rat sperm for the first time. We were also able to show that the expression of its protein in the testes is mainly located in the early spermatogonia. Still concerning *TMEM18*, we demonstrated that its expression in testes was increased in the rats subjected to CR and to GLP-1 administration.

Was already found that there is a link between *TMEM18* and *PPARG* in human adipose tissue, existing a positive correlation between *TMEM18* and *PPARG* expression. *TMEM18* affects adipogenesis and this occurs potentially by direct or indirect functional interaction with *PPARG*.[285] Thus, we hypothesized that the function of *TMEM18* in the testes might be mediated by *PPARG*. Was already reported that PPARG has the potential to improve insulin resistance since its activation increases peripheral tissue sensitivity to insulin.[263,264] We in fact observed a negative correlation between the HOMA-IR, which was significantly lower in the CR group, and the unchanged *TMEM18* expression in sperm of these rats. Insulin resistance is known to negatively affect the sperm quantity and quality[263] and we accordingly observed a negative correlation between the higher HOMA-IR and the sperm concentration of the rats subjected to CR. We also found a positive correlation between *TMEM18* expression in sperm of the CR rats and its sperm concentration. Although both the expression of *TMEM18* in the sperm and the sperm concentration did not differ in the animals from the CR group compared to those from control group, all this suggests that the expression of *TMEM18* in sperm in rats subjected to CR becomes associated with the sperm concentration and this could be mediated by the impact in insulin resistance. Furthermore, a study demonstrated that prostaglandin J2, an agonist of PPARG, increases the viability of sperm, whereas all these events are reduced by the irreversible PPARG antagonist GW9662 confirming the involvement of PPARG in sperm viability.[263] Once again assuming and taking into account that the expression of *TMEM18* and *PPARG* are closely linked, we observed that the *TMEM18* expression in the testes of the rats subjected to CR has a positive correlation with its sperm viability. That correlation did not exist in the animals from the control group, so this suggests that, despite sperm viability did not show a significant difference in CR rats when compared to control rats, the expression of *TMEM18* in the testes of CR rats becomes associated with a higher sperm viability. Thus, if the CR were more intense or longer lasting, perhaps a significant increase in the expression of *TMEM18* in sperm could be observed (as it is already noticeable a tendency to increase in relation to the control group) and further increase the expression of *TMEM18* in the testes, and from this way, it would be possible to see a significant increase in both viability and sperm concentration.

There is a reciprocal transcriptional regulation between genes of *Nrf2* and *PPARG*. Experiments with *Nrf2* null mice show that expression of *PPARG* is reduced due to direct effect of *Nrf2* knockdown, and *Nrf2* expression is weakened in mice with decreased *PPARG*. A positive feedback loop between *PPARG* and *Nrf2* seem to exist, being that *PPARG* may act directly or through upstream pathway for *Nrf2* activation. Nuclear receptor *PPARG* and transcription factor *Nrf2* may act synergically in the activation of antioxidant defense genes.[264] In this study we observe that *TMEM18* expression in the testes of rats subjected to CR had a positive correlation with the *NFE2L2* (a transcription factor also known as *Nrf2*) expression also in its testes, being that both expressions were significantly higher in the animals of the CR group, when compared to the control group. This same correlation also occurred in sperm of these rats, but in this case the expression of both *TMEM18* and *NFE2L2* had no significant differences compared to the control group. Furthermore, we perceived that the expression of *TMEM18* in CR rats' testes was positively correlated with their sperm total antioxidant capacity. This leads us to believe that *TMEM18* may also have a beneficial role in the oxidative state of both the testes and sperm of rats that were subjected to CR, through the activation of the Nrf2-mediated antioxidant system. Interestingly, we also observed that there is a positive correlation between the expression of *TMEM18* and *FTO* in both the testes and sperm of rats subjected to CR, being that *FTO* also seemed to us be linked to the decrease in oxidative stress, especially in the testes.

Similarly to what was observed in the CR group, the expression of *TMEM18* in sperm from rats subjected to GLP-1 administration is positively correlated with the expression of *NFE2L2* also in sperm. This leads us to suppose that the administration of GLP-1 may promote greater antioxidant capacity through its impact on the *TMEM18* gene, also due to the activation of the Nrf2-mediated antioxidant system.

6. Conclusions

The optimum body energy homeostasis is necessary for normal physiological activities, including reproductive function. The tight link between energy metabolism and reproduction raises the concern regarding the effects of nutritional approaches based on negative energy balance used to achieve weight loss, particularly in male fertility. Energy homeostasis has been related to some genes linked to energy metabolism control, namely the obesity-related genes that are associated with a predisposition to conditions like obesity. Thus, we focused on 4 genes previously correlated to the development of obesity – *FTO*, *MC4R*, *GNPDA2* and *TMEM18* - and investigated their presence and expression in sperm and testes of Wistar rats under two protocols that are known to promote weight loss due to an imbalance in the body energy balance: caloric restriction and GLP-1 administration.

In this study we described that CR despite not promoting weight loss, promoted a lower weight gain compared to the animals of the control group. That lower weight gain must have been a result of the lower caloric intake inherent to the restriction applied, while regarding to appetite control, we could observe by the increased ghrelin levels and the abruptly decreased leptin/ghrelin ratio, suggesting an enhancement of appetite in CR animals. Nevertheless, CR was beneficial in terms of promoting a decrease in insulin resistance. On the other hand, administration of GLP-1, beyond the expected increase in active GLP-1 levels, did not promote any change in glucose metabolism, hormonal profile and consequently in body weight. Thus, regarding the purpose for which these two approaches are used - weight loss - in this study that was not observed. Despite that, we observed an impact of both interventions on sperm quality. CR promoted an increase in head defects, while the GLP-1 administration promoted an improvement in terms of sperm morphology. Having this in mind, we proceeded to the identification of the ORGs in testes and spermatozoa of Wistar rats and to the analysis of their expression, in the testes and spermatozoa, in response to CR and to the administration of GLP-1.

Regarding to the ORGs we observed the presence of *FTO*, *MC4R* and *TMEM18* transcripts in rat testes and we also identified those transcripts, for the first time, in rat sperm. Additionally, we were able to identify, for the first time, the presence of *GNPDA2* transcripts in both rat testes and sperm. Furthermore, we identified the corresponding protein, by IHC, in Wistar rat testes, and found that they all have distinct cellular locations. In the future, to better understand the role of ORGs on testes, the reasoning for their different testes cellular location should be further investigated. Concerning the response of the expression of these genes to the two interventions, we observed that CR and administration of GLP-1 promote an increase in the expression of all of them in the testes, however in the spermatozoa the expression of all these ORGs was not altered.

Based on the literature we found that FTO is associated with the regulation of testicular damage by oxidative stress and considering that in this animal model a lower incidence of this damage under CR conditions had already been observed, we also decided to go deeper into this aspect. So, we firstly determined that the total antioxidant capacity is not altered, either in the testes or spermatozoa, under CR or GLP-1 administration. It would be interesting in future to determine if there are differences in the formation of ROS, another aspect involved in the oxidative status. We found that CR and GLP-1 administration promote, in testes, an increase in *NFE2L2*, that encodes for an important antioxidant transcription factor. Then we searched for correlations between the ORGs expression and the results obtained regarding hormonal profile, neuropeptides, oxidative stress and sperm quality parameters.

We managed to start exploring, individually, the role the ORGs at the testicular and sperm levels, which to this date has not been studied. Regarding FTO, under CR, it becomes directly and/or indirectly correlated in a positive manner with improvements linked to oxidative stress in the testes, with the total antioxidant capacity of sperm and sperm concentration and viability. With respect to MC4R, under CR, we suggested that we are facing a negative feedback between MC4R and one of its inhibitors, the neuropeptide NPY, since these become positively correlated. In addition, it also appears to be linked directly and indirectly with improvements in the oxidative status of the testes and sperm. The effect of CR on GNPDA2 seems to reverse the HSP flow in the opposite direction to the formation

of UDP-GlcN6P, which on the one hand is beneficial by decreasing insulin resistance but on the other hand seems to be associated with increased of head defects in sperm, probably due to the decreased glycosylation that is essential for spermatogenesis and sperm maturation. Finally, TMEM18, assuming its known link with PPARG, was positively associated with concentration and sperm viability, both directly and indirectly, under CR conditions. Furthermore, it seems to promote improvements related to oxidative stress in the testes, with the total antioxidant capacity of sperm. Overall, we were able to perceive that in the CR group there is a general association of all ORGs with an improvement in oxidative status both in testes and sperm. This coincides with the fact that the expressions of all genes seem to become intimately associated with each other due to CR, since a generalized correlation was observed between the ORGs, both in testes and in sperm.

Although the expression of these ORGs in sperm has not undergone significant changes, under GLP-1 administration conditions, the expression of three of them - *MC4R*, *GNPDA2* and *TMEM18* - is strongly positively correlated with the expression of *NFE2L2* also in sperm. The expression of *GNPDA2* in sperm of the rats subjected to GLP-1 administration also seems to be become positively correlated with sperm concentration. Still regarding the effect on male reproductive function, the administration of GLP-1 promoted an improvement in sperm quality as there was a statistically significant increase in sperm with normal morphology, that seems to be indirectly associated with FTO. Thus, the administration of GLP-1, despite not promoting weight loss, seems to have some beneficial potential regarding to the male fertility. GLP-1 analogues could be the target of future studies to try to circumvent the so fleeting pharmacokinetic/pharmacodynamic profile of native GLP-1 and thus perhaps achieve weight loss and continue or accentuate the beneficial effects in the sperm shown in this study for native GLP-1.

This work highlights that, especially CR, but also the administration of GLP-1, despite not reaching the expected negative energy balance and consequent weight loss to which they are so related, shown to have an essentially beneficial role. This is evident in the decrease in insulin resistance and also in male fertility particularly through the improvement in the oxidative status of the testes and sperm that appears to be associated with the expression of the selected ORGs. In addition, these two interventions promote correlations between the ORGs and aspects related to male fertility that could be a study target for improvements in this major male function.

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