UNIVERSIDADE D
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Daniela Rosendo da Silva

THE GUT-ADIPOSE TISSUE CROSSTALK:
UNRAVELLING THE MECHANISMS OF GHRELIN-MEDIATED REGULATION OF ADIPOSE TISSUE ANGIOGENESIS

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular orientada pelo Professor Doutor Paulo Nuno Centeio Matafome e pela Professora Doutora Emília da Conceição Pedrosa Duarte e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra, Departamento de Ciências da Vida

Outubro de 2020

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The experimental research was performed at the Institute of Physiology and Coimbra Institute for Clinical and Biomedical Research, Faculty of Medicine, University of Coimbra. The clinical study and sample collection were performed at Centro Hospitalar e Universitário de Coimbra – Hospital Geral – Covões, Coimbra.

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INDEX

RESUMO

Introdução e Objetivos: A disfunção do tecido adiposo e a desregulação da secreção de hormonas intestinais constituem dois dos principais pilares da síndrome metabólica e da dibetes mellitus tipo 2. Terapêuticas que têm como base a remodelação do trato gastrointestinal resultam numa melhoria substancial da função do tecido adiposo, ao mesmo tempo que induzem a normalização do perfil de secreção das hormonas intestinais, nomeadamente da grelina, uma hormona associada à fome e à ativação de neurónios orexigénicos que expressam neuropéptido Y. Recentemente, a grelina foi proposta como um sensor de nutrientes, visto que a sua acilação depende de ácidos gordos provenientes da alimentação, o que pode sugerir um papel desta hormona no armazenamento de nutrientes no tecido adiposo. Assim, a nossa hipótese é que o sistema grelina/neuropéptido Y esteja alterado no tecido adiposo de indivíduos obesos com síndrome metabólica, e que possa ser modelado através da cirurgia metabólica. Para além disso, temos como objetivo explorar o efeito da grelina nos adipócitos e, naturalmente, no *adipo-vascular coupling.*

Materiais e Métodos: O perfil de expressão genética do eixo grelina/neuropéptido Y no tecido adiposo visceral de indivíduos obesos com desregulação metabólica foi avaliado através de uma reação em cadeia de polimerase em tempo real. Adicionalmente, avaliámos a ação da cirurgia metabólica neste eixo em ratos Goto-Kakizaki mantidos numa dieta altamente calórica e submetidos a gastrectomia vertical em *sleeve*. O efeito direto da grelina acilada e não acilada em adipócitos foi investigado na linha celular 3T3-L1 e o efeito em células endoteliais foi estudado em celúlas endoteliais microvasculares humanas.

Resultados: O nosso grupo mostrou que o sistema neuropéptido Y e recetores está profundamente alterado no tecido adiposo visceral de indivíduos obesos ao longo da progressão da desregulação metabólica. A expressão dos recetores Y1 e Y5 diminuiu com o desenvolvimento da diabetes tipo 2 e o recetor Y2 com o aparecimento da insulinoresistência. Mais ainda, os intermediários deste sistema estão correlacionados com vários genes envolvidos no metabolismo, plasticidade e função endócrina do tecido adiposo. Ambos os recetores Y1 e Y2 sofreram um aumento em ratos submetidos à gastrectomia vertical em *sleeve,* enquanto o recetor da grelina acilada se encontrou diminuído. A grelina acilada e não acilada induziram

acumulação lipídica e adipogénese em adipócitos e não alteraram a viabilidade de células endoteliais microvasculares.

Conclusão: O sistema neuropéptido Y e recetores correlaciona-se com fatores envolvidos na regulação do metabolismo, angiogénese e função endócrina no tecido adiposo visceral, e sofre alterações com o agravamento da desregulação metabólica, em indivíduos obesos, constituindo assim um interessante alvo terapêutico para a síndrome metabólica e diabetes tipo 2. Ambas as formas da grelina são capazes de induzir acumulação lipídica e adipogénese, corroborando a ideia desta hormona como um sensor metabólico. Por fim, o nosso trabalho mostrou que a gastrectomia vertical em *sleeve* é uma ferramenta que permite modelar o eixo grelina/neuropéptido Y no tecido adiposo visceral.

Palavras-chave: síndrome metabólica, tecido adiposo, gastrectomia vertical em *sleeve*, grelina, neuropéptido Y

ABSTRACT

Introduction and Objectives: Adipose tissue dysfunction and gut secretome dysregulation constitute two of the main pillars of metabolic syndrome and type 2 diabetes mellitus. Gut remodelling-based therapies for metabolic syndrome and type 2 diabetes mellitus, result in substantial improve on adipose tissue health while, inducing a normalization of gut hormones secretion, namely ghrelin's, a hormone highly associated to hunger and orexigenic neuropeptide Y-expressing neurons activation. Recently, ghrelin has been proposed to display a role as a nutrient sensor, since its acylation is dependent on fatty acid chains derived from ingested food, which might suggest a role for ghrelin in nutrient storage in adipose tissue. Thus, we hypothesized that the adipose tissue ghrelin/neuropeptide Y system could be altered in obese subjects with metabolic syndrome and could be modulated by metabolic surgery. Furthermore, we aimed to explore the effect of ghrelin in adipocytes and, naturally, in the adipo-vascular coupling.

Materials and Methods: Genetic expression profiling of the ghrelin/neuropeptide Y axis in the visceral adipose tissue of obese subjects with metabolic dysregulation was performed by a high-throughput real-time polymerase chain reaction. Additionally, we assessed the effect of metabolic surgery in ghrelin/neuropeptide Y axis in Goto-Kakizaki rats fed a high caloric diet and submitted to vertical sleeve gastrectomy. The direct effects of ghrelin and des-acyl ghrelin in adipocytes were investigated in a 3T3-L1 fibroblasts cell line and the effects in endothelial cells were studied in human microvascular endothelial cells.

Results: Our group showed that the neuropeptide Y/Y receptors system is deeply altered in visceral adipose tissue, with the progression of metabolic dysregulation in obese individuals. Y1 and Y5 receptors were downregulated with type 2 diabetes development and the Y2 receptor with insulin resistance onset. Furthermore, intermediates of this system are correlated with several genes involved in adipose tissue metabolism, plasticity and endocrine behaviour. Both Y1 and Y2 receptors were increased in the peri epididymal adipose tissue of rats submitted to vertical sleeve gastrectomy, while acylated ghrelin receptor levels were decreased. Acylated and non-acyl ghrelin induced lipid accumulation and adipogenesis in adipocytes and did not alter the viability of human microvascular endothelial cells.

Conclusion: The neuropeptide Y/Y receptors system correlates with factors involved in the regulation of metabolism, angiogenesis and adipokine secretion regulation in visceral adipose tissue and is deeply altered with the aggravation of metabolic dysregulation, in obese individuals, thus constituting an interesting therapeutical target for metabolic syndrome and type 2 diabetes. Ghrelin (both forms) is able to directly induce lipid accumulation and adipogenesis, supporting a role as a nutrient sensor. Lastly, our work showed that vertical sleeve gastrectomy is a tool to remodel the ghrelin/neuropeptide Y axis in visceral adipose tissue.

Key-words: metabolic syndrome, adipose tissue, vertical sleeve gastrectomy, ghrelin, neuropeptide Y

MSc – PUBLICATIONS AND SCIENTIFIC COMMUNICATIONS

Scientific Communication

March 2020 – Rosendo-Silva, D., Eickhoff, H., Seiça, R., Matafome, P., A gastrectomia em *sleeve* modela os componentes do eixo grelina-NPY no tecido adiposo de um modelo animal obeso e diabético tipo 2, March 2020, 16º Congresso da Sociedade Portuguesa de Diabetologia, Vilamoura

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ABBREVIATION LIST

Metabolic Syndrome **(MetS)**

Type 2 Diabetes Mellitus **(T2DM)**

Cardiovascular Disease **(CVD)**

Body Mass Index **(BMI)**

Metabolically Healthy Obesity **(MHO)**

Metabolically Unhealthy Obesity **(MUO)**

Adipose Tissue **(AT)**

White Adipose Tissue **(WAT)**

Subcutaneous Adipose Tissue **(SAT)**

Visceral Adipose Tissue **(VAT)**

Sterol Regulatory Element-Binding Transcription Factor 1 **(SREBP-1)**

Hypoxia Inducible Factor-1 alpha **(HIF-1α)**

Vascular Endothelial Growth Factor **(VEGF)**

Tumour Necrosis Factor alpha **(TNF-α)**

Interleukin 6 **(IL-6)**

AMP-activated Protein Kinase **(AMPK)**

Glucose Transporter 4 **(GLUT4)**

Peroxisome Proliferator-Activated Receptor gamma **(PPARγ)**

Free Fatty Acids **(FFAs)**

Glucagon-Like Peptide 1 **(GLP-1)**

Peptide YY **(PYY)**

Cholecystokinin **(CCK)**

Glucose-dependent Insulinotropic Peptide **(GIP)**

Growth Hormone Secretagogue Receptor 1α **(GHSR1a)**

Vertical Sleeve Gastrectomy **(VSG)**

Dipeptidyl Peptidase IV **(DPP-4)**

Agouti-Related Peptide **(AgRP)**

Neuropeptide Y **(NPY)**

Mammalian Target of Rapamycin **(mTOR)**

α-Melanocyte-Stimulating Hormone **(α-MSH)**

Knockout **(KO)**

Ghrelin O-Acyl Transferase **(GOAT)**

Human Umbilical Vascular Endothelial Cells **(HUVEC)**

Human Microvascular Endothelial Cells **(HMVEC)**

Goto-Kakizaki **(GK)**

Isobutyl-1-methylxanthine **(IBMX)**

D-Lys3-GHRP-6 **(DLS)**

Phosphate-buffered saline **(PBS)**

Wistar **(W)**

Standard Diet **(SD)**

High Caloric Diet **(HCD)**

Peri epididymal Adipose Tissue **(pEAT)**

Glycated Haemoglobin **(HbA1c)**

Homeostasis Model Assessment 2 Insulin Resistance Index **(Ox-HOMA2IR)**

Insulin Sensitive **(IS)**

Insulin Resistant **(IR)**

Quantitative Real-Time Polymerase Chain Reaction **(qRT-PCR)**

LIST OF FIGURES

LIST OF TABLES

Chapter 1 – INTRODUCTION

I. METABOLIC SYNDROME: A MULTIFACTORIAL PRECURSOR OF TYPE 2 DIABETES

The first official definition for metabolic syndrome (MetS) was provided by the World Health Organization in 1988 and mainly relied on the presence of insulin resistance or hyperinsulinemia. Years later, the National Cholesterol Education Program/Adult Treatment Panel III, presented a new definition, which supported a diagnosis dependent on the presence of, at least, three of the following risk factors: abdominal adiposity, hypertriglyceridemia, hypercholesterolemia, hypertension and impaired fasting glucose [1].

Beyond its increasing worldwide levels, MetS is already acknowledged as a major economic burden, which prevalence is growing side by side with the increasing incidence of obesity and type 2 diabetes mellitus (T2DM) [2]. In fact, MetS is acknowledged as a major risk factor for the onset and development of cardiovascular diseases (CVD) [3] and is also indicated as a predictor of T2DM, a chronic hyperglycaemic condition, that derives from a deficit in insulin secretion and/or progressive insulin resistance [1]. According to the World Health Organization, more than 650 million adults were obese in 2016, and the International Diabetes Federation estimated that 1 in 11 adults suffer from T2DM [4, 5]. In Portugal, 28.7% of the adult population was obese in 2015 and the estimated prevalence of T2DM was around 13.3% in the same year [6, 7]. In spite of no such data is available for MetS, a quarter of the worldwide adult population is estimated to be affected [2].

Obesity is mainly characterized as an abnormal excess of body fat, which might impair health, and most commonly identified through the body mass index (BMI), obtained by dividing the weight by the square of the height. In order to be considered obese, one should have a BMI equal, or superior, to 30 kg/m^2 [8]. The fact that obesity itself increases the risk for development of T2DM and CVD often results in being mistaken with MetS. Due to that it is important to distinguish metabolically healthy obesity (MHO) - presented by obese individuals without hypertension and hyperlipidaemia, and with normal insulin sensitivity - from metabolic unhealthy obesity (MUO), characterized by several of the metabolic abnormalities that define MetS [2, 8]. The prevalence of MHO changes when using different criteria. Recurring to BMI, Velho *et al.* showed that the

prevalence of MHO was in-between 3.3 and 32.1% in men and 11.4 and 43.3% in women, which exposes the necessity of establishing a solid definition for MHO. Furthermore, MHO was more frequently found in females and decreased with age for both sexes [9]. However, some doubts persist on using a BMI-based diagnosis, since the BMI itself can be a faulty measure, due to the non-discrimination between fat and lean mass, and fat distribution [8]. It is still not clear if MHO is an initial state, that sooner or later, will eventually transit into MUO and therefore, MetS, or if such evolution can be stopped, being MHO a steady state [8]. Thus, understanding the molecular mechanisms that distinguish MHO from MUO might be helpful in delaying, or preventing, the onset of obesity-related MetS, and associated complications, such as CVD and T2DM, two major causes of mortality. To note, MHO and MUO also differ in fat distribution, with MHO subjects presenting higher subcutaneous/visceral fat ratio than MUO individuals, and with lower ectopic fat accumulation [8], which draws attention to the importance of fat redistribution and function of adipose tissue to the development of metabolic disorders. Moreover, besides the amount and distribution of fat, adipose tissue dysfunction seems to be an important determinant for the deterioration of the metabolic status in obesity.

II. ADIPOSE TISSUE (DYS)FUNCTION

The adipose tissue (AT) is mainly composed by adipocytes and stromovascular cells, essentially mesenchymal progenitor cells, endothelial cells, preadipocytes, pericytes, macrophages and fibroblasts [10, 11]. The crosstalk between adipocytes and the stromovascular cells renders the AT with a high dynamicity and plasticity, which confers it ability to remodel accordingly to the energetic status and environmental changes [12].

Depending on morphology and function, two different types of AT can be distinguished: white adipose tissue (WAT) and brown AT. The latter is abundant in mitochondria and dissipates energy in the form of heat. The adipose cells in this tissue are smaller and triglycerides are arranged as small vacuoles. On the other hand, WAT has the main function of storing energy as triglycerides and its adipocytes are usually large cells composed by a single lipid droplet [10]. WAT can be divided, according to its distribution, in subcutaneous (SAT) and visceral adipose tissue (VAT).

The visceral depots can even be subdivided into mesenteric (around the intestine) or omental (from the stomach to the ventral abdomen) [10]. In WAT, hormones like glucagon, cortisol, and thyroid hormones, favour the hydrolysation of triglycerides into fatty acids and glycerol (lipolysis), in order to fulfil the energetic demands. Conversely, after meals, triglyceride synthesis occurs upon insulin stimulation, as a result of lipolysis inhibition and sterol regulatory element-binding transcription factor 1 (SREBP-1)-induced lipogenesis [13].

Lipid anabolism will force the expansion of the tissue in order to promote an adequate fat storage, which may occur through adipocyte hypertrophy and/or hyperplasia, and thus resulting in an increased adipocyte size and/or number, respectively. Adipogenesis, the differentiation of preadipocytes into mature adipocytes, is mutually regulated by angiogenesis, the growth of blood vessels, in what we may call an adipo-vascular coupling. This coupling results from the differentiation of preadipocytes along the vascular wall, being angiogenesis regulated by adipocyte-derived factors and adipogenesis also dependent of capillarization, so that each adipocyte is irrigated by one, or more, capillaries [10, 12]. Different arrangements in the vasculature lead to distinct outcomes from adipocytes. For instance, hyperplasia happens upon neo-vascularization, whereas hypertrophy requires dilation of the existent capillaries [14]. Thus, the plasticity of the vasculature may determine the type of AT expansion upon triglyceride synthesis. The vascular network provides the tissue with oxygen, nutrients, hormones and growth factors, and, allows the removal of waste products. Furthermore, the blood vessels have several fenestrations, permitting the passage of adipokines to the blood [10, 11]. AT remodelling requires an alteration in the vasculature, in order to avoid the existence of hypoxic regions, usually associated with hypertrophic growth. The vasculature is regulated by several mediators with either pro, or anti angiogenic effects, and so, angiogenesis will depend on the balance between them in the AT [10, 12]. Acute hypoxia, as the main stimuli for angiogenesis induction, triggers hypoxia inducible factor-1 (HIF-1α)-dependent gene expression of vascular endothelial growth factor (VEGF) family, determinant regulators of endothelial cells growth [10, 12, 14]. Angiogenesis might occur either by sprouting, which forms entirely new vessels, or splitting, in which a new blood vessel is created by the division of an existing one. In the sprouting type, VEGF-A, the most secreted VEGF-like peptide by WAT, acts like a chemoattractant signal, upon which stimulation, endothelial cells adopt a tip cell phenotype, with numerous filopodia, that allow migration. Notch

signalling acts as a negative regulator of sprouting, since when activated by delta-like 4 in a tip cell, leads to suppression of VEGF signalling in the adjacent one and contributes to vessel stabilization [10, 15].

WAT is the largest endocrine organ of the body, with a secretome which is currently known to include more than six hundred factors. AT secretome varies with the depot subtype. For instance, leptin and adiponectin are mainly secreted by SAT, while the proinflammatory cytokines tumour necrosis factor α (TNFα), monocyte chemoattractant protein-1 and interleukin 6 (IL-6) are essentially released by VAT [16]. Beyond VEGF, several other AT-secreted factors participate in the regulation of angiogenesis, such as the proangiogenic hepatocyte and fibroblast growth factors, transforming growth factor β, TNF-α, matrix metalloproteinases, leptin and angiopoietins, as well as the anti-angiogenic adiponectin and thrombospondins [12]. However, this large panoply of AT products not only regulates its vascular function, but is also involved in lipid and glucose metabolism, appetite regulation, coagulation, blood pressure, immune function, among many other functions [13, 17]. For instance, leptin, one of the main and firstly identified adipokines, plays a major role in appetite and energy homeostasis regulation in the hypothalamus [13]. Another major adipokine is adiponectin, which is known to increase liver and skeletal muscle fatty acid oxidation and energy expenditure [18]. Adipokines also act in WAT, given that leptin and adiponectin activate AMP-activated protein kinase (AMPK) while inhibiting SREBP-1c, and thus supress lipid synthesis, promoting fatty acids oxidation instead [13]. Moreover, adiponectin, but not leptin signalling, results in glucose transporter 4 (GLUT4)-mediated glucose uptake by adipocytes, thus contributing to glucose homeostasis [13].

In the context of MetS, the majority of these AT characteristics and mechanisms are either altered or impaired, suggesting a close relationship between AT dysfunction, MetS and its comorbidities.

a. ADIPOSE TISSUE DYSFUCTION IN METABOLIC SYNDROME

Data collected from longitudinal cohort studies, confirms that excessive prevalence of VAT over SAT constitutes a major risk factor for obesity and MetS, by leading to proinflammatory adipokine abundance and alterations in lipid metabolism, that may contribute to insulin resistance [19]. In

fact, a study in C57BL/6 mice revealed that SAT transplantation into the visceral cavity resulted in reduced body weight and adipocytes size, as well as improved glucose homeostasis [20].

Overfeeding and excessive nutrient uptake will enforce AT expansion, and eventually lead to adipocytes hypertrophy. Enlargement of adipose cells will give rise to persistent local inflammation and hypoxia, key drivers of the downregulation of peroxisome proliferator-activated receptor gamma (PPARγ), the master regulator of adipogenesis, thus favouring hypertrophic growth [13, 14, 21]. Concomitantly, increased nutrient availability and impaired β-oxidation trigger ceramide and diacylglycerol accumulation, thus contributing to insulin resistance, caused by signalling defects in the insulin pathway and ending up in disturbed glucose transporter, GLUT-4, translocation [13, 15]. Furthermore, uncontrolled lipid oxidation will lead to dysregulated free fatty acids (FFAs) inwards and outwards flux. This will give rise to dyslipidaemia, increased FFAs levels in circulation, that will accumulate ectopically in several organs, leading to lipotoxicity and widespread insulin resistance, a major abnormality that characterizes MetS [13].

The necessity of AT to expand needs to be accompanied by an adaptative response from the vascular network. However, in certain cases the persistent challenge to expand is associated with impaired vasculature remodelling. It seems that in obese subjects, *VEGF* expression and capillary density are decreased, when compared to lean controls, during weak oxygenation conditions. This may mean that the observed low oxygenation levels (3.8% oxygen, in this case) were not low enough to induce hypoxia-dependent VEGF expression in the AT of these obese individuals, or that, they present a weak angiogenic capacity [22]. In other study, higher VEGF-A levels were detected in both SAT and VAT of MHO subjects, but decreased with the aggravation of insulin resistance (MUO individuals) [23]. This suggests that in different stages of the pathophysiology we may have distinct angiogenic capacity, which deteriorates as progressing to MUO. A defective angiogenic response could generate a vicious circle of low oxygenation and, consequently, hypoxia-induced chronic activation of inflammatory and stress pathways, ultimately leading to cell death. Sustained hypoxia can also trigger transforming growth factor β production, that allows vascular growth at an initial stage. However, its permanent synthesis leads to dysregulated increase in extracellular matrix components, mainly collagen. Collagen depots will excessively accumulate in AT, giving rise to fibrosis, another major player in the tissue dysfunction and characteristic of the majority of obese patients' AT [14, 15]. Indeed, AT from insulin-resistant

obese individuals presented increased fibrosis, which was negatively correlated with insulin sensitivity and positively correlated with macrophage M2 type infiltration [24].

Adipocytes' hypertrophy will, sooner or later, also lead to an augment in necrosis, even under a proper angiogenic response. Such happens when cells reach a limit of physiological cell growth. As a direct consequence, there is an increase in immune cells infiltration and the triggering of pro-inflammatory pathways. The non-resolution of such inflammation, that eventually develops into a chronic low-grade inflammation state, constitutes another major hallmark of AT dysfunction [15]. Adipocytes' size strongly correlates with the presence of inflammation markers, such as TNFα and IL-6. Coincidentally, the presence of these markers was exacerbated in obese subjects with or without T2DM, that, as expected, also exhibited enlarged adipose cells in SAT [25]. Moreover, macrophage recruitment is augmented in SAT and VAT depots of obese individuals, with further increase as patients develop insulin resistance [15]. These evidences emphasise the probable involvement of adipocytes hypertrophic growth in the development of the low-grade inflammatory state, that seems to be activated way before MetS-related TD2M onset.

Altogether, chronic hypoxia, impairment in angiogenesis, fibrosis, necrosis and sustained inflammation constitute the solid rock pillars that drive and support unhealthy AT expansion, thus being considered the major hallmarks of AT dysfunction. As covered above, tissue malfunction leads to changes in nutrient uptake and endocrine function, giving rise to metabolic stress and disorders, characterized by the phenotype of MUO (**Figure 1**).

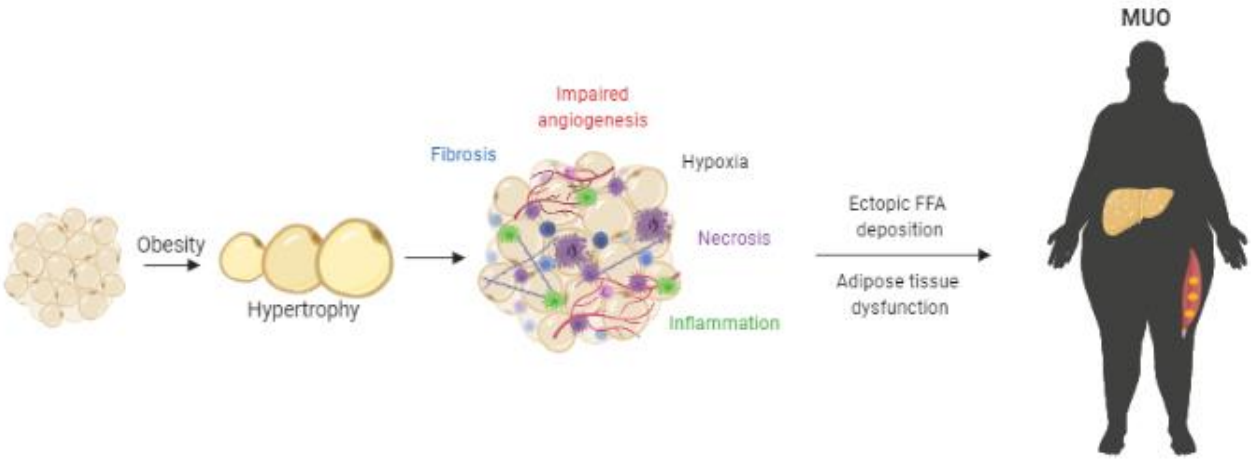

Figure 1 – **The adipo-vascular coupling.** Obesity-induced adipocytes' hypertrophy requires a proper AT remodelling to allow healthy expansion of the tissue. However, alterations in the microenvironment of adipocytes can originate a set of events that contribute to AT dysfunction, and thus cause a dysregulation in lipid fluxes, leading to MUO. Image created with Biorender.com

Therefore, strategies that allow the modulation of such events have been developed to improve or restore AT function in MetS patients. As a starter, anti-angiogenic drugs were developed, since it was thought that impairing angiogenesis could avoid overexpansion of AT, thus improving obesity and MetS. Inhibition of VEGF-A receptor 2 was, in fact, proven to restrict AT growth in diet-induced obese mice, resulting in decreased body weight [26]. Furthermore, this same strategy results in weight loss and increased insulin sensitivity, in the *ob/ob* background [15]. Notwithstanding such favourable evidences, angiogenesis impairment and sustained inhibition of AT expansion might induce lipotoxicity-associated ectopic FFAs deposition in peripheral organs. Indeed, VEGF ablation, in mice, generated, as expected, a reduction in vascularity and AT expansion. However, it also resulted in increased hypoxia, inflammation, apoptosis and in alteration in the tissue secretome, leading to a rapid development of insulin resistance during a high-fat diet consumption [27]. Collectively, this data suggests that modulation on the angiogenic process may come across as a potential therapeutic target for obesity-related metabolic complications. However, the controversy in the beneficial outcomes of whether to inhibit or promote angiogenesis is not yet clarified. One valid strategy may be tissue depot-specific angiogenic modulation, favouring angiogenesis in the SAT to allow healthy fat storage and expansion and the secretion of a more benign secretome. Modulation of the chronic low-grade inflammatory state has been also addressed to combat the pathophysiology of obesity and MetS. A clinical trial in obese individuals with MetS submitted to Etanercept (an immunoneutralizer of TNFα) treatment revealed improved fasting glucose levels and adiponectin levels, which is described to decrease the risk for MetS-related T2DM [25, 28]. However, repression of TNFα signalling recurring to a dominant negative, led, in high fat fed mice, both to reduced adiponectin secretion and glucose tolerance [15]. Thus, the contradictory data reveals the weaknesses of therapeutic strategies that aim to manage individually angiogenesis and inflammation in AT. Synergistic combination of angiogenesis and inflammation modulators with anti-fibrotic compounds could constitute an efficient therapeutic approach to improve AT dysfunction, being, therefore, potentially valuable for the development of strategies to combat obesity and MetS.

III. GUT SECRETOME DYSREGULATION IN METABOLIC SYNDROME

The gastrointestinal tract is an organ system that essentially complies the mouth, oesophagus, stomach, intestines and the anal canal, and that is frequently addressed as gut [29]. The gut is recognized as one of the largest endocrine organs in the organism, with more than thirty hormone genes identified to be expressed until the date [30]. The enteroendocrine cells, located along the gut epithelium, produce and secrete signals in response to oscillations in lumen nutrients. Such signals, mostly hormones, can act either locally or in several organs such as brain, AT and pancreas, in order to control appetite, energy expenditure and glucose and lipid metabolism [29, 30]. Some examples of these hormones are glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), secreted by L-cells located in the ileum, cholecystokinin (CCK), released by K cells from the duodenum, glucose-dependent insulinotropic hormone (GIP) secreted by I cells and ghrelin, that is mainly secreted by the X/A-like cells from the stomach **(Figure 2)** [30].

Fasting was shown to trigger a sharp secretion of ghrelin levels, a hormone highly associated to hunger and meal initiation [30]. Anorexigenic peptides GLP-1, CCK and PYY are released after nutrient ingestion and absorption and act through stimulation of vagal sensory nerves to induce satiety [30, 31]. However, they can also act via systemic circulation, reaching specific brain nuclei that contain several gut hormones receptors, through which they control appetite. Such hormones can also exert peripheric effects as they seek to promote glucose homeostasis and inhibition of gastric emptying [30, 31].

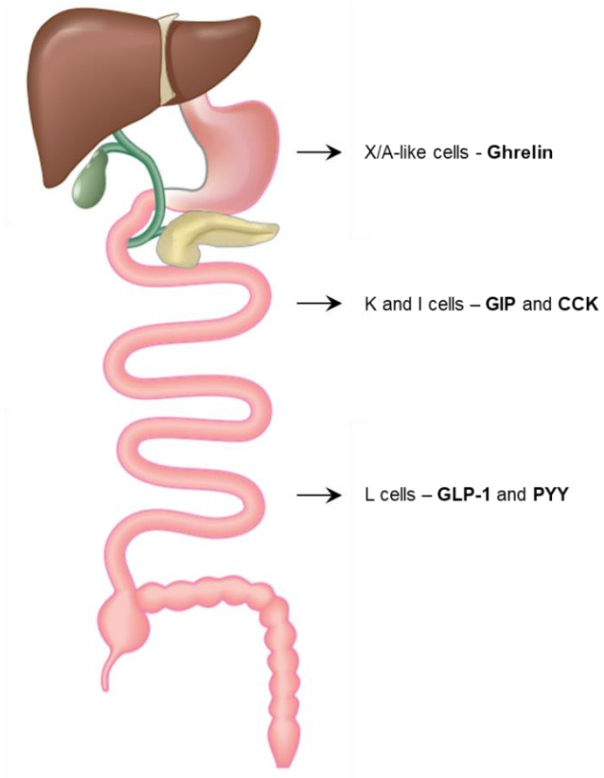

Figure 2 – **Enteroendocrine cells subtypes.** The gastrointestinal tract is rich in enteroendocrine cells that secrete gut hormones in response to lumen nutrients' oscillation. The X or A-like cells, located in the stomach are responsible for the secretion of ghrelin. The duodenum houses the populations of K and I cells, that mainly secrete GIP and CCK, whereas the distal small intestine, is rich in L cells, the main source of GLP-1 and PYY. Illustration adapted from *Gribble et al., 2016.*

Gut hormones secretion and bioavailability during pre- or post-meal periods differ naturally among individuals. However, similar secretion profiles have been found in individuals with obesity and MetS. The anorexigenic hormone GLP-1, with a determinant role in mediating glucose-stimulated insulin secretion, was reported to be significantly decreased in overweight and obese subjects thirty minutes after a test meal when compared to normal-weight controls even though pre-prandial GLP-1 levels were similar in both [32]. Zwirska-Korczala *et al*. described weaker postprandial PYY responses in obese women with MetS compared with lean control subjects, as well as lower fasting and postprandial CCK plasma levels in morbidly obese women, also with MetS [33]. Ghrelin dysregulation is also a major endocrine dysfunction often associated with the pathophysiology of obesity, and therefore, MetS [34]. Lower fasting plasma total ghrelin levels were reported in obese subjects, in comparison to normal-weight controls. Moreover, in the postprandial period, the progressive decline in ghrelin levels found in healthy individuals is also

absent in obese ones [35]. In other study, thirty minutes after meal ingestion there was a reduction in about 30% of postprandial total and acylated ghrelin plasma levels in lean women, but no significant changes were found in obese women with MetS [33].

In sum up, the described hormonal deregulation, that seems to lead to the unbalance between these hormones, that mediate anorexigenic (GLP-1, CCK and PYY) and orexigenic (ghrelin) effects, is still not clearly established as a cause, or consequence, of obesity and eventually MetS. Nevertheless, compounds that work in order to restore or revert this unbalance have been developed, such as GLP-1 long-lasting analogues, dual agonists combining GLP-1 and PYY and pharmacological inhibitors of ghrelin and its receptor, the growth hormone secretagogue receptor 1 alpha (GHSR1a) [36, 37].

IV. METABOLIC SURGERY: UNVEILING THE GUT-ADIPOSE TISSUE CROSSTALK

Bariatric surgeries were performed for the first time in the 1950s decade. Nowadays, they are one of the most recommended therapeutic strategies for obesity, MetS and T2DM, with 394,431 surgeries being performed worldwide in 2018 [38, 39]. Vertical sleeve gastrectomy (VSG) and Roux-en-Y gastric bypass are the most frequently performed surgical approaches, accounting for 37% and 45% of total bariatric surgeries, respectively, but the resultant rearrangement of the gastrointestinal tract is substantially different [38, 40, 41] (**Figure 3**).

Briefly, Roux-en-Y gastric bypass consists in creating a small gastric pouch in the upper part of the stomach, and then, dividing the small intestine in two parts, pulling one upwards, on top of the colon, to be connected to the pouch. On the other hand, in VSG the gastric volume is reduced by resecting approximately 80% of the stomach, in a vertical fashion, but the anatomical route of food is maintained [40, 41]. Despite the relative low rate of associated complications, both these procedures might lead to intestinal obstruction symptoms [40].

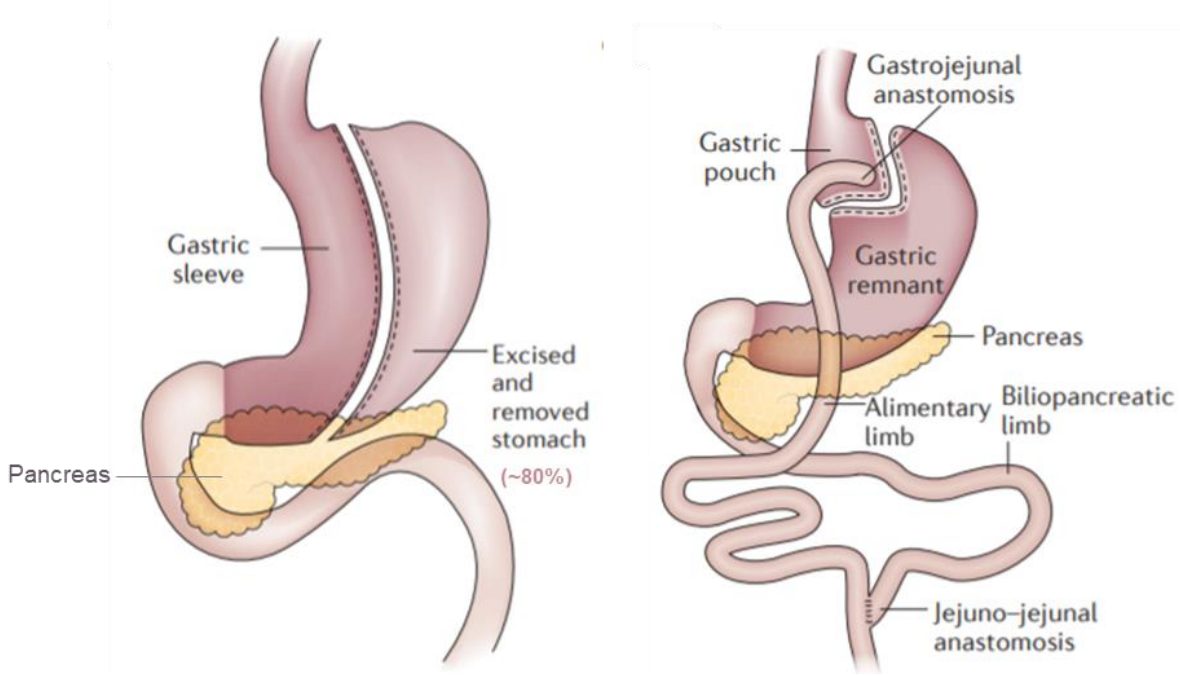

Figure 3 – Gut remodelling metabolic surgery. The two most common bariatric procedures: Vertical sleeve gastrectomy (left) and Roux-en-Y gastric bypass (right). Adapted from *Nguyen et al. 2016.*

VSG provides some advantages when compared to the gastric bypass, such as the decreased complexity and difficulty of the surgical techniques, faster hospital discharge, decreased perioperative complications, lower probability of developing vitamin deficiencies (caused by duodenum bypassing), and last but not least, VSG can be converted in most of the other types of bariatric surgeries, if necessary [40]. Patients submitted to VSG present a significant reduction in weight and BMI in the very next month, an effect that is usually extended up for one year [41]. Beyond leading to significant weight reduction, surgery also ameliorates glucose metabolism, dyslipidaemia and hypertension [38, 41]. In fact, improvement of insulin sensitivity and T2DM remission happens few days after surgery, even before significant weight loss is achieved, suggesting both events to be, at least, partially independent [38]. The term “metabolic surgery”, widely accepted as the most accurate terminology when referring to these procedures, arose precisely from the observation that there was a direct effect on metabolic control after surgery that did not necessarily result from weigh loss. Thus, metabolic surgery is nowadays recommended as a therapeutic strategy with the main goal of leading to metabolic improvements, rather than weight loss itself [38].

The awareness of the importance of the gut in the context of metabolic diseases was only realized upon observation of metabolic surgeries effects. Initially, reduction of stomach's expandability, and/or promotion of nutrient malabsorption by rearranging the intestines were accepted as the main causes underlying the benefits of metabolic surgery [38]. However, soon it was noticed that the surgery could alter gut hormones secretion profile. The anorexigenic hormones GLP-1, CCK and PYY suffer major increases after meal consumption in VSG-submitted patients [41, 42-44]. The central actions of these hormones cannot account alone for the amelioration of glycaemic control, since this effect is independent from weight loss. In turn, surgery is believed to improve glycaemic control through the robust increase in the incretin effect played by the GLP-1, that determines insulin excursion from pancreatic β cells. The debate is still open on how VSG induces selective enhancement of postprandial GLP-1, CCK and PYY: is it due to a selective modulation of enteroendocrine cells types or, to increased rates of gastric emptying and nutrient flow into the intestines? In fact, one can argue that the increased and rapid nutrient exposition, might acutely result in enhanced nutrient-sensing and CCK, GLP-1 and PYY secretion, chronically favouring an increase in the cellular types responsible for their secretion [45]. However, more studies addressing such questions are in need, to fully understand and clarify the origin and cause of the altered gut secretome profile seen after surgery.

Postoperative postprandial total ghrelin levels are decreased in a vast number of studies in humans and rodents, even in long-term follow-up. Regarding fasting levels, some controversy still remains, although most studies also point towards a reduction in ghrelin secretion [42-44, 46]. To what concerns VSG, the diminished ghrelin secretion might be due to the resection of the stomach's gastric fundus, the main location of X cells [47].

Not neglecting the obvious importance of the increased hypothalamic anorexigenic activity inherent to metabolic surgery, one cannot exclude the putative effects that gut hormones can play directly in metabolic effector organs, such as the AT, in such conditions. Indeed, some studies in rodents have been claiming that surgery-induced beneficial effects cannot be exclusively attributed to the decreased caloric intake [48, 49]. Gut hormones are already acknowledged to regulate some key events in AT, in physiological conditions. For instance, GLP-1 promotes lipolysis, whereas ghrelin and CCK are thought to be lipogenic hormones, while all being adipogenesis inducers [Reviewed by Rosendo-Silva 2020, 45]. In fact, it can be speculated that

a shift into a more catabolic profile of AT could underlie the improvement in glucose homeostasis and body weight. Many are the alterations seen in AT of VSG-submitted rodents and humans. Beyond reducing VAT weight, VSG led, in diabetic obese rats, to an increase in adipogenesis, vascular differentiation and angiogenesis, that might improve microvasculature. The also observed increased levels of AMPK, that point to a raise in fatty acids oxidation, reflect a metabolic shift in AT that may underlie the amelioration of triglyceridaemia, cholesterolaemia and weight loss also found in operated animals [50]. In humans, VSG, however, decreases adipogenesis markers in SAT [51]. However, biopsies were done 1 week after the procedure and another recent study has shown that reshaping of AT morphology might take longer. In fact, hyperplasia of SAT adipocytes was only seen in the patients 5 years after Roux-en-Y gastric bypass [52]. In this depot, metabolic surgery induces a huge increase in lipid oxidation, suggesting that, after the procedure, the organism adopts a more energetic dissipation-prone status rather than favouring its storage [50]. Given the excessive triglyceride surplus of diabetic obese patients, it is possible that this tremendous increase in SAT β-oxidation can be harmful to mitochondria, by augmentation in ROS production. Post-operative human SAT reveals a marked decrease in angiopoietin and Tie expression, which suggests a decrease in angiogenic capacity. Macrophage recruitment to the SAT also decreases after VSG, as well as the expression of several pro-inflammatory mediators [53, 54]. VSG induces a decrease in leptin and a raise in adiponectin levels, and the latter highly correlates with enhanced postoperative AT insulin sensitivity [55]. In fact, GLUT4 is increased in the epididymal fat tissue of gastrectomized rats with improved glycaemic profile [50]. Overall, these results reinforce the crucial role of AT for the surgery-induced glucose homeostasis. The fact that surgery induces both improvement of AT function and alteration in gut hormones suggests the existence of a crosstalk between the gut and AT. Gut hormones are described to regulate several events in AT in physiological conditions, such as metabolism, endocrine behaviour and plasticity [Reviewed by Rosendo-Silva 2020, 45]. As well, the modulation of gut signals elicited by these therapeutic approaches used in MetS, results in numerous adaptations in AT function and remodelling, that might contribute, at least in part, to the resolution of T2DM and decreased risk of CVD (**Figure 4**). Further studies, are then, in need, in order to clarify this interaction, which complies a difficult task, since the relation between these organs in the pre-operative disease context is still not completely understood.

Having in mind that AT dysfunction is a major risk factor for MetS and MetS patients present alterations in gut hormones secretion patterns (**Figure 4**), the causality, however, remains to be determined. Are such events parallel in the development of MetS or the unbalanced gut hormonal milieu, caused by dietary habits, underlies AT malfunction?

The suspicion that metabolic surgery-induced alterations in gut secretome could be underlying the beneficial results of the procedure, led to the development of pharmacological mimetics. The vast majority are GLP-1 analogues, such as liraglutide, dulaglutide and semaglutide, that preserve the incretin ability while surpassing GLP-1's short half-life, and result in long-term lowering effects in fasting plasma glucose, glycated haemoglobin and weight loss [45]. Recently, by combining GLP-1 and PYY with oxyntomodulin (a gut hormone co-secreted with GLP-1 and PYY by L-cells) in concentrations that allow to reach the post-prandial excursions seen after surgeries, Tan *et al.* developed a triple agonist that aims to achieve the metabolic improvements of VSG without submitting the patients to the risks of surgeries [56]. Preliminary results showed a marked decrease in food intake, as well as decreased postprandial blood glucose levels in obese individuals. However, fasting glucose and insulin were not changed, but, it's worth to note that the individuals were not diabetic, and the time-frame of the study (3 days with no follow-up) didn't allow body weight monitoring [56].

However, the possible involvement of other gut hormones, such as ghrelin or PYY, should not be discarded. Y2 receptor deficient mice did not differ in terms of body weight and glycaemic control from wild-type mice upon Roux-en-Y gastric bypass [57]. Being Y2 the receptor for PYY_{3-36} (generated upon PYY cleavage by dipeptidyl peptidase 4 (DPP-4)), such evidences suggest that the results of surgery are independent from PYY. However, despite constituting a powerful scientific tool, one cannot exclude the possibility of evolutionary development of compensatory mechanisms in these animal models that might be masquerading the results.

The marked alteration seen in total ghrelin plasma levels after metabolic surgery might suggest a putative role in contributing to the weight loss and increased insulin sensitivity seen after VSG, and possibly a determinant role in post-operative AT function [44, 47]. After surgery AT itself experiences, as covered, several adaptations, and the molecular mechanisms that drive them are still to uncover. Nonetheless, ghrelin is already acknowledged to regulate a variety of events in the AT in normal physiological conditions, which will be further elucidated.

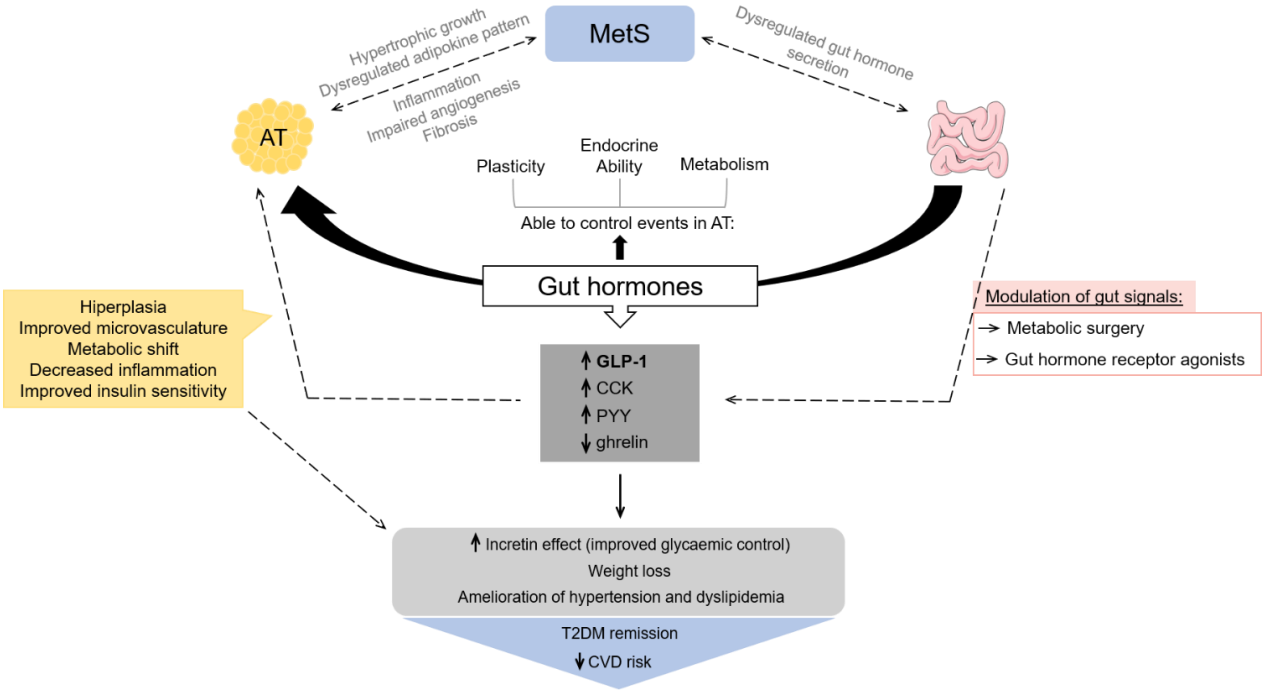

Figure 4 - An integrated view on the gut-adipose tissue crosstalk. Dysfunctional AT and dysregulated gut hormone profile are both features of MetS or T2DM. Given that gut hormones are known to regulate several aspects of AT function, restoration of gut hormone profile through bariatric surgery or the use of analogues, may be a promising strategy in the amelioration of MetS-associated AT dysfunction. Adapted from *Rosendo-Silva, 2020.*

V. A SPECIAL GLANCE AT GHRELIN

Ghrelin is the major orexigenic peptide identified until the date and is mainly recognized as an appetite and growth hormone secretagogue stimulant [31, 58]. In 1999 ghrelin was identified for the first time as the endogenous ligand for the GHSR1a. The truncated isoform 1b is also a product of the human *GHSR* gene transcription, but there is not a clear clue on its function and ligands [58]. In approximately 20 years of ghrelin research several biological actions have been attributed to this hormone, such as stimulation of growth hormone secretion, hedonic and homeostatic feeding, reward-seeking behaviour, regulation of glucose and energy metabolism, cognition and stimulation of gastrointestinal motility, to name a few [58, 59].

a. HYPOTHALAMIC EFFECTS OF GHRELIN IN ENERGY HOMEOSTASIS

In the brain, GHSR1a is highly expressed in the hypothalamus, ventral tegmental area, predominantly in dopaminergic neurons, and hippocampus, being therefore associated with food intake, motivation and drive to ingest food and learning and memory performance [31, 58, 59]. Within the hypothalamus, arcuate nucleus' agouti-related peptide (AgRP)/neuropeptide Y (NPY)-expressing neurons display the higher density of GHSR1a expression, but proopiomelanocortin neurons also express the receptor, however, in a lower extent [31]. GHSR1a belongs to the family of G-protein coupled receptors, and thus, after ghrelin binding, suffers alterations in the conformation of the intracellular domains, and activates G proteins through exchange of guanosine bi- for guanosine tri-phosphate in the α subunit. GHSR1a is a multifaceted receptor, since it can activate a variety of signalling cascades [60] (**Figure 5**).

Activation of Gq protein leads to phospholipase C activation and consequent diacylglycerol and inositol (1,4,5) triphosphate formation, leading to protein kinase C activation and Ca^{2+} release from the endoplasmic reticulum, respectively. The most well described result from this signalling pathway is growth hormone secretion from the anterior pituitary [60]. Beyond activating the classical pathways involving Akt and mitogen-activated protein kinases, the complex ghrelin-GHSR1a was also described to activate AMPK in both arcuate and ventromedial nucleus. Such event contributes to fatty acid oxidation, thus fulfilling the bioenergetic needs for sustained AgRP/NPY activity [58, 60].

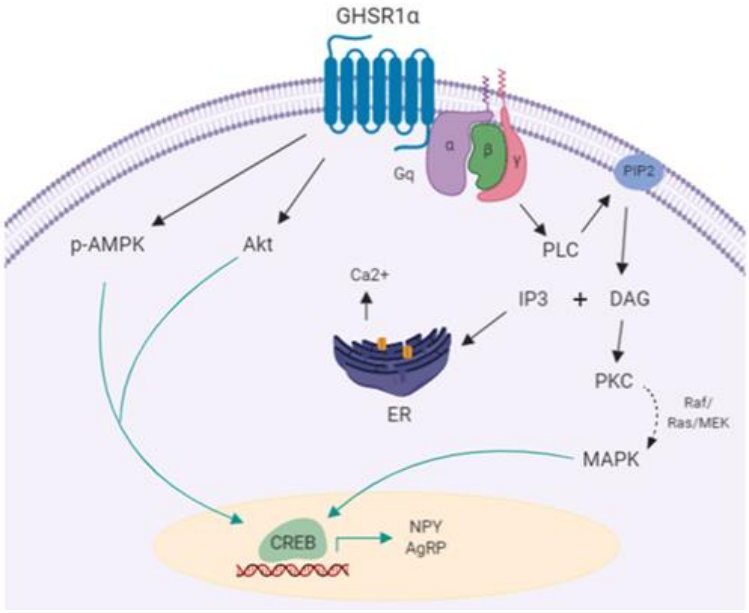

Figure 5 – Molecular mechanisms downstream GHSR1a activation. Activation of GHSR1a results in AMPK activation, Ca^{2+} release and PKC activation, that in turn leads to the stimulation of MAPKs pathway. Akt pathway is also activated. AMPK, MAPK and Akt induce transcription of NPY and AgRP.

One common result of the activation of aforementioned pathways is the transcription of both NPY and AgRP [61]. Evidences also point to a ghrelin-dependent mammalian target of rapamycin (mTOR) activation, which can come up as a contradictory evidence since AMPK is a major negative regulator of mTOR [60]. Despite the molecular mechanisms underlying the interaction of GHSR1a and mTOR are still unknown, it was shown in rats, that ghrelin administration elicited upregulation of mTOR mainly in the arcuate nucleus, and that, inhibition with rapamycin attenuated ghrelin's orexigenic effect [62]. Such might suggest that ghrelin-GHSR1a complex may be able to differentially activate either AMPK or mTOR, depending of the hypothalamic nuclei and/or the energetic profile. Ghrelin, in opposition to the anorexigenic gut-derived hormones GLP-1, PYY and CCK, stimulates arcuate nucleus' AgRP/NPY-expressing neurons, which directly synapse on proopiomelanocortin neurons or activate inhibitory interneurons that innervate them, thus resulting in attenuated secretion of alpha melanocyte-stimulating hormone (α-MSH) [31, 61] (**Figure 6**). The concurrent activation and upregulation of NPY and AgRP (but mainly NPY) and AgRP-mediated inhibition of melanocortin signals, both directly and indirectly, mediates ghrelin-induced increase in food intake [61] (**Figure 6**). Ghrelin's orexigenic effect is GHSR1a-dependent,

since ablation of the receptor, in mice, leads to unaltered food intake upon ghrelin administration, when in comparison to the increased nutrient intake seen in wild-type littermates [63].

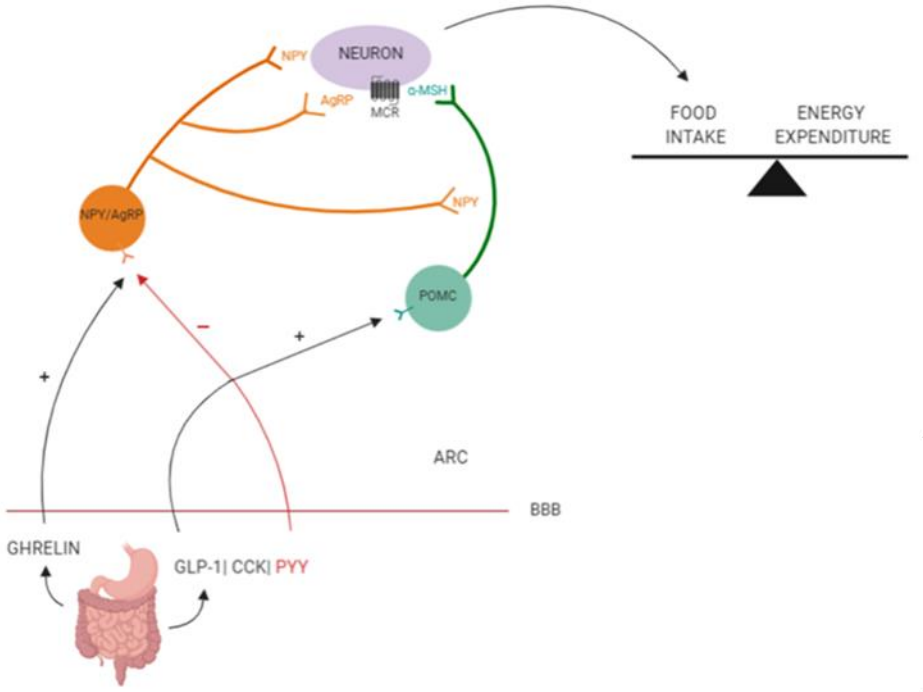

Figure 6 – **Ghrelin opposes anorexigenic signals in the hypothalamus**. Ghrelin, in opposition to the other gut-derived but, anorexigenic hormones (GLP-1, CCK and PYY), leads to the stimulation of orexigenic neurons, and thus, to an inhibition of melanocortin signals. BBB- Blood brain barrier; MCR- Melanocortin receptors; POMC – Proopiomelanocortin neuron.

NPY, also known to be involved in the regulation of metabolism and energy homeostasis, may act through six subtypes of receptors (Y1 – Y6). Differentially truncated NPY peptides, generated essentially by DPP-4 through hydrolysis of the post-proline bond between positions 2 and 3, bind with different affinity to the various receptor subtypes. For instance, while NPY is thought to bind preferentially to Y1 or Y5 receptors, NPY_{3-36} is a high affinity endogenous ligand of Y2 instead [64]. Y receptors are all coupled to a Gi protein, leading to decreased cyclic adenosine monophosphate production. Y1 and Y5 receptors seem to be the subtypes mainly involved in mediating orexigenic effects. In fact, Nguyen *et al.* conducted a study in mice with a double knockout (KO) for Y1 and Y5 receptors that revealed that food intake mechanisms depend on both receptors [65].

b. ACYLATION: A CRUCIAL STEP FOR GHRELIN ACTIVATION

The uniqueness of this gut hormone is not only dependent on the events that it determines, starting way before, right after the transcription of the preproghrelin gene that encodes for both the 28 and 23 amino acid peptides, ghrelin and obestatin, respectively. Little is known regarding obestatin's functions, although some authors defend it may counteract ghrelin's orexigenic properties [58]. Once translated, ghrelin may suffer acylation, a *sui generis* modification mediated by ghrelin O-acyl transferase (GOAT), that consists in the addition of an octanoyl (most common) or dodecanoyl side chain from a fatty acid to its third serine residue [58]. GOAT is, to the date, the only enzyme capable of accomplish ghrelin activation, a fact that is corroborated by GOAT-deficient mice, which present total absence of octanoylated/dodecanoylated ghrelin species [66]. Furthermore, Tschöp *et al.* and others have been confirming that the lipidic moiety that extensively contributes to this post-translational modification of ghrelin is mainly derived from the pool of ingested lipids rather than from those stored in fat reservoirs [66]. More precisely, mid chain fatty acids are the most reliable source, providing both C8 and C10 side chains, while being readily to use without being cleaved by lipases [58].

However, in physiological conditions, non-acylated ghrelin makes up between 80-90% of total circulating ghrelin levels [58]. Despite being still under debate, des-acyl ghrelin has some recognized functions, such as contributing to glucose homeostasis and preventing muscle atrophy. In fact, mice overexpressing the preproghrelin gene, thus presenting increased des-acyl ghrelin levels, display improved glucose tolerance and insulin sensitivity [67]. However, it's quite uncertain whether des-acyl ghrelin contributes to, or supresses, ghrelin's orexigenic effect [58]. Nonetheless, des-acyl ghrelin is unable to activate the GHSR1a, at least in physiological conditions, since only acylation renders ghrelin the ability to bind and activate it. Other receptors des-acyl ghrelin may bind to remain unidentified [31, 58].

c. REGULATION OF GHRELIN SECRETION

Ghrelin is mainly secreted by X/A-like cells located in the mucosa of the stomach's gastric fundus, which are of the closed type, not directly contacting with luminal nutrients. Within the gut, ghrelin-producing cells are also found in the duodenum, where they are believed to be the opened-type, thus having direct access to gut lumen nutrients [58]. Ghrelin plasmatic levels are elevated during fasting and its secretion is attributed to sympathetic innervation-dependent β-adrenergic receptors activation in X/A-like cells. Ghrelin's release can also be stimulated by gut motility that is triggered by external food cues, such as smell or taste [47, 58]. During and after food intake, circulating ghrelin total levels decrease in proportion to the caloric intake, due nutrient uptake-induced somatostatin and gastrin release that decreases gastric ghrelin secretion [47, 58]. Thus, the regulation of ghrelin secretion seems to be dependent, at a first stage (fasting) on sympathetic nervous system activation, while is suppression appears to be essentially commanded by other gut-derived peptides involved in nutrient digestion. Interestingly, anorexigenic hormones, highly involved in the regulation of energy homeostasis, and that oppose ghrelin's effect, have no particular effect on its secretion [58]. Depending on the diet, ghrelin levels attenuation may be more, or less, pronounced, since a study in healthy individuals showed that a high-protein breakfast resulted in a more marked decrease in ghrelin concentration than an equally caloric high-carbohydrate one [68]. Nonetheless, lipids are the weakest macronutrient at suppressing postprandial ghrelin levels [58].

Recently, Tschöp *et al.* unexpectedly found out that fasting triggers a sharp secretion of the non-acylated form of ghrelin, whereas acyl ghrelin levels remained constant over a 36-hour fasting, in mice [66]. It is to say that this is one of the few reports that discriminates between the acylated and non-acylated ghrelin's blood levels, since the vast majority only addresses total ghrelin levels. This may mean that, at least in mice, the so well accepted increase in ghrelin levels during fasting might actually reflect a major increase in des-acyl ghrelin levels rather than a raise in the acylated form. The relatively recent discovery of the GOAT/ghrelin system and the complexity and uniqueness of the acylation process might have been contributing to the report mostly of ghrelin total levels.

d. GHRELIN, BEYOND A HUNGER HORMONE

Obesity usually results from an unbalance between energy intake and consumption. Such misalignment may be the outcome from an interplay of genetic and environmental factors and ultimately, a failure of the body's homeostatic mechanisms that control food intake and energy expenditure, combined which excess fat storage [31]. The obesity-associated hyperphagic behaviour is critically governed by the antagonism between AgRP/NPY neurons and proopiomelanocortin neurons, and in fact, there is some evidence that these mechanisms may not be totally functional in the context of this pathology. Gao *et al.* revealed that in diet-induced obese Sprague–Dawley rats, hypothalamic NPY and AgRP expression was increased, suggesting a probable increase in ghrelin-induced food intake [69]. As aforementioned in chapter III., MetS and obese subjects present lower fasting ghrelin total levels than lean controls, the same being verified in diet-induced obese mice [33, 35, 70], which, at first, may seem incompatible with increased NPY/AgRP expression. Nonetheless, the permanent positive energy balance characteristic of obesity might be an explanation for the reduction in fasting ghrelin levels. However, some evidences point to an increase in fasting acylated ghrelin in human obesity and T2DM, with concomitant reduction in the des-acyl ghrelin form [79]. Since des-acyl ghrelin concentration in plasma is usually around 95% of total ghrelin, that accounts for the reduction in total ghrelin levels that most authors claim to happen.

In the post-prandial state, both patients and obese rodents show an incapacity for decreasing ghrelin levels [33, 35, 70] as seen in healthy controls, which is thought to lead to an inefficient suppression in appetite. However, obese individuals with binge-eating disorder, present an even harsher decline in fasting but not in postprandial ghrelin levels than those observed in obese non-binge eaters, which suggests that, intriguingly, ghrelin levels might come as consequence of overeating instead of actually being a contributor to increase in food intake, as previously thought [71].

Tschöp *et al.* gathered important piece of evidence that question the narrow view of ghrelin simply as a hunger hormone. Unexpectedly, fasting was shown to trigger a sharp secretion of the non-acylated form of ghrelin, whereas acyl ghrelin levels remained constant over a 36-hour fasting, in mice [65]. Furthermore, the authors verified that GOAT gene (*Mboat4*) expression was reduced

in fasted animals when compared to *ad libitum*-fed ones, and GOAT/ghrelin transgenic mice do not present increased food consumption [66]. Following the same rational, ghrelin KO models lack significant alterations in food intake and the same is verified under adult-onset ablation of ghrelin cells [72], whereas GHSR1a deficient mice show hypophagia [58]. Considering these results, one may argue that GHSR1a may be able to induce feeding independently of ghrelin activation or ghrelin acylation may be dependent of dietary signals.

Accordingly, another intriguing finding was that ghrelin acylation is highly dependent on diet-derived medium-chain fatty acids, but not from those stored in fat depots [66]. Furthermore, ghrelin/GOAT overexpressing mice fail to generate acyl ghrelin species under a low-fat diet chow [58], highlighting the dependence of GOAT-mediated acylation on readily available fatty acids. If the bulk of acyl ghrelin is released upon fasting, as early research suggested, the processes of ghrelin acylation and secretion cannot be simultaneously regulated.

Altogether, such evidences raise awareness to a novel perspective in ghrelin's involvement in energy homeostasis. One cannot call in doubt ghrelin's orexigenic effects and the classical view of ghrelin as a hunger hormone, but such might be an event exclusively of short-term fasting, as suggested by the decrease in *GOAT* gene expression and unaltered acyl ghrelin levels during longer periods. Apparently and surprisingly, the GOAT/ghrelin axis seems to behave as a metabolic sensor of lipids, with acyl side chain groups being conferred by dietary fatty acids. Furthermore, ghrelin was shown to induce GLP-1 secretion (*in vitro* and *in vivo*), that in turn initiates a rigorous anabolic program by stimulating insulin secretion. As described in chapter III., fasting ghrelin and postprandial GLP-1 levels are reduced in human obesity, and a study as uncovered that ghrelin administration rescues GLP-1 post-prandial plasmatic levels in obese mice [73]. This newly proposed role of ghrelin is even more credible when taking in consideration the other well-known effects of ghrelin, such as growth hormone secretion, hepatic lipogenesis and, as it will be covered bellow, adiposity [58, 73]. In fact, it seems unreasonable that organisms would invest in growth and nutrient storage during food-deprivation periods. Thus, it is imperative to understand precisely the functions of both acyl and des-acyl ghrelin, the relation between ghrelin secretion and post-translational modification (acylation), and dissect such effects in an energetic-status dependent manner, since ghrelin seems to be involved in both the preparation

of the organism for incoming food/feeding, but also in the sensing of nutrients after a lipid-rich meal.

e. GHRELIN/NPY-AXIS EFFECTS IN ADIPOSE TISSUE

The GHSR1a is not only expressed in the brain, but also in the periphery. For instance, there is evidence for its expression essentially in the stomach, liver, kidney and also in AT [74]. In similarity, NPY receptor Y1/Y2/Y5 subtypes are also expressed in the AT [75, 76]. As aforementioned, ghrelin has a preponderant effect in adiposity, that is not necessarily associated with its food intake stimulatory effect. Theander-Carrillo *et al.* showed that central ghrelin administration, in mice, was able to stimulate enzymes involved in lipogenesis, independently of food intake, thus leading to triglyceride storage in white adipocytes and weight gain. Furthermore, the authors used a triple KO mice model for β-adrenergic receptors to demonstrate a sympathetic innervation dependence in central ghrelin-mediated adipose tissue metabolism regulation [77]. Years later, a similar study but in animals maintained in high fat diet, revealed that this diet was capable of blocking hyperphagia but not WAT lipogenesis, suggesting, that the lipogenic role of ghrelin is governed independently of its orexigenic activity [78]. Furthermore, such result might reflect a protective mechanism to promote tissue expansion in order to avoid ectopic fat accumulation, at least at an early stage. Given the tight regulation between AT growth and increased vascularization, it would be interesting to understand if central ghrelin administration induces AT angiogenesis, and how. These findings supporting ghrelin's activation of AT lipogenic genetic machinery independently of hunger/feeding regulation, reinforce a probable role of ghrelin as an energetic sensor.

Ghrelin is able to directly influence AT metabolism and behaviour. Both acylated and des-acyl forms of ghrelin (10^{-15} to 10^{-7} mol/L) increase PPARγ expression in human VAT adipocytes and in 3T3-L1 cells, a murine cell line of pre-adipocytes, thus stimulating adipogenesis [79, 80]. Furthermore, stimulation of human adipocytes undergoing differentiation, with both forms of ghrelin, in a range of concentrations that contains those found in subjects with or without obesity and T2DM, resulted in SREBP1 activation, increased expression of lipoprotein lipase and

lipogenic enzymes and reduced glycerol outflow [79]. However, in human SAT adipocytes, ghrelin administration (0.1 to 1000 ng/mL or 3 x 10^{-11} to 3 x 10^{-7} mol/L) led to a decrease in PPARγ [81]. A possible explanation might be a differential effect of ghrelin according to the depot origin of the adipocytes, either VAT or SAT. Nonetheless, ghrelin effects' in 3T3-L1 cell line are also incoherent among studies, with some evidences pointing at a possible inhibition of PPARγ-induced adipogenesis at the concentration of 0.1, 10 and 1000 ng/mL. Ghrelin-induced adiposity is a GHSR1a-dependent effect, since ablation of the receptor resulted in decreased body weight due to loss of fat mass and downregulation in adipogenesis-related genes. Nonetheless, such effects might also occur through non-GHSR1a-related mechanisms, since des-acyl ghrelin also induced adipogenesis while lacking the ability to activate it. GHSR1a ablation also resulted in improved insulin sensitivity, thus protecting against insulin resistance, while decreasing AT lipogenesis and adipocyte's area [82]. Furthermore, Rodríguez *et al.* found out that acylated and des-acyl ghrelin could play an anti-inflammatory role by decreasing both TNF-α-induced apoptosis and autophagy in human adipocytes through reduction of the activation of caspase-8 and caspase-3 and downregulation of genes involved in autophagy [83]. However, evidence gathered in GHSR1a KO aged mice models suggests, in contrary, a pro-inflammatory role for ghrelin in AT, since it was reported a decreased WAT inflammation and macrophage recruitment in the absence of the receptor [84].

NPY was described to be an antifibrotic agent in obese mice AT. Through activation of Y1 receptor, NPY leads to decreased fibronectin and collagen, even upon transforming growth factor β stimulation, thus preventing obesity-associated fibrosis [85]. Adipogenesis seems also to be favoured by NPY, but essentially through Y2 receptor activation instead. Stressor factors, combined with a high fat diet, induce NPY release, that in turn leads to NPY/Y2R upregulation in WAT, and adipocyte proliferation and differentiation. Ultimately, such conditions led, in mice, to the development of obesity and, few months later, MetS [76]. Rosmaninho-Salgado and colleagues showed that NPY-induced adipogenesis was dependent not only in Y2R, but also Y5R-mediated PPARγ increased expression in 3T3-L1 cells [75].

α-MSH from both circulation and sympathetic neurons is a potent lipolytic inducer in adipocytes through activation of melanocortin receptor 5, leading also to the suppression of re-esterification

and adipogenesis. In antagonism with NPY and ghrelin, the melanocortin system results in decreased fat mass and, consequently, weight loss [86].

Altogether, these data suggest that the opposite hypothalamic actions displayed by NPY and α-MSH in controlling food intake may also be relevant in the context of AT metabolism regulation. Furthermore, ghrelin may, as well be an interesting target for obesity/MetS by allowing regulation of this antagonistic relationship and a direct modulation of AT biology and behaviour, in similarity to its role in the arcuate nucleus that favours NPY-dependent activity over melanocortin-dependent one.

f. GHRELIN/NPY-AXIS IN THE REGULATION OF ANGIOGENESIS

Endothelial cell dysfunction is a major contributor for angiogenesis impairment, and, therefore, for improper AT remodelling. Ghrelin has been implicated in the avoidance of endothelial cells apoptosis. Zhao *et al.* showed that ghrelin prevents high-glucose-induced human umbilical vascular endothelial cells (HUVECs) apoptosis, through inhibition of caspase 3 [87]. Furthermore, acylated ghrelin was implied in the protection of HUVECs from apoptosis through mTOR activation and upon GHSR1a binding [88]. Thus, such evidences may suggest an indirect role for ghrelin in ameliorating angiogenesis, through the prevention of apoptosis-derived endothelial dysfunction.

Nonetheless, some studies have also been elucidating a direct action of ghrelin in the regulation of angiogenesis. Katare *et al.* conducted a study where they found that acylated ghrelin administration leads to an increase in angiogenesis in a hindlimb ischemia mice model [89]. Similar findings were presented by Wang *et al.*, but through induction of a myocardial infarction in a diabetic animal model, in which ghrelin improved angiogenesis. Ghrelin-GHSR1a interaction results in increased AMPK/nitric oxide signals, through upregulation of VEGF and HIF-1α [90]. Studies in human microvascular endothelial cells (HMVECs) also corroborate the stimulatory effects of ghrelin in cell migration and angiogenesis, at physiological concentrations [91]. Recently, a study revealed that GHSR1a KO mice present decreased AT mass and vascularity. Moreover, ghrelin was a significant angiogenesis inducer, in both HUVECs and bone marrow-

derived endothelial progenitor cells from wild-type mice, through the activation of GHRS1a-dependent extracellular regulated protein kinases signalling pathway. Deletion of the receptor caused ghrelin's inability to induce such protein phosphorylation on endothelial cells, and thus, inability to induce the angiogenic process [92]. Nonetheless, in this study, GHRS1a deletion was not conditional, meaning that hypothalamic ablation of the receptor could result in decreased food intake, and probably, a consequent loss of weight and fat mass in the KO mice. Thus, the decrease in adipose tissue vascularity could be a secondary outcome resultant from decreased food intake-induced shrinkage of fat depots rather than due to local inability of ghrelin-GHSR1a-mediated angiogenesis. Nevertheless, the involvement of ghrelin in tumour growth and injury healing suggests a probable role in angiogenesis [91].

However, in contrary to this study, several other studies failed to observe such proangiogenic effect of ghrelin [93-95]. Matrigel plug injection in SAT, in the presence or absence of acylated ghrelin, showed no effects in the angiogenic response of the tissue either in obese or lean mice [95]. Some authors even deny the existence of a proangiogenic role mediated by ghrelin. For instance, in human coronary artery endothelial cells, ghrelin seems to inhibit oxidized low-density lipoprotein-induced angiogenesis [96]. Conconi *et al.*, also showed that ghrelin could inhibit fibroblast growth factor-2-mediated angiogenesis in HUVECs and in the chick embryo chorioallantoic membrane [97].

Despite seeming controversial, these evidences may suggest that ghrelin regulates angiogenesis according to the cell type, thus exerting a pro or anti angiogenic action. In fact, the majority of these studies used different concentrations of ghrelin either in physiological or supraphysiological doses, and during different incubation times, that may lead to different angiogenic responses, even when in the same cellular type. Furthermore, the majority of these studies did not assess the effect of des-acyl ghrelin in regulating angiogenesis. Another limitation, is that, some studies did not address the dependence in GHSR1a, or in any other target of ghrelin, upon its administration.

The hypothalamic target of ghrelin, NPY, is also known to stimulate endothelial cells proliferation and migration, and is a strong angiogenesis inducer, either in vitro and in vivo models. In HUVECs, such effects seem to be mediated by Y1, Y2 and Y5 receptors [98]. Kuo *et al.*, provided evidence for a proangiogenic role of NPY in mice AT, triggered by a combination of stress and

high-fat diet-induced increase in glucocorticoids in the tissue, upregulating both NPY and Y2 receptor [76]. Furthermore, and opposing to what was expected, the sympathetic nerves were not the main source of NPY in AT, and the cells that account for the significant expression of NPY in AT were not yet identified [76].

Overall, ghrelin's effect in the regulation of adipose tissue angiogenesis remains controversial and further research needs to approach this aspect in order to understand what is the role of this hormone in the adipo-vascular coupling regulation, as well as, investigate a possible involvement of NPY-dependent mechanisms.

Chapter 2 – SCIENTIFIC FRAMEWORK AND OBJECTIVES

SCIENTIFIC FRAMEWORK

The awareness of AT as multifactorial tissue capable of communicating with several organs, especially the insulin-sensitive ones, rather than just a lipid storage depot, opened several doors of investigation and answered some questions regarding the pathophysiology of metabolic diseases. In particular, AT dysfunction proved to be a crucial contributor to the onset and development of MetS and T2DM. Another hallmark of MetS is the dysregulation of the gut secretome (**Figure 4**). The putative gut-AT crosstalk gains a special relevance knowing that gut hormones are acknowledged to regulate AT metabolism, plasticity and endocrine behaviour [Reviewed by Rosendo-Silva 2020, 45], thus highlighting their determinant role in controlling overall metabolism. Given the escalating prevalence and hazardous risk that MetS and T2DM comply to the human life it is urgent to understand how this crosstalk works and how it is jeopardized in the disease context.

Metabolic surgery is one of the most recurrent therapeutics for MetS/T2DM, and in particular VSG is one of the most popular and effective procedures. In the last few years our group has demonstrated that VSG is able to ameliorate AT dysfunction, in Goto-Kakizaki (GK) rats with T2DM, partly by improving AT microvasculature and increasing angiogenesis [50]. Moreover, VSG-operated rats presented a marked decrease in postprandial total ghrelin levels, thus shifting towards a normalization of the usual secretion profile characteristic of healthy animals and subjects [46].

Ghrelin stimulates adipogenesis, thus contributing to the growth of AT, either via sympathetic mechanisms or through a direct action in AT, an effect that seems compatible with the acylation of ghrelin that happens at the expense of dietary fatty acids and acts a signal of hypercaloric diets [58, 66]. AT expansion needs to be tightly synchronized with a proper rearrangement in the vascularization. The involvement of ghrelin in angiogenesis has been addressed in some models, but however, there's still a lot of controversy, and a lack of attribution of such effects to the different forms of ghrelin. Similarly, the hypothalamic target of ghrelin, NPY, is also acknowledged to be an adipogenic inducer and is described to induce angiogenesis, but when stimulated by stress and a high-fat diet consumption [76].

Given the hypothalamic interaction between ghrelin/GHSR1a and NPY to induce food intake, we hypothesised that, in the AT, the adipo-vascular coupling might be, as well, regulated by ghrelin

through an NPY-dependent mechanism, an axis that would ultimately contribute to the coordinated response that drives AT adaptation after metabolic surgery, and that might contribute, at least in part, to the resolution of T2DM and decreased risk of CVD.

Considering the already known roles of ghrelin in AT, as well as the restoration of its levels after VSG to those found in healthy animal models, we wondered whether ghrelin/NPY axis was altered in VAT of obese individuals with or without MetS, obtained through a collaboration with *Centro Hospitalar e Universitário de Coimbra – Hospital Geral – Covões.*

MAIN OBJECTIVE

To study the putative alterations of ghrelin/NPY-axis in the AT of obese patients with several degrees of insulin resistance and metabolic dysregulation, as well as the involvement of ghrelin/NPY in tissue microvasculature and expansion.

SPECIFIC OBJECTIVES

- **i.** Characterization of ghrelin/NPY-axis in WAT from obese individuals with several degrees of insulin resistance and metabolic dysregulation;
- **ii.** Characterization of ghrelin/NPY-axis in WAT from animal models submitted to VSG;
- **iii.** Determine the effects of ghrelin and des-acyl ghrelin in adipocytes *in vitro;*
- **iv.** Determine the effect of ghrelin and des-acyl ghrelin in angiogenesis in HMVECs;

Chapter 3 – MATERIALS AND METHODS

REAGENTS

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

ANTIBODIES

Calnexin (AB0037, Sicgen, Portugal), GHSR1a, DPPIV, NPY2R (ab85104, ab129060, ab31894, Abcam, Cambridge, United Kingdom), GAPDH (AB0049-20, Sicgen, Portugal), PPARγ (#2443, Cell Signaling, USA), NPY1R (6732-0150, Bio-Rad, California, EUA).

Table 1 - **Primary antibodies used in Western Blotting.**

Antibody	Molecular Weight (kDa)	Dilution	Manufacturer
Anti-DPP-4	110 kDa	1:1000	Abcam
Anti-GHSR1α	48 kDa	1:500	Abcam
Anti-NPY2R	~ 42 kDa	1:500	Abcam
Anti-Calnexin	83 kDa	1:2000	Sicgen
Anti-PPARγ	55 kDa	1:1000	Cell Signaling
Anti-GAPDH	37 kDa	1:1000	Sicgen
Anti-NPY1R	~52 kDa	1:500	BioRad

IN VITRO STUDY

Materials

3T3-L1 pre-adipocytes were a kind gift from Professor Claúdia Cavadas and obtained from the American type Culture Collection – LGC Promochem (Barcelona, Spain); HMVECs were a kind gift from *Instituto de Biofísica, Faculdade de Medicina da Universidade de Coimbra.* Fetal Bovine Serum was purchased from Gibco (Barcelona, Spain) and Dubecco's Modified Eagles Medium high glucose from Sigma (St. Louis, MO, USA). Endothelial Growth Medium was purchased from Lonza (Illinois, USA). Cell plates were obtained from Corning (Tewksbury, Massachusetts, USA); Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, Oil Red O staining and isopropanol were obtained from Sigma (St. Louis, Missouri, USA).

CELL CULTURE, DIFFERENTIATION AND TREATMENT OF 3T3-L1 CELLS

3T3-L1 Culture

3T3-L1 preadipocytes were received as a kind gift from Professor Claúdia Cavadas and were cultured in Dulbecco's Modified Eagles Medium (DMEM) high glucose supplemented with 1% antibiotic/antimycotic (Gibco, Barcelona, Spain) and sodium pyruvate (Gibco, Barcelona, Spain) at 400 mM, pH 7.4, in a 5% CO_2 95% air atmosphere incubator. The cells reached complete confluence after 48 hours of culture.

3T3-L1 Viability

3T3-L1 preadipocytes were seeded in a 96-well plate with a density of 2×10^4/well in 10% FBS/DMEM medium. After 24 hours, preadipocytes were added 0.1, 1, 10, 30 and 100 ng/ml ghrelin (Tocris, Bristol, United Kingdom) or des-acyl ghrelin (Tocris, Bristol, United Kingdom). Twenty-four hours later, the Alamar blue assay was performed. The medium was replaced by a solution of Dulbecco's Modified Eagles Medium high glucose/10% Fetal Bovine Serum with 10% of resazurin ($0.1\ mg \cdot mL^{-1}$) and cells were incubated overnight. All samples were assayed in triplicate, and each experiment was repeated at least three times. Afterwards, the absorbances at 570 and 600 nm were measured with an ELISA reader (Synergy HT - Biotek, USA). The data obtained by the Gen5 program was used to calculate cell viability, according to the following equation:

$$\text{Cell Viability} = \frac{(Absorbance570 - Absorbance600) of\ treated\ cells}{(Absorbance570 - Absorbance600)\ of\ control\ cells} \text{ x } 100$$

3T3-L1 Differentiation

Preadipocytes were seeded in multi-well plates according to the assay to be performed. After reaching confluence (usually after 2/3 days), the medium was removed and replaced for fresh one supplemented with the differentiation cocktail: IBMX (0.5 mM), insulin (10 µg/mL) and dexamethasone (1 µM) (day 0). At the third day, the medium was removed and replaced for fresh one supplemented with the respective drugs, yet without the differentiation cocktail (day 3). Two days after (day 5), the medium was renewed as in day 3 and the cells were harvested or stained at day 7 (**Figure 7**). Control condition cells were those incubated with IBMX and dexamethasone at day 0 and then only with non-supplemented fresh medium on the following days.

The positive control were cells that at days 0, 3 and 5 were supplemented with insulin (10 µg/mL). In order to assess ghrelin's effect in adipogenesis, pre-adipocytes were incubated with growing concentrations of ghrelin and des-acyl ghrelin (0.1, 1, 10, 30 and 100 ng/ml) and with GHSR1a or GOAT inhibitors, D-Lys3-GHRP-6 (DLS) (1 µM) (Abcam, Cambridge, United Kingdom) and GO-CoA-Tat (1 µM) (Phoenix Pharmaceuticals, Belmont, California, USA), respectively.

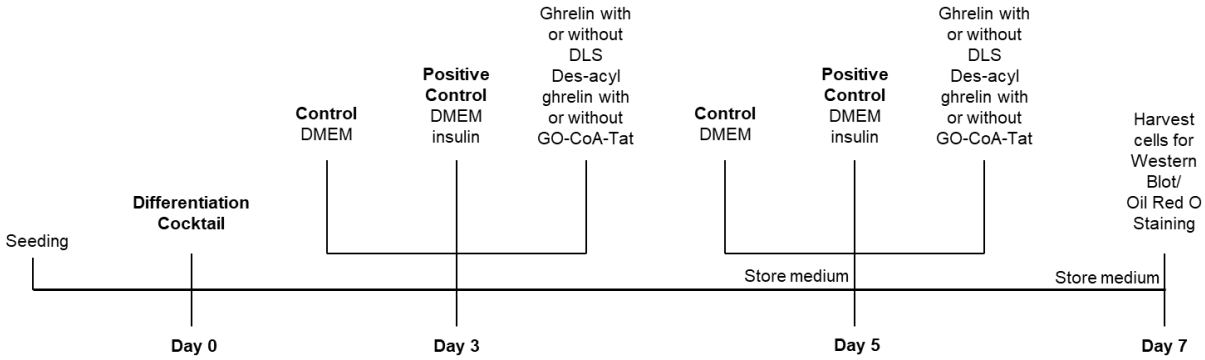

Figure 7 – **Timeline for 3T3-L1 pre-adipocytes differentiation and experiments.** After reaching confluency cells were added differentiation cocktail (day 0). At days 3 and 5 cells were treated/supplemented, and harvested for Western Blot or stained with Oil Red O at day 7.

WESTERN BLOTTING OF CELLULAR EXTRACTS

Cells were seeded in 6-well plates and submitted to the differentiation protocol. At day 7, cells were washed with ice-cold phosphate-buffered saline (PBS) and disrupted in lysis buffer (0.25 M Tris-HCl, 125 mM NaCl, 1% Triton-X-100, 0.5% SDS, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 2 mM Na_3VO_4, 10 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 10 mM phenylmethylsulfonyl, and 40 μL of protease inhibitor, pH 7.7). Afterwards, cells underwent 3 cycles of freezing/thawing and were sonicated (5 seconds at 50% amplitude) and centrifuged at 14,000 rpm at 4ºC during 20 minutes. The supernatant was collected and protein concentration was determined by the bicinchoninic acid method (Alfa Aesar, USA). Samples were added 2x Laemmli buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8), re-sonicated and boiled at 95ºC for 3 minutes. Vertical electrophoresis was carried in 8% polyacrylamide gel with the following composition: resolving (0.75 M Tris-HCl, 0.2% SDS, pH 8.8), stacking (0.25 M Tris-HCl, 0.2% SDS, pH 6.8) plus acrylamide, Milli-Q water, ammonium persulfate and tetramethlyethylenediamine. Electrophoresis system (Bio-Rad, USA)

was filled with running buffer (125 mM Tris-base, 480 Mm glycine, 1% SDS, pH 8.8), equal amounts of protein were loaded, as well as a protein standard (GRiSP, Research Solutions, Portugal), and voltage was kept constant during protein migration (120 V). SDS-polyacrylamide gels were transferred electrophoretically to polyvinilydene difluoride membranes (GRiSP, Research Solutions, Portugal) at 750 mA for 2 hours after being activated in methanol, hydrated in Milli-Q water and washed 15 minutes in transfer buffer (50 mM CAPS, 2% NaOH, 10% methanol, pH 11). Afterwards, membranes were blocked for 2 hours in 5% albumin in wash buffer (250 mM Tris, 1.5 mM NaCl pH=7.6 plus 0.5% Tween20), incubated overnight with primary specific antibodies (**Table1**) and then probed with biotin-conjugated specific antibodies for 2 hours at room temperature. Antibodies were diluted in wash buffer plus 0.01% Tween20 and 1% bovine serum albumin. Membranes were revealed through chemiluminescent method using a ECL substrate 1:1 (Advansta, EUA) and the luminescence detection system VersaDoc with Quantity One software (BioRad, Hercules, CA, USA) or ImageQuant LAS500 Software (GE Healthcare, United Kingdom). Image Lab software (Bio Rad, USA) was used for image data processing.

OIL RED O STAINING

Cells were seeded in 96-well plates and submitted to the differentiation protocol. At day 7, cells were washed with PBS and fixed with p-formaldehyde 4% for 30 minutes at room temperature. During this step, the Oil Red O working solution was prepared from a stock solution (0.5% Oil Red O in 100% isopropanol), by mixing 6 parts of stock with 4 parts of distilled water, followed by filtration in a 0.2 µm syringe filter. Cells were re-washed twice with PBS and then once with distilled water, and added 60% isopropanol for 5 minutes. Staining with Oil Red O was carried for 30 minutes in a plate shaker at room temperature, after removing the isopropanol. Cells were washed with distilled water to remove excess staining and the wells dried, in order to extract Oil Red O with 100% isopropanol. All samples were assayed in triplicate, and each experiment was repeated at least three times. Afterwards, the absorbances at 492 nm were measured with an ELISA reader (Synergy HT - Biotek, USA). The data obtained by the Gen5 program.

CELL CULTURE AND TREATMENT OF HUMAN MICROVASCULAR ENDOTHELIAL CELLS

HMVECs Culture

HMVECs were cultured in Endothelial Growth Medium supplemented according the manufacturer recommendations, in a 5% CO_2 95% air atmosphere incubator. The cells reached complete confluence after 48 hours of culture.

HMEVCs Viability

HMVECs were seeded in a 96-well plate with a density of 2 × 104/well in Endothelial Growth Medium. The rest of the protocol was the exact one described above for 3T3-L1 cells.

IN VIVO STUDY

ANIMALS AND SURGICAL PROCEDURES

One-month old Wistar (W) and GK rats from local breeding colonies were housed in a controlled environment with day-night cycles of 12 h, temperature between 22–24 °C, and a relative humidity of 50–60%. Animals had ad libitum access to water and to either standard rat chow (AO3, Charles River, SAFE, France) (SD) or high-caloric diet (AO3- derived) enriched with sucrose (20%) and fat (20%) (HCD) (Charles River, SAFE, France). At 4 months old part of the group GKHCD was subdivided in: sham surgery (GKHCD_Sh) or sleeve gastrectomy (GKHCD_Sl) (**Figure 8**). The animals assigned to surgery were operated under intramuscular anesthesia using ketamine (75 mg/kg body weight, Pfizer Inc., New York, USA) and chlorpromazine (3 mg/kg body weight, Laboratórios Vitória, Amadora, Portugal). The abdomen was shaved and the skin prepped with a 4 % polividone-iodine solution (MEDA Pharma, Lisbon, Portugal). A midline incision was performed and the abdominal cavity entered. VSG was achieved by dissection of the greater curvature including the lower part of the stomach and the aglandular forestomach with ligation of the short gastric vessels, accordingly to a technique described before [99]. The stomach was sectioned over a bulldog clamp to fashion the gastric sleeve. The operative specimen, including most of the forestomach, was removed. The gastric wound was closed with a continuous invaginating extramucosal suture with a 4/0 midterm absorbable synthetic glyconate monofilament thread. Animals assigned to the sham operation were incised in the anterior gastric wall. To resemble a more complex surgical technique, an interval of 20 min was observed before closing the gastric incision with a continuous extramucosal technique, as described above. At 6

months old, the animals were sacrificed by cervical displacement and peri epididymal white adipose tissue (pEAT) was collected, cleaned, weighted, and immediately frozen in liquid nitrogen to be stored at -80 °C to perform Western Blotting analysis. The study protocol regarding the use of laboratory animals was approved by the Ethics Committee of the Faculty of Medicine of the University of Coimbra, Coimbra, Portugal.

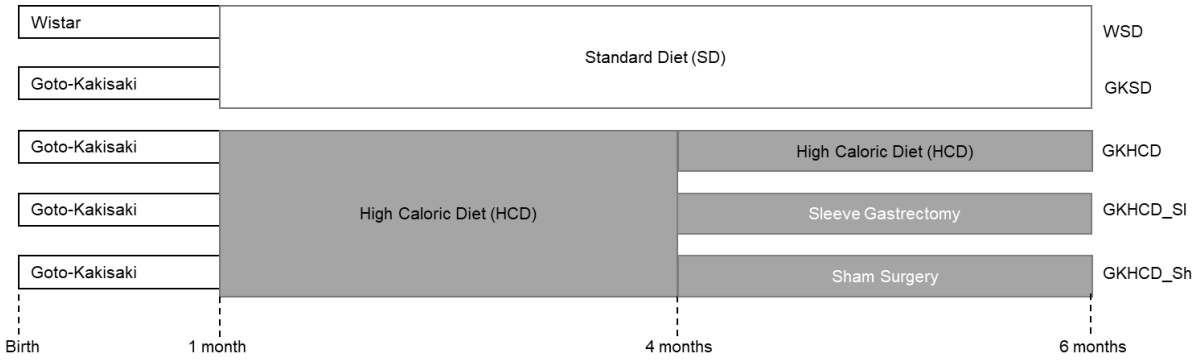

Figure 8 - ***In vivo*** **studies experimental design.** Wistars rats were fed standard diet. Goto-Kakizaki rats were fed either standard or high caloric diet. At 4 months of age part of diet-induced obese Goto-Kakizaki rats were submitted either to sleeve gastrectomy or sham surgery.

WESTERN BLOTTING OF TISSUE HOMOGENATES

Recurring to dry ice to avoid thawing, 150 mg of pEAT were collected and homogenised in 0.5 mL of lysis buffer in Tissue Lyser II (Qiagen, Germany). The lysates were cleared by centrifugation for 15 minutes at 14000 rpm, 4ºC, allowing a separation of the fat (top layer) from the protein fraction (supernatant) and from the membranes and nucleic acids present on the pellet. The supernatant was collected and the following protocol is the same as the one described above for the cellular extracts.

HUMAN STUDY

PATIENT SELECTION AND CHARACTERIZATION

A cohort of patients with obesity with or without diabetes aged from 25 to 65 years was recruited from the obesity surgery consultation at the Hospital Geral de Coimbra (Covões) to participate on a prospective longitudinal study entitled “Estudo Anatomo-Morfológico do Tecido Adiposo na Obesidade”. The study was approved by the Ethical Committee of Centro Hospitalar e

Universitário de Coimbra and Faculdade de Medicina da Universidade de Coimbra and patients signed an informed consent according to the principles outlined in the Declaration of Helsinki. The exclusion criteria were active inflammatory and/or chronic diseases, previous submission to restrictive/malabsorptive surgical procedures, and T2DM medication other than metformin.

CLINICAL DATA AND SAMPLE COLLECTION

One day before surgery, fasting blood samples were collected. Serum and plasma were isolated and all samples were stored and kept at -80ºC. Several clinical blood parameters were evaluated by an automatic analyser, such as fasting glucose, insulin, glycated haemoglobin (HbA1c), cholesterol and triglycerides levels. Both fasting glucose and insulin levels were then used to calculate the percentage of insulin sensitivity and β-cell function as well as the homeostasis model assessment 2 insulin resistance index (Ox-HOMA2IR) (Oxford, United Kingdom). Ox-HOMA2IR was described by Jonathan Levy in 1998 and differs from the usual HOMA by accounting for variations in hepatic and peripheral insulin resistance, increases in insulin secretion curve for plasma glucose concentrations superior to 180 mg/dL and also the contribution of circulating proinsulin [100]. VAT biopsies were collected from the obese patients while undergoing metabolic surgery and kept in liquid nitrogen during transportation to our facilities, to be then stored at -80ºC. All the generated clinical data was properly organized in a database in SPSS Statistics Software, version 24.0 (IBM, USA).

SUBJECT GROUPS ASSIGNEMENT

In total, 140 patients (113 women and 27 men) were divided in groups according to glycaemic profile: fasting glucose levels, HbA1c and Ox-HOMA2IR. Such subject characterization resulted in four different groups: 1 – insulin sensitive group (IS) (n=20), composed by individuals that were both IS and normoglycemic (NG) (Ox-HOMA2IR<1); 2 – insulin resistant (IR) and NG group (n=66), with insulin resistant patients (Ox-HOMA2IR>1) that were normoglycemic (fasting glucose<100 mg/dL and HbA1c<5.7%); 3 – pre diabetic group (n=34), that allocated IR patients with fasting glucose levels from 100 to 125mg/dL or HbA1c between 5.7 and 6.4%; 4 – T2DM group (n=20), constituted by IR subjects diagnosed with T2DM (fasting blood glucose above 125mg/dL or HbA1c>6.4%).

TOTAL RNA EXTRACTION FROM HUMAN VISCERAL ADIPOSE TISSUE

Total RNA from 100mg of human VAT was isolated with a RNeasy Lipid Tissue Mini Kit (Qiagen, Germany). Upon isolated, RNA samples were analysed by NanoDrop One/One spectrophotometer (ThermoFisher, Waltham, MA, USA) at 260nm to evaluate RNA concentration and checked for possible protein or phenol contamination. RNA samples were also analysed through capillary electrophoresis with an Agilent RNA 6000 Nano Kit and the results obtained with the Agilent 2100 Bioanalyser (Agilent Technologies, CA, USA) at *Laboratório de Biomedicina Mitocondrial e Teranóstica, Centro de Neurociências e Biologia Celular*, to provide information on RNA integrity.

QUANTITAIVE REAL-TIME POLYMERASE CHAIN REACTION USING THE HIGH-THROUGHPUT PLATFORM BIOMARK™ HD SYSTEM

Each RNA sample was diluted to the same concentration (25 ng/µL). Reverse transcriptase enzyme (1 µL) [qScript cDNA super mix (Quanta BioSciences)] was added to 4 µL of each RNA sample to obtain cDNA. Each cDNA sample was pre-amplified. Briefly, 1.25 µL of each cDNA sample was mixed with 0.5 µL of a mix of pooled primers (500 nM final concentration each), 2.25 µL water and 1 µL of PreAmp Master Mix enzyme (Fluidigm). Thermal cycling was performed according to the enzyme manufacturer for 12 cycles. The samples were then treated with Exonuclease I (New England Biolabs) to remove unincorporated primers. After Exonuclease treatment the samples were diluted 5x in TE buffer (10 mM Tris-HCl, 1 mM EDTA). For each sample (2.25 µL) a Pre-Mix was prepared with 2.5 µL SsoFast Eva Green Supermix (BioRad), 0,25 µL of 20x DNA binding dye sample reagent (Fluidigm). The samples (5 µl) were pipetted into the respective inlet of a Fluidigm® 96.96 Gene expression IFC. For each assay (gene) a mix of 12 µL was individually prepared: 6 µL of 2x Assay loading reagent (Fluidigm), 5.4 µL of TE buffer, 1.2 µL from a stock of 50 µM each mixed forward and reverse primers. The assays (5 µL) were pipetted into their respective assay inlets on the chip. The assay and sample mixes were loaded with the Load mix (136x) script of the HX controller (HD Biomark). After loading the chip qRT-PCR was carried out using the BioMark HD™, accordingly to the cycling parameters recommended by Fluidigm® for 96.96 Gene expression Integrated Fluidic Circuit. Data were

collected with Data Collection Software and were analyzed using Fluidigm® Real Time PCR Analysis v2.1 software. Genes with melting curves displaying more of one peak (amplification of non-specific products) were not included in the analysis. The data were normalized for the reference gene *ATCB*. The primers were obtained from Sigma and reconstituted to a final concentration of 100 µM in water. **Table 2** resumes the genes and primer sequences relevant for this project.

Table 2 – Genes of interest and respective sense and antisense sequences.

Gene	Sense	Antisense
NPY	CAGGCAGAGATATGGAAAAC	TTACACGATGAAATATGGGC
NPY1R	TTTCCATCGGACTCTCATAG	TTCTTTTGGTTTCACTGGAC
NPY2R	AGGTCAGTTGTAGACTCTTG	TGGTACTATCTATAAGCTCTGG
NPY5R	AAAGGGTGTTACAAGGAAAG	CAGCAGTATTATTCTCTGTGG
PYY1R	TCACTTAACTACATCTCCTGG	GCCTCTCAGATTCTCTAGTC
MBOAT4	CTCCTTCTGTGAGGTTCTG	GAGCAAGTAGCTGAAATAGG
GHSR	ATCTGCTCATCTTCCTCTG	TGACGAATTGGAAGAGTTTG

Chapter 4 - RESULTS

THE METABOLIC HEALTHY TO UNHEALTHY STATUS TRANSITION IS PARALLEL WITH ADIPOSE TISSUE DYSFUNCTION

As previously stated in the Materials and Methods section, obese subjects were assigned to 4 different groups: 1 – insulin sensitive and normoglycemic group (IS NG) (n=20), composed by subjects with MHO, that presented normal fasting glycemia (86.4 ± 11.7 mg/dL), HbA1c [34.4 ± 1.29 (5.3 ± 0.2)] and Ox-HOMA2IR (0.6 ± 0.2); 2 – insulin resistant and normoglycemic group (IR NG) (n=66), with insulin resistant patients with higher Ox-HOMA2IR levels (2.7 ± 1.3) that were normoglycemic (86.7 ± 8.3 mg/dL), with HbA1c [34.4 ± 1.29 (5.3 ± 0.2)]; 3 – pre diabetic group (IR Pre-Diabetic) (n=34), that allocated insulin resistant patients with increased HOMA2 index (2.4 ± 1.2) and with increased fasting glucose levels (96.1 ± 11.6 mg/dL) or HbA1c [41 ± 1.38 (5.9 ± 0.2)]; 4 – T2DM group (IR Diabetic) (n=20), constituted by insulin resistant subjects with high Ox-HOMA2IR index (3.0 ± 1.9) and diagnosed with T2DM, thus having fasting blood glucose above 125 mg/dL (126.3 ± 28.3 mg/dL) and HbA1c>6.4% [54.1 ± 5.33 (7.1 ± 0.7)]. The full characterization of patient's metabolic parameters among the groups is detailed in Rodrigues *et al.* 2020, a recent paper published by our research group [101].

Plasma leptin levels were similar among groups, whereas adiponectin was drastically reduced in group 4 in comparison to group 1 ($p<0.01$) and groups 2 and 3 ($p<0.05$) [101]. Furthermore, both groups 2 and 4 had increased triglycerides levels when comparing to the IS NG patients ($p<0.05$ and $p<0.01$, respectively). Additionally, individuals from group 4 presented lower high-density lipoprotein (HDL) cholesterol than groups 1 and 2 ($p<0.001$ and $p<0.05$, respectively), and the IR Pre-Diabetic group (3) lower than group 1 ($p<0.05$) [101].

The hypoadiponectinemia seen in obese pre-diabetic and diabetic patients is a clear marker of AT dysfunction, and so are the lower HDL cholesterol and increased fasting triglycerides, corroborating the strong relation between AT dysfunction and the transition from MHO to MUO as well as the development of T2DM.

NPY/Y RECEPTORS SYSTEM IS ALTERED IN THE VISCERAL ADIPOSE TISSUE OF OBESE INSULIN RESISTANT/DIABETIC PATIENTS

Alteration of visceral adipose tissue NPY/Y receptors system in obese subjects at several stages of metabolic dysregulation

When analysing the expression of the Y receptors, which all bind NPY in its native or cleaved forms, despite with different affinity, a similar behaviour was detected in *NPY1R* and *NPY5R*. Both receptors were significantly increased in group 3 (IR Pre-Diabetic) in relation to insulin-sensitive patients, and decreased then in patients of group 4 (IR Diabetic). In detail, *NPY1R* was significantly increased in group 3 ($p<0.05$ vs IS NG (1)) and decreased then in group 4, but when comparing to the IR Pre-Diabetic (3) group ($p<0.05$ vs IR Pre-Diabetic (3)), whereas *NPY5R* was augmented in group 3 when comparing to group 1 ($p<0.05$ vs IS NG (1)) and its expression was again reduced group 4 in comparison to both groups 2 and 3 ($p<0.05$ vs IR NG (2), $p<0.01$ vs IR Pre-Diabetic (3)) (**Figure 9A and 9D**). In contrast, *NPY2R* had a different expression profile among groups, being significantly decreased, right in the IR NG group, when comparing to group 1 ($p<0.05$ vs IS NG (1)), a result that was extended also to group 3 ($p<0.05$ vs IS NG (1)) (**Figure 9B**). Regarding *PPY1R* expression, or *NPY4R*, as often addressed, no significant alteration was found among the four groups (**Figure 9C**). The expression of the *NPY* gene, despite not reaching statistical significance, showed a tendency to decrease in the IR Pre-Diabetic group (3), in comparison to group 1 ($p=0.067$) (**Figure 9E**).

Given the similar pattern of gene profile expression of *NPY1R* and *NPY5R* among the groups (**Figure 9A and 9D**), a Spearman correlation analysis was performed to access behaviour similarities between the different types of Y receptors (**Table 3**). In fact, a strong positive correlation was found between *NPY1R* and *NPY5R* ($r= 0.870$, $p=.000$) and *PPY1R* and *NPY2R* ($r= 0.659$, $p=.000$) (**Table 3**). Low positive correlations were found between *NPY1R* and *PPY1R* ($r= 0.349$, $p=0.001$) and *DPP4* and *PPY1R* ($r= 0.319$, $p=0.002$) and an even weaker, but still significant, positive correlations were found in *NPY* versus *DPP4* ($r= 0.242$, $p=0.021$).

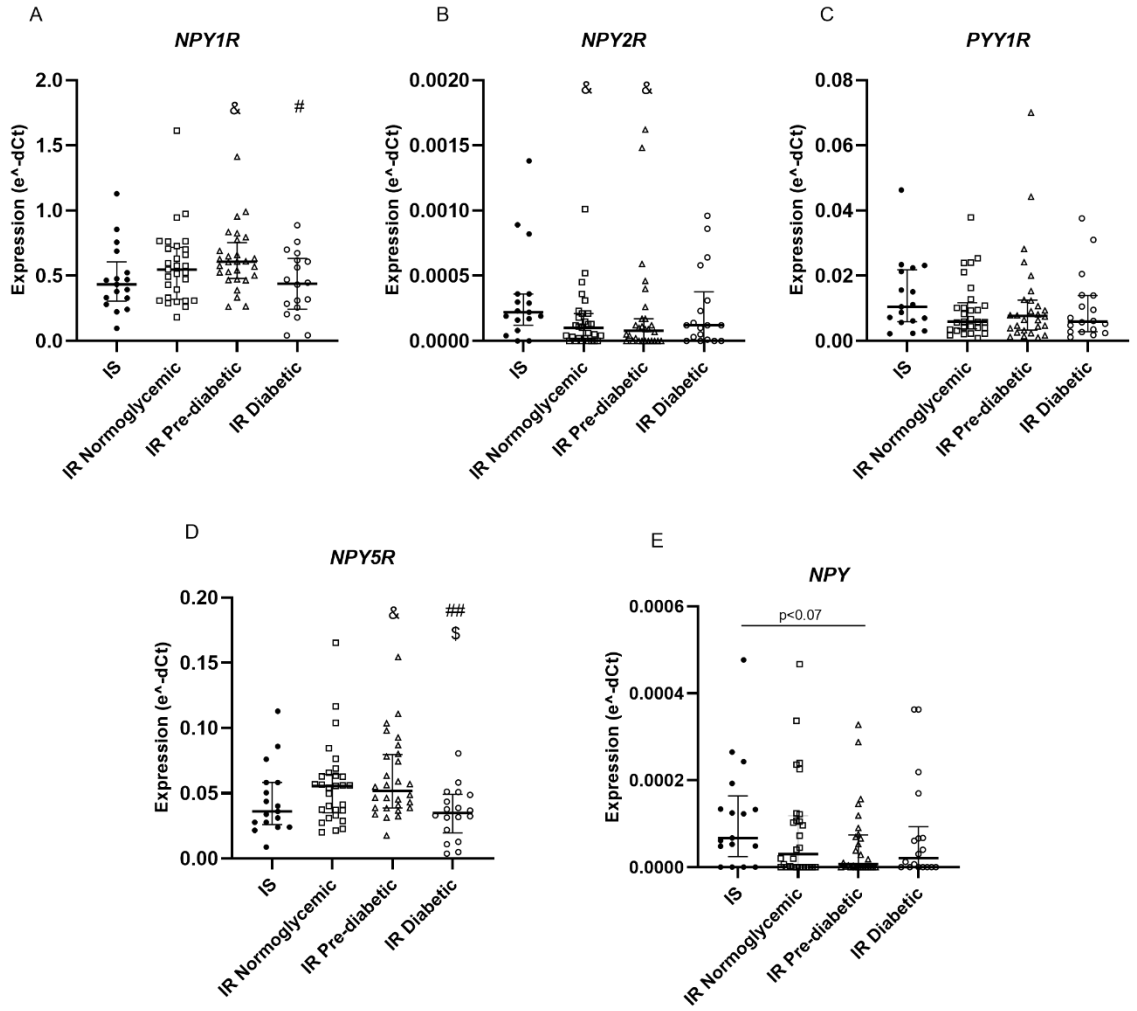

Figure 9 – **Gene expression profile of the NPY/Y receptors system in the VAT of obese individuals at different stages of metabolic dysregulation.** *NPY1R* and *NPY5R* expression was significantly increased in pre-diabetic patients and lowered with the development and aggravation of T2DM in group 4 (**A and D**). The expression of *NPY2R* was decreased in insulin-resistant normoglycemic patients and in pre-diabetics (**B**). PPY1R was not altered in the different groups (**C**), and so was not NPY expression, despite a slight tendency to decrease in the pre-diabetic group (**E**). Data is presented as median and interquartile range per group, and Kruskal-Wallis comparisons were conducted to compare among the groups, since groups had reduced sample size (<30). p<0.05 was considered significant. & vs IS NG; $ vs IR NG; # vs IR Pre-Diabetic. 1 symbol: p<0.05; 2 symbols: p<0.01; 3 symbols: p<0.001.

Table 3 – Spearman correlation analysis of NPY/Y receptors system.

	PPY1R	*NPY2R*	*NPY5R*	*DPP4*	*NPY*
NPY1R	r= 0.349 p= **0.001** n= 92	r= 0.059 p= 0.575 n= 92	r= 0.870 p= **.000** n= 92	r= 0.157 p= 0.136 n= 92	r= 0.077 p= 0.471 n= 91
PPY1R		r= 0.659 p= **.000** n= 92	r= 0.197 p= 0.059 n= 92	r= 0.319 p= **0.002** n= 92	r= 0.206 p= **0.05** n= 91
NPY2R			r= 0.016 p= 0.876 n= 92	r= -0.002 p= 0.987 n= 92	r= 0.08 p= 0.452 n= 91
NPY5R				r= 0.034 p= 0.744 n= 92	r= 0.047 p= 0.655 n= 91
DPP4					r= 0.242 p= **0.021** n= 91

Regarding DPP4, the enzyme responsible by NPY cleavage, its expression was consistent among groups, not revealing any significant alteration (**Figure 10 left**) and so was the expression of *MBOAT4*, the gene that encodes for the GOAT enzyme, that is responsible for ghrelin acylation (**Figure 10 right**). The *GHSR* gene, that encodes for the GHSR1a was, unexpectedly, not found in the VAT of these patients. The gene was impossible to detect in the number of thermal cycles performed in the qRT-PCR analysis.

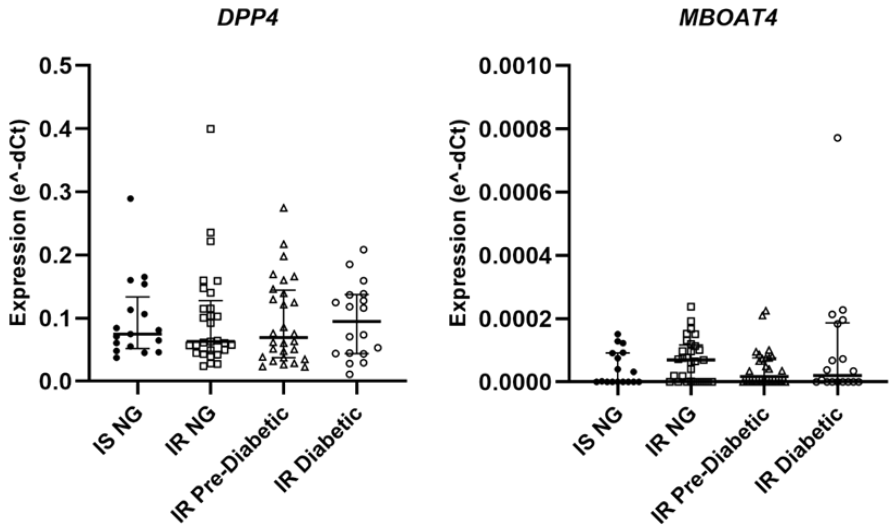

Figure 10 - Gene expression profile of *DPP4* and *MBOAT4* was not altered in the VAT of obese individuals at different stages of metabolic dysregulation. Data is presented as median and interquartile range per group, and Kruskal-Wallis comparisons were conducted to compare among the groups, since groups had reduced sample size (<30). $p<0.05$ was considered significant. & vs IS NG; $ vs IR NG; # vs IR Pre-Diabetic. 1 symbol: $p<0.05$; 2 symbols: $p<0.01$; 3 symbols: $p<0.001$.

Alteration of visceral adipose tissue NPY/Y receptors system in obese insulin resistant patients

Given the observed alteration of the system in patients with some degree of metabolic dysregulation, we then asked if there were any prominent alterations in the genetic profile expression parallel with insulin-resistance development. Therefore, we conducted a two-group analysis: IS vs IR (being the IS group composed by insulin-sensitive patients, and thus the ones from group 1, and the IR group constituted by all insulin-resistant patients, that were previously allocated in groups 2, 3 and 4). The expression of *NPY2R* was significantly decreased in the IR individuals when compared to the IS group (**Figure 11A**), while *NPY* showed a trend to decrease in the same group (**Figure 11B**). The expression pattern of the other Y receptors, *DPP4* and *MBOAT4* was unaltered in this two-group analysis (data not shown).

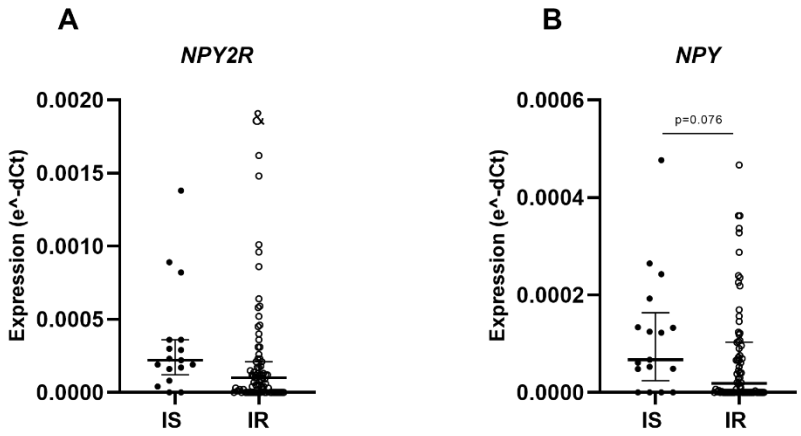

Figure 11 - **Gene expression profile of the *NPY2R* and *NPY* in the VAT of insulin-sensitive or resistant obese individuals.** *NPY2R* expression decreases in the IR group (A), as well as *NPY's* (B), although the latter did not reach statistical significance. Data is presented as median and interquartile range per group, and Kruskal-Wallis comparisons were conducted to compare among the groups, since IS group had reduced sample size (<30). p<0.05 was considered significant. & vs IS. 1 symbol: p<0.05; 2 symbols: p<0.01; 3 symbols: p<0.001.

THE ALTERATION OF VISCERAL ADIPOSE TISSUE NPY/Y RECEPTORS SYSTEM CORRELATES WITH THE PROGRESSION OF METABOLIC DYSFUNCTION MARKERS IN OBESE PATIENTS

We then pursued to analyse the relation between the ghrelin/NPY axis components and several anthropometric parameters and components of the metabolic syndrome evaluated in the cohort of obese patients, that have been described to suffer alterations among the groups, as metabolic dysregulation aggravates, from group 1 to 4 [101]. Despite all being weak, we observed negative correlations between *NPY* and weight and *NPY2R* and BMI (r= -0.339, p=0.001 and r= -0.233, p=0.038, respectively) (**Table 4**). Furthermore, a subtle positive correlation was found between NPY and HDL cholesterol (r= 0.277, p=0.009) (**Table 4**). *NPY1R, NPY5R* and *PPY1R* expression did not correlate with any of the parameters (**Table 4**), as well as *DPP4* and *MBOAT4* (data not shown).

Table 4 - Spearman correlation analysis of NPY/Y receptors system and several metabolic parameters evaluated in the total cohort of obese patients.

	NPY1R	*PPY1R*	*NPY2R*	*NPY5R*	*NPY*
Weight (kg)	r= -0.086	r= -0.113	r= -0.158	r= -0.105	r= -0.339
	p= 0.428	p= 0.293	p= 0.141	p= 0.329	**p= 0.001**
	n= 88	n= 88	n= 88	n= 88	n= 87
BMI (kg/m^2)	r= -0.099	r= -0.177	r= -0.233	r= -0.120	r= -0. 199
	p= 0.361	p= 0.101	**p= 0.038**	p= 0.268	p= 0.066
	n= 87	n= 87	n= 87	n= 87	n= 86
Leptin (ng/mL)	r= o.150	r= -0.009	r= -0.007	r= 0.139	r= 0.031
	p= 0.201	p= 0.942	p= 0.951	p= 0.236	p= 0.794
	n= 74	n= 74	n= 74	n= 74	n= 73
Adiponectin (ug/mL)	r= -0.099	r= -0.101	r= -0.055	r= 0.045	r= 0.061
	p= 0.363	p= 0.350	p= 0.614	p= 0.680	p= 0.577
	n= 87	n= 87	n= 87	n= 87	n= 86
HDL cholesterol (mg/dL)	r= -0.067	r= -0.029	r= 0.094	r= 0.125	r= 0.277
	p= 0.532	p= 0.786	p= 0.383	p= 0.242	**p= 0.009**
	n= 89	n= 89	n= 89	n= 89	n= 88
OxHOMA2IR	r= 0.139	r= -0.004	r= -0.063	r= 0.091	r= -0.095
	p= 0.187	p= 0.970	p= 0.555	p= 0.393	p= 0.375
	n= 91	n= 91	n= 91	n= 91	n= 90
Fasting glycemia (mg/dL)	r= -0.045	r= 0.032	r= -0.055	r= -0.166	r= -0.073
	p= 0.673	p= 0.766	p= 0.601	p= 0.113	p= 0.490
	n= 92	n= 92	n= 92	n= 92	n= 91
HbA1c (mmol/mol) (%)	r= -0.152	r= -0.057	r= 0.002	r= -0.234	r= -0.199
	p=0.247	p= 0.663	p= 0.987	p= 0.072	p= 0.127
	n= 92	n= 92	n= 92	n= 92	n= 91

However, the analysis of the correlations in the total population (**Table 4**) did not allow to understand if and how are these relationships altered during the onset and development of the metabolic complications that are present as we move along from group 1 to 4. Thus, a new Spearman correlation analysis was performed considering the four-group division: IS NG (group 1), IR NG (group 2), IR Pre-Diabetic (group 3) and IR Diabetic (group 4) (**Table 5**).

Moderate and low negative correlations were found between *NPY* and weight and BMI in IR patients with normal fasting glycemia (2) (r= -0.522, p=0.005 and r= -0.381, p=0.05), albeit not reaching statistical significance for BMI. In pre-diabetic patients (3), *NPY* negatively correlates with HbA1c (r= -0.474, p=0.017). Interestingly, *NPY* expression seemed to increase with plasma adiponectin increase, in group 1 (r= 0.467, p=0.068), an effect that was the opposite of what was verified for group 3 (r= -0.395, p=0.051), although neither of the correlations reached statistical differences. Additionally, *NPY* tended to correlate with HDL cholesterol, only in the IS NG group (r= 0.513, p=0.05) (**Table 5**). In the IS NG group, moderate positive correlations were present in *NPY1R* and *NPY5R* versus plasmatic leptin, which vanished in the other groups (r= 0.613, p=0.02 and r= 0.543, p=0.045 vs IS NG, respectively). Moreover, plasma adiponectin levels were negatively correlated with *NPY1R*, only in group 3 (r= -0.484, p= 0.014) (**Table 5**). *PPY1R* and *NPY2R* were not correlated to any parameter in the four-group analysis, and the same was verified for *DPP4* and *MBOAT4* (data not shown for the latter).

Table 5 - Spearman correlation analysis of NPY/Y receptors system and several metabolic parameters evaluated in the obese patients, presented by groups.

		IS NG (1)			IR NG (2)			IR Pre Diabetic (3)			IR Diabetic (4)		
NPY	**Weight (kg)**	r= -0.174	p= 0.553	n= 14	r= -0.522	**p= 0.005**	n= 27	r= -0.125	p= 0.526	n= 28	r= -0.386	p= 0.113	n= 18
	BMI (kg/m²)	r= -0.016	p= 0.958	n= 14	r= -0.381	p= 0.05	n= 27	r= 0.046	p= 0.821	n= 27	r= -0.188	p= 0.454	n= 18
	Leptin (ng/mL)	r= 0.164	p= 0.574	n= 14	r= 0.211	p= 0.323	n= 24	r= -0.259	p= 0.283	n= 19	r= 0.107	p= 0.692	n= 16
	Adiponectin (ug/mL)	r= 0.467	p= 0.068	n= 16	r= 0.223	p= 0.253	n= 28	r= -0.395	p= 0.051	n= 25	r= -0.264	p= 0.306	n= 17
	HDL cholesterol (mg/dL)	r= 0.513	p= 0.050	n= 15	r= 0.311	p= 0.107	n= 28	r= -0.212	p= 0.279	n= 28	r= 0.108	p= 0.680	n= 17
	OxHOMA2IR	r= -0.001	p= 0.996	n= 16	r= -0.030	p= 0.881	n= 28	r= -0.040	p= 0.841	n= 28	r= 0.265	p= 0.288	n= 18
	Fasting glycemia (mg/dL)	r= -0.404	p= 0.107	n= 17	r= 0.252	p= 0.196	n= 28	r= 0.063	p= 0.752	n= 28	r= -0.050	p= 0.845	n= 18
	HbA1c (mmol/mol) (%)	r= 0.223	p= 0.564	n= 9	r= -0.461	p= 0.180	n= 10	r= -0.474	**p= 0.017**	n= 25	r= 0.047	p= 0.863	n= 16
NPY1R	**Weight (kg)**	r= 0.332	p= 0.246	n= 14	r= -0.073	p= 0.711	n= 28	r= -0.222	p= 0.257	n= 28	r= -0.053	p= 0.836	n= 18
	BMI (kg/m²)	r= 0.288	p= 0.318	n= 14	r= 0.253	p= 0.193	n= 28	r= -0.126	p= 0.530	n= 27	r= -0.106	p= 0.675	n= 18
	Leptin (ng/mL)	r= 0.613	**p= 0.020**	n= 14	r= 0.091	p= 0.666	n= 25	r= 0.198	p= 0.416	n= 19	r= -0.229	p= 0.393	n= 16
	Adiponectin (ug/mL)	r= -0.147	p= 0.587	n= 16	r= -0.044	p= 0.819	n= 29	r= -0.484	**p= 0.014**	n= 25	r= -0.074	p= 0.779	n= 17
	HDL cholesterol (mg/dL)	r= -0.075	p= 0.790	n= 15	r= -0.180	p= 0.350	n= 29	r= -0.223	p= 0.253	n= 28	r= 0.074	p= 0.779	n= 17
	OxHOMA2IR	r= -0.143	p= 0.598	n= 16	r= 0.065	p= 0.738	n= 29	r= 0.036	p= 0.857	n= 28	r= 0.313	p= 0.206	n= 18
	Fasting glycemia (mg/dL)	r= 0.016	p= 0.562	n= 17	r= 0.060	p= 0.757	n= 29	r= -0.012	p= 0.951	n= 28	r= 0.173	p= 0.493	n= 18
	HbA1c (mmol/mol) (%)	r= -0.137	p= 0.895	n= 9	r= -0.386	p= 0.270	n= 10	r= -0.223	p= 0.283	n= 25	r= 0.043	p= 0.875	n= 16
NPY5R	**Weight (kg)**	r= 0.499	p= 0.069	n= 14	r= -0.123	p= 0.533	n= 28	r= -0.237	p= 0.244	n= 28	r= -0.110	p= 0.663	n= 18
	BMI (kg/m²)	r= 0.327	p= 0.253	n= 14	r= -0.194	p= 0.323	n= 28	r= -0.074	p= 0.714	n= 27	r= -0.212	p= 0.399	n= 18
	Leptin (ng/mL)	r= 0.543	**p= 0.045**	n= 14	r= 0.088	p= 0.674	n= 25	r= -0.281	p= 0.244	n= 19	r= -0.126	p= 0.641	n= 16
	Adiponectin (ug/mL)	r= -0.038	p= 0.888	n= 16	r= 0.042	p= 0.828	n= 29	r= -0.309	p= 0.133	n= 25	r= 0.071	p= 0.786	n= 17
	HDL cholesterol (mg/dL)	r= 0.147	p= 0.602	n= 15	r= -0.008	p= 0.967	n= 29	r= 0.101	p= 0.539	n= 28	r= 0.145	p= 0.579	n= 17
	OxHOMA2IR	r= -0.065	p= 0.812	n= 16	r= 0.034	p= 0.859	n= 29	r= -0.041	p= 0.766	n= 28	r= 0.284	p= 0.254	n= 18
	Fasting glycemia (mg/dL)	r= -0.120	p= 0.645	n= 17	r= 0.017	p= 0.932	n= 29	r= -0.170	p= 0.387	n= 28	r= 0.069	p= 0.785	n= 18
	HbA1c (mmol/mol) (%)	r= -0.052	p= 0.895	n= 9	r= -0.224	p= 0.533	n= 10	r= -0.266	p= 0.199	n= 25	r= -0.056	p= 0.837	n= 16

		IS NG (1)			IR NG (2)			IR Pre Diabetic (3)			IR Diabetic (4)		
PPY1R	**Weight (kg)**	r= 0.009	p= 0.976	n= 14	r= -0.128	p= 0.518	n= 28	r= -0.044	p= 0.825	n= 28	r= -0.188	p= 0.455	n= 18
	BMI (kg/m²)	r= 0.125	p= 0.670	n= 14	r= -0.117	p= 0.555	n= 28	r= -0.264	p= 0.183	n= 27	r= -0.158	p= 0.531	n= 18
	Leptin (ng/mL)	r= 0.415	p= 0.140	n= 14	r= 0.204	p= 0.328	n= 25	r= -0.186	p= 0.446	n= 19	r= -0.306	p= 0.249	n= 16
	Adiponectin (ug/mL)	r= -0.276	p= 0.300	n= 16	r= 0.079	p= 0.683	n= 29	r= -0.257	p= 0.215	n= 25	r= -0.313	p= 0.222	n= 17
	HDL cholesterol (mg/dL)	r= 0.047	p= 0.869	n= 15	r= 0.079	p= 0.682	n= 29	r= -0.243	p= 0.213	n= 28	r= -0.137	p= 0.599	n= 17
	OxHOMA2IR	r= -0.265	p= 0.321	n= 16	r= -0.061	p= 0.404	n= 29	r= 0.290	p= 0.135	n= 28	r= 0.224	p= 0.372	n= 18
	Fasting glycemia (mg/dL)	r= -0.151	p= 0.562	n= 17	r= 0.188	p= 0.328	n= 29	r= 0.251	p= 0.198	n= 28	r= 0.039	p= 0.877	n= 18
	HbA1c (mmol/mol) (%)	r= 0.052	p= 0.895	n= 9	r= -0.181	p= 0.617	n= 10	r= -0.082	p= 0.699	n= 25	r= 0.025	p= 0.927	n= 16
NPY2R	**Weight (kg)**	r= 0.065	p= 0.825	n= 14	r= 0.080	p= 0.687	n= 28	r= -0.155	p= 0.431	n= 28	r= -0.395	p= 0.105	n= 18
	BMI (kg/m²)	r= -0.135	p= 0.647	n= 14	r=0.083	p= 0.674	n= 28	r= -0.326	p= 0.097	n= 27	r= -0.428	p= 0.076	n= 18
	Leptin (ng/mL)	r= -0.033	p= 0.911	n= 14	r= 0.377	p= 0.063	n= 25	r= -0.324	p= 0.176	n= 19	r= 0.035	p= 0.896	n= 16
	Adiponectin (ug/mL)	r= -0.053	p= 0.845	n= 16	r= -0.032	p= 0.869	n= 29	r= -0.071	p= 0.736	n= 25	r= -0.309	p= 0.227	n= 17
	HDL cholesterol (mg/dL)	r= -0.095	p= 0.736	n= 15	r= 0.159	p= 0.411	n= 29	r= 0.082	p= 0.677	n= 28	r= 0.041	p= 0.876	n= 17
	OxHOMA2IR	r= -0.050	p= 0.854	n= 16	r= -0.024	p= 0.903	n= 29	r= 0.302	p= 0.119	n= 28	r= 0.041	p= 0.873	n= 18
	Fasting glycemia (mg/dL)	r= 0.048	p= 0.855	n= 17	r= 0.085	p= 0.662	n= 29	r= 0.002	p= 0.993	n= 28	r= -0.186	p= 0.459	n= 18
	HbA1c (mmol/mol) (%)	r= 0.156	p= 0.689	n= 9	r= -0.524	p= 0.120	n= 10	r= 0.151	p= 0.471	n= 25	r= -0.315	p= 0.234	n= 16

In order to further study if the observed correlations were altered upon insulin resistance development, we conducted an analysis as before, considering the two groups IS and IR (being the IS group composed by insulin-sensitive patients, and thus the ones from group 1, and the IR group constituted by all insulin-resistant patients, that were previously allocated in groups 2, 3 and 4). In this new two-group analysis, no alterations were verified for the majority of the interest genes (*NPY, PPY1R, NPY2R, DPP4* and *MBOAT4*). However, *NPY5R* showed negative correlations, although weak, with HbA1c in the IR group (r= -0.384, p=0.005) (**Figure 12A**) but not in the IS one (r= -0.053, p=0.895) (**Figure 12B**), and fasting glycemia in IR group (r= -0.245, p=0.034) (**Figure 12C**), but not in the IS one (r= -0.120, p=0.645, respectively) (**Figure 12D**). Similarly, *NPY1R* showed the same behaviour, with a negative correlation with HbA1c (r= -0.281, p=0.046) in the IR group (**Figure 12E**), but not in the IS group (r= -0.137, p=0.724) (**Figure 12F**).

Moreover, both *NPY5R* and *NPY1R* had a strong correlation with plasma leptin levels in IS patients (r= 0.543, p=0.045 and r= 0.613, p=0.020, respectively) (**Figure 13A and 13C**), which was not observed in IR patients (r= 0.029, p=0.823 and r= 0.021, p=0.872, respectively) (**Figure 13B and 13D**).

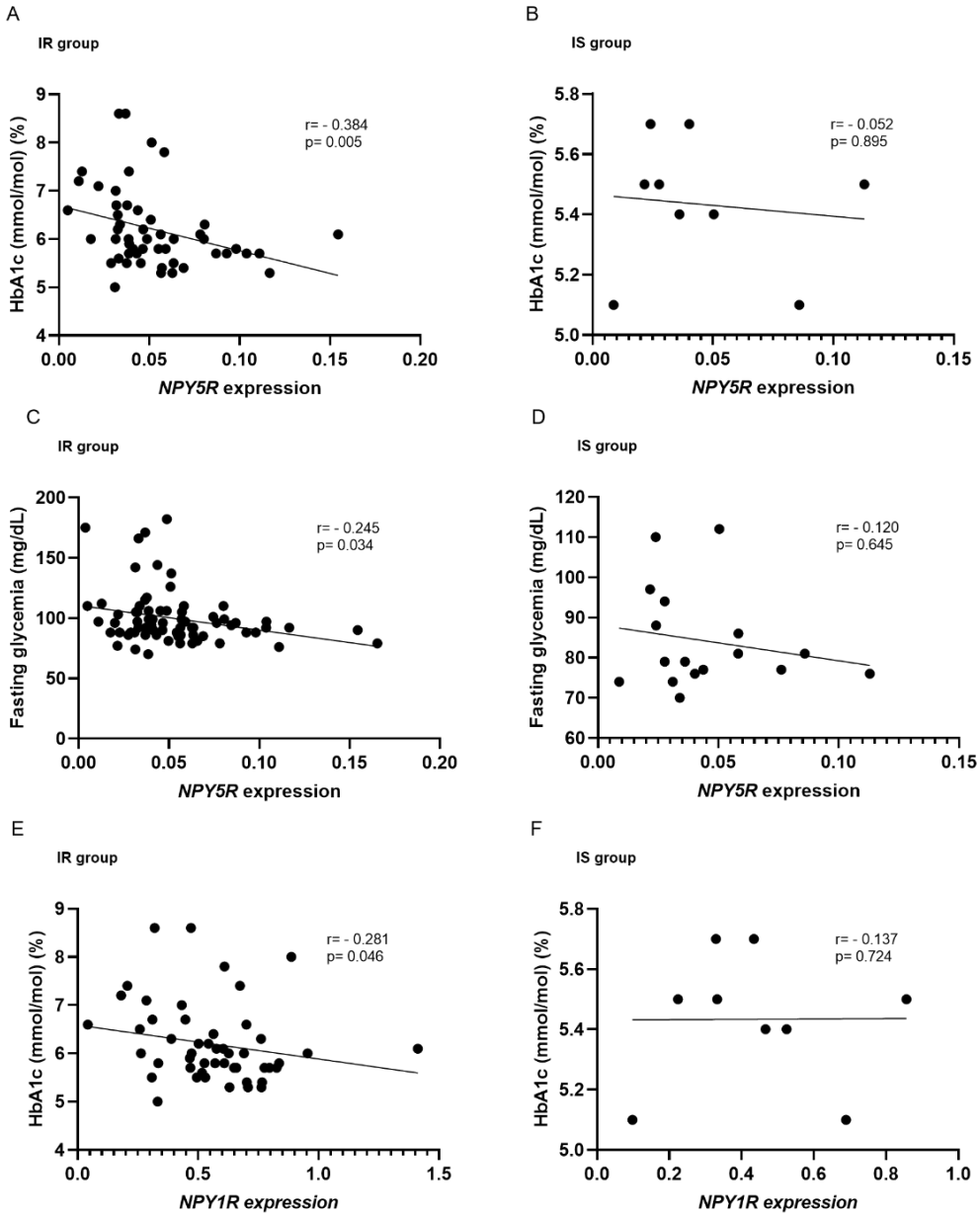

Figure 12 - Spearman correlation analysis of *NPY5R* and *NPY1R* with HbA1c and fasting glycaemia in obese subjects with or without insulin resistance. *NPY5R* VAT expression was negatively correlated with both HbA1c (r= -0.384, p=0.005) and fasting glycemia (r= -0.245, p=0.034) in the IR group (A and C) but not in the IS one (B and D). *NPY1R* VAT expression was negatively correlated with both HbA1c (r= -0.281, p=0.046) in IR patients (E), but not in the IS group (F).

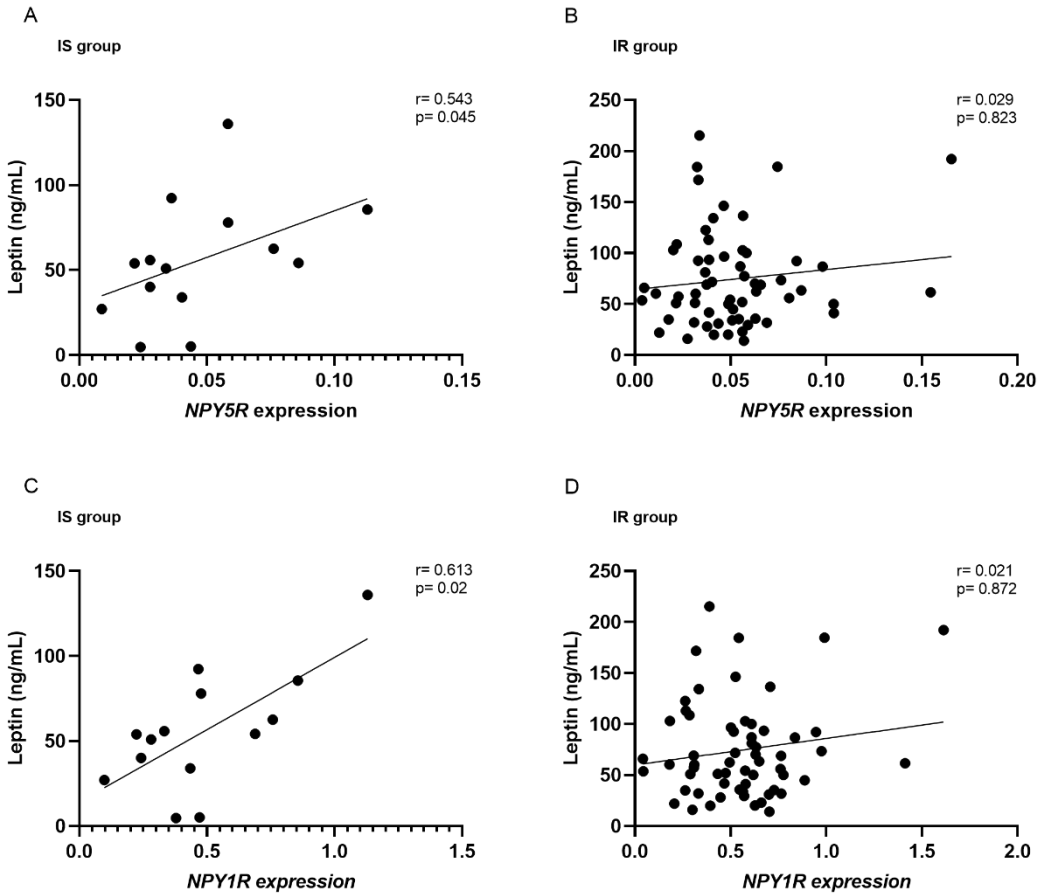

Figure 13 - Spearman correlation analysis of *NPY5R* and *NPY1R* with leptin plasma levels in obese subjects with or without insulin resistance. *NPY5R* and *NPY1R* VAT expression were positively correlated with leptin levels in the IS group (r= 0.543, p=0.045) (A) and (r= 0.613, p=0.02) (C), but not in the IR one (B and D).

Altogether, such results suggest that NPY/Y receptors system correlates with markers of adipose tissue function, such as plasma leptin and adiponectin levels, and negatively with HbA1c in patients with insulin sensitivity or at very early stages of metabolic dysregulation. In the other hand, patients with alterations of glucose metabolism (including prediabetic ones) show a loss of such correlations, denoting an association between the alteration of the NPY/Y receptors system with markers of metabolic dysregulation from MHO to MUO, such as hypoadiponectinemia, leptin resistance and dysglycaemia.

NPY SIGNALLING MACHINERY CORRELATES WITH VISCERAL ADIPOSE TISSUE METABOLISM AND PLASTICITY IN HUMAN OBESITY

Given the recently proposed role of ghrelin as a lipid sensor [66], which suggests a determinant role of ghrelin in nutrient partitioning after meals, we asked whether the ghrelin/NPY axis was actually involved in the regulation of AT metabolism, the main organ responsible for the storage and breakdown of lipids.

A Spearman correlation analysis revealed that, in the cohort of obese subjects, both *NPY1R* and *NPY5R* showed moderate to high positive correlations with several well-known markers of AT metabolism. In detail, *PPARG*, that encodes for the master regulator of adipogenesis, was highly positively correlated with *NPY5R* and at a lesser extent, with *NPY1R* (r= 0.779, p=.000 and r= 0.679, p=.000, respectively) (**Table 6**). Lipid droplet-associated proteins Cidea and perilipin A are encoded by *CIDEA* and *PLIN1* genes, whose expression was observed to be also positively correlated to both *NPY1R* and *NPY5R* expression (*CIDEA* vs *NPY1R*: r= 0.457, p=.000; *CIDEA* vs *NPY5R*: r= 0.579, p=.000; and *PLIN1* vs *NPY1R*: r= 0.430, p=.000; *PLIN1* vs *NPY5R*: p= 0.484, p=.000) (**Table 6**). In contrast, *NPY2R* reveals no correlations and *PYY1R* a very low positive correlation with *PPARG* and *PLIN1* expression levels (r= 0.271, p=0.009 and r= 0.208, p=0.046, respectively) (**Table 6**).

The expression of *PPARA*, an important transcriptional regulator of genes involved in β-oxidation, was verified to be positively correlated with all Y receptors (vs *NPY1R*: r= 0.596, p=.000; vs *PPY1R*: r= 0.633, p=.000; vs *NPY2R*: r= 0.403, p=.000 and vs *NPY5R*: r= 0.593, p=.000) (**Table 6**). Similarly, although being positively correlated with all Y receptors, AT browning-associated *UCP1*, the gene that encodes for the mitochondrial uncoupling protein 1, was drastically differently related to *PYY1R/NPY2R* (high correlation index: r= 0.787, p=.000 and r= 0.711, p=.000, respectively) and *NPY1R/NPY5R* expression (low correlation index: r= 0.394, p=.000 and r= 0.336, p=0.001, respectively) (**Table 6**).

The gene encoding for the insulin receptor, *INSR*, was observed to be positively correlated with the different Y receptors, but at a lower extent with *NPY2R* (vs *NPY1R*: r= 0.562, p=.000; vs *PPY1R*: r= 0.513, p=.000; vs *NPY2R:* r= 0.327, p=0.001; vs *NPY5R*: r= 0.548, p=.000) (**Table 6**). The *NPY*, *DPP4* and *MBOAT4* genes were not correlated with any of the analysed genes.

Table 6 – Spearman correlation analysis of NPY/Y receptors system with genes responsible to regulate metabolism, in the VAT of the total population of obese subjects.

	NPY1R	*PPY1R*	*NPY2R*	*NPY5R*	*NPY*
	r= 0.430	r= 0.208	r= 0.096	r= 0.484	r= -0.099
PLIN1	**p= .000**	**p= 0.046**	p= 0.363	**p= 0.000**	p= 0.350
	n= 92	n= 92	n= 92	n= 92	n= 91
	r= 0.457	r= -0.063	r= -0.154	r= 0.579	r= -0.124
CIDEA	**p= .000**	p= 0.554	p= 0.144	**p= 0.000**	p= 0.242
	n= 92	n= 92	n= 92	n= 92	n= 91
	r= 0.679	r= 0.271	r= 0.155	r=0.779	r= .000
PPARG	**p= .000**	**p= 0.009**	p= 0.140	**p= 0.000**	p= 0.999
	n= 92	n= 92	n= 92	n= 92	n= 91
	r= 0.596	r= 0.633	r= 0.403	r= 0.593	r= 0.190
PPARA	**p= .000**	**p= .000**	**p= 0.000**	**p= 0.000**	p= 0.072
	n= 92	n= 92	n= 92	n= 92	n= 91
	r= 0.562	r= 0.513	r= 0.327	r= 0.548	r= 0.562
INSR	**p= .000**	**p= .000**	**p= 0.001**	**p= 0.000**	p= 0.330
	n= 92	n= 92	n= 92	n= 92	n= 91
	r= 0.394	r= 0.787	r= 0.711	r= 0.336	r= 0.394
UCP1	**p= .000**	**p= .000**	**p= 0.000**	**p= 0.001**	p= 0.384
	n= 92	n= 92	n= 92	n= 92	n= 91

PLIN1 – Perilipin 1 | *CIDEA* - Cell Death Inducing DFFA Like Effector A | *PPARG* – Peroxisome Proliferator-Activated Receptor Gamma | *PPARA* - Peroxisome Proliferator-Activated Receptor Alpha | *INSR* – Insulin Receptor | *UCP1* – Uncoupling Protein 1

The AT secretome reflects the function of its own adipocytes, and thus, the expression of some of the main adipokines were assessed in terms of putative relationships with the ghrelin/NPY axis (**Table 7**). Once again, in the total population, the *NPY1R* and *NPY5R* genes showed a very similar behaviour, presenting positive correlations with *LEP* (r= 0.308, r=0.003 and r= 0.358, p=0.012) and *ADIPOQ* (r= 0.542, p=.000 and r= 0.701, p=.000) (**Table 7**). The *NPY2R* and *PPY1R* genes did not correlate with any of the analysed genes, and so did not *MBOAT4* (data

not shown for the latter). A negative weak correlation was present between *NPY* and *LEP* (r= -0.262, p=0.012), and also between *DPP4* and *TNF* (r= -0.340, p= 0.001) (**Table 7**).

Table 7 - Spearman correlation analysis of NPY/Y receptors system with genes that encode some of the most relevant adipokines, in the VAT of the total population of obese subjects.

	NPY1R	***PPY1R***	***NPY2R***	***NPY5R***	***NPY***	***DPP4***
	r= 0.308	r= -0.081	r= -0.070	r= 0.358	r= -0.262	r= -0.154
LEP	**p= 0.003**	p= 0.445	p= 0.510	**p= .000**	**p= 0.012**	p= 0.144
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92
	r= 0.542	r= 0.047	r= -0.098	r= 0.701	r= 0.013	r= 0.026
ADIPOQ	**p= .000**	p= 0.658	p= 0.352	**p= .000**	p= 0.904	p= 0.808
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92
	r= -0.077	r= 0.012	r= 0.112	r= -0.101	r= -0.047	r= -0.171
IL6	p= 0.466	p= 0.909	p= 0.288	p= 0.340	p= 0.655	p= 0.102
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92
	r= -0.178	r= -0.032	r= 0.072	r= -0.165	r= -0.092	r= -0.109
IL1B	p= 0.089	p= 0.761	p= 0.495	p= 0.116	p= 0.388	p= 0.300
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92
	r= -0.041	r= -0.174	-0.118	r= -0.033	r= -0.013	r= -0.340
TNF	p= 0.697	p= 0.097	p= 0.264	p= 0.751	p= 0.901	**p= 0.001**
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92

LEP – Leptin | *ADIPOQ* – Adiponectin | *IL6* – Interleukin 6 | *IL1B* – Interleukin 1 beta | *TNF* – Tumour Necrosis Factor

Having in mind the inhibitory effect of leptin in hypothalamic NPY, we investigated further the relationship between *NPY-LEP* by conducting a four-group analysis, according to the initial division of the groups: IS NG (group 1), IR NG (group 2), IR Pre-Diabetic (group 3) and IR Diabetic (group 4). Apparently, in groups 1 and 2, *NPY* expression was negatively correlated with *LEP* expression (**Figure 14A and 14B**), albeit not reaching statistical significance in the IS NG (1) group (IS NG: r= -0.434, p=0.082 and IR NG: r= -0.444, p=0.018), an effect that was lost in groups 3 and 4 (IR Pre-Diabetic: r= -0.031, p=0.877 and IR Diabetic: r= 0.191, p=0.867) (**Figure 14C and 14D**).

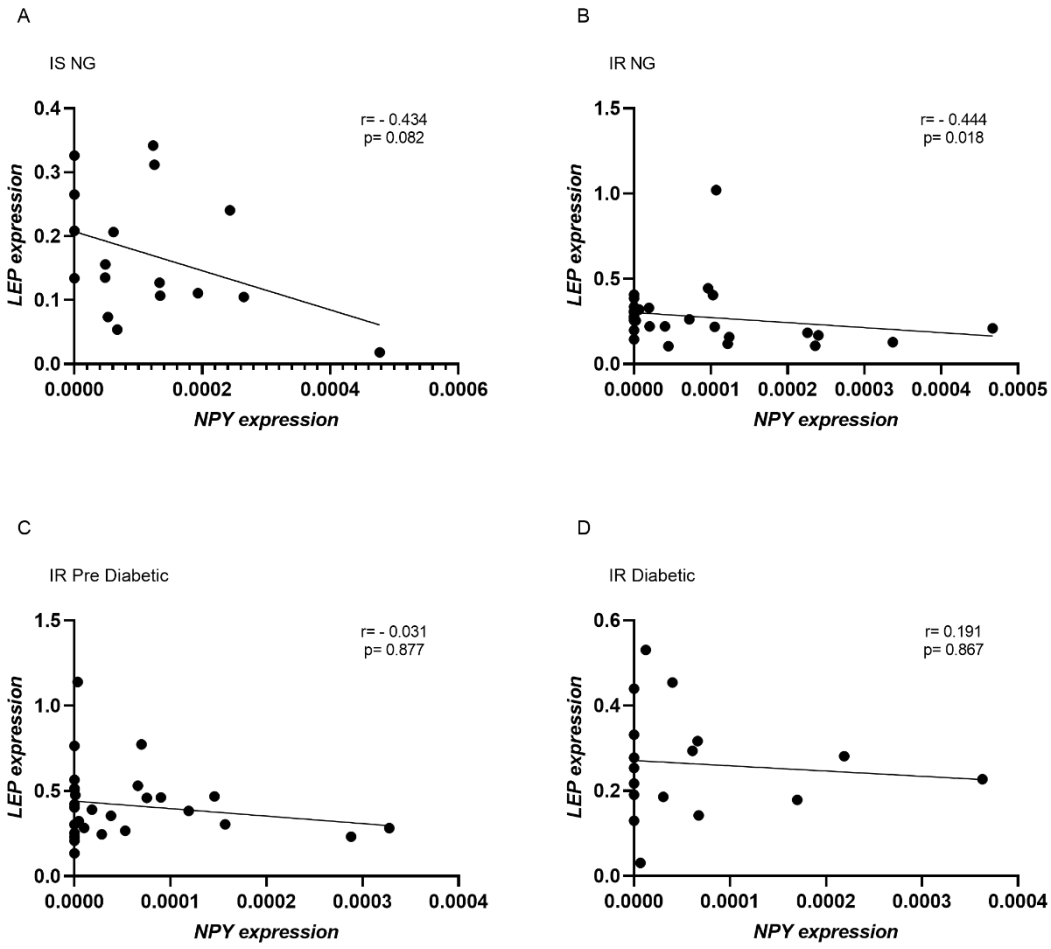

Figure 14 - Spearman correlation analysis of NPY versus LEP expression in the VAT of obese individuals at different stages of metabolic dysregulation. *NPY* VAT expression was negatively correlated with LEP expression in the IR NG group (r= -0.444, p=0.018) (B). A trend for a negative correlation was found in IS NG group (A). The effect seen in IR NG group was vanished in the IR Pre Diabetic (C) and IR Diabetes group (D).

AT lipid metabolism, either favouring nutrient storage or breakdown, requires a proper and function rearrangement of the vascular network. Thus, having in mind the results from the Spearman correlation analysis between the NPY/Y receptors system and several key genes regulating AT metabolism (**Table 6**) we wondered whether there was any relationship between this system and several markers of microvascular function in AT (**Table 8**).

The angiopoietin/Tie system is a crucial regulator of vessels remodelling. Overall, all Y receptors were positively correlated to the genes we analysed that encode proteins from the angiopoietin

signalling, such as *ANGPT1* (angiopoietin 1), *ANGPTL4* (angiopoietin-like 4) and *TEK* (Tie2, the receptor for angiopoietin 1). Nonetheless, NPY1R and NPY5R expression was most prominently positively correlated with *ANGPT1* (r= 0.777, p=.000 and r= 0.812, p=.000, respectively), *ANGPTL4* (r= 0.254, p=0.018 and r= 0.332, p=0.001, respectively) and *TEK* (r= 0.613, p=.000 and r= 0.503, p=.000) (**Table 8**), with ANGPT1 showing the highest values. Neither *PPY1R* nor *NPY2R* correlated with *ANGPTL4*, but showed moderate positive correlations with *TEK* (r= 0.707, p=.000 and r= 0.542, p=.000, respectively) (**Table 8**). Furthermore, *PPY1R* showed a weak positive relation with *ANGPT1* (r= 0.283, p=0.006), but not *NPY2R*, and *DPP4* and *NPY* were negatively correlated, at a lower extent, with *ANGPTL4* (r= -0.254, p=0.014 and r= -0.273, p=0.009, by order).

The *PECAM1* gene, that encodes for the CD31 protein, a member of the immunoglobulin family highly involved in angiogenesis [76] revealed positive correlations with all Y receptors (vs *NPY1R*: r= 0.561, p=.000; vs *PPY1R*: r= 0.370, p=.000; *NPY2R*: r= 0.284, p=0.006; *NPY5R*: r= 0.490, p=.000) (**Table 8**).

Similarly, *VWF*, that encodes for the von Willebrand factor, another participant in the angiogenic process, is positively correlated with the Y receptors (vs *NPY1R*: r= 0.393, p=.000; vs *PPY1R*: r= 0.413, p=.000; *NPY2R*: r= 0.402, p=0.000; *NPY5R*: r= 0.349, p=0.001), also showing a weak negative correlation with *DPP4* (r= -0.311, p=0.003) (**Table 8**). Still on the evaluation of angiogenesis/vascular function markers, we found positive correlations between *VEGFA* and *NPY1R, PPY1R* and *NPY5R* (r= 0.388, p=.000, r= 0.309, p=0.003 and r= 0.514, p=.000, by order). Moreover, all Y receptors were positively correlated with VEGF receptor, *FLT1*, with the *PPY1R/NPY2R* duo revealing the strongest associations (vs *PPY1R*: r=0.512, p=.000; vs *NPY2R*: r=0.510, p=.000; vs *NPY1R* r=0.375, p=.000 and vs *NPY5R*: r=0.294, p=0.004) (**Table 8**).

Finally, the Y receptors showed positive, but weak, correlations with *EPAS1*, that encodes for HIF-2α, a factor that activates target genes crucial for angiogenesis (vs *NPY1R*: r= 0.447, p=.000; vs *PPY1R*: r= 0.235, p=0.024; vs *NPY2R*: r= 0.221, p=0.024; vs *NPY5R*: r= 0.425, p=.000). Furthermore, *EPAS1* was negatively correlated with *DPP4* (r= -0.305, p=0.002) (**Table 8**). No correlations were found between *MBOAT4* and the batch of genes analysed with regards to vascular/angiogenic function (data not shown).

Table 8 – Spearman correlation analysis of NPY/Y receptors system with genes that encode some of the most relevant vascular/angiogenic markers, in the VAT of the total population of obese subjects.

	NPY1R	***PPY1R***	***NPY2R***	***NPY5R***	***NPY***	***DPP4***
	r= 0.777	r= 0.283	r= 0.117	r= 0.812	r= -0.062	r= 0.026
ANGPT1	**p= .000**	**p= 0.006**	p= 0.268	**p= .000**	p= 0.558	p= 0.803
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92
	r= 0.254	r= 0.086	r= -0.045	r= 0.332	r= -0.273	r= -0.254
ANGPTL4	**p= 0.018**	p= 0.416	p= 0.671	**p= 0.001**	**p= 0.009**	**p= 0.014**
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92
	r= 0.613	r= 0.707	r= 0.542	r= 0.503	r= 0.135	r= -0.004
TEK	**p= .000**	**p= .000**	**p= .000**	**p= .000**	p= 0.201	p= 0.972
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92
	r= 0.561	r= 0.370	r= 0.284	r= 0.490	r= 0.017	r= -0.172
PECAM1	**p= .000**	**p= .000**	**p= 0.006**	**p= .000**	p= 0.871	p= 0.102
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92
	r= 0.393	r= 0.413	r= 0.402	r= 0.349	r= -0.043	r= -0.311
VWF	**p= .000**	**p= .000**	**p= .000**	**p= 0.001**	p= 0.685	**p= 0.003**
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92
	r= 0.388	r= 0.309	r= 0.202	r= 0.514	r= 0.093	r= 0.122
VEGFA	**p= .000**	**p= 0.003**	p= 0.054	**p= .000**	p= 0.382	p= 0.248
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92
	r= 0.375	r= 0.512	r= 0.510	r= 0.294	r= -0.022	r= -0.314
FLT1	**p= .000**	**p= .000**	**p= .000**	**p= 0.004**	p= 0.823	p= 0.087
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92
	r= 0.447	r= 0.235	r= 0.221	r= 0.425	r= -0.125	r= -0.305
EPAS1	**p= .000**	**p= 0.024**	**p= 0.034**	**p= .000**	p= 0.238	**p= 0.002**
	n= 92	n= 92	n= 92	n= 92	n= 91	n= 92

ANGPT1 – Angiopoietin 1 | *ANGPTL4* – Angiopoietin-like 4 | *TEK* – Tie2 receptor | *PECAM1* – Platelet and Endothelial Cell Adhesion Molecule | *VWF* – von Willebrand Factor | *VEGFA* – Vascular Endothelial Growth Factor A | *FLT1* – fms Related Receptor Tyrosine Kinase 1 | *EPAS1* – Endothelial PAS domain protein 1

VERTICAL SLEEVE GASTRECTOMY MODULATES GHRELIN/NPY AXIS IN THE ADIPOSE TISSUE OF A DIABETIC OBESE ANIMAL MODEL

VSG deeply modulates the gut secretome while inducing a drastic improvement in AT microvasculature health. Our group has, in fact, demonstrated that VSG restored ghrelin secretion profile [46], and efficient vascular function in HCD-fed GK rats [50]. We hypothesized that the improvement of AT microvasculature health could be related to the normalization of the secretion profile of ghrelin, induced by the surgery.

In fact, in the pEAT, VSG induced alterations in intermediates of ghrelin/NPY axis (**Figure 15**). The GHSR1a was significantly increased in both HCD-submitted groups, GKHCD and GKHCD_Sh ($p<0.01$ and $p<0.05$ vs WSD, respectively) (**Figure 15A**). Despite with no statistical significance, GKHCD_Sl rats had a decrease in GHSR1a levels, when compared to the GKHCD, normalizing its levels in relation to WSD rats ($p<0.06$ vs GKHCD) (**Figure 15A**).

Regarding DPP4, GKHCD rats showed a decrease in comparison to the WSD group ($p<0.01$ vs WSD), that were recovered in the GKHCD_Sl group ($p<0.01$ vs GKHCD and $p<0.05$ vs GKHCD_Sh) (**Figure 15B**).

The NPY receptors system was also modulated by VSG. The NPY1R levels are increased in the GKHCD_Sl, when compared to all other groups ($p<0.005$ vs WSD, $p<0.05$ vs GKSD, $p<0.01$ vs GKHCD and $p<0.01$ vs GKHCD_Sh) (**Figure 15C**). Furthermore, NPY2R levels also showed a drastic increase in the VSG-submitted group (GKHCD_Sl) in comparison to WSD ($p<0.001$), to GKSD ($p<0.05$) and GKHCD ($p<0.05$) (**Figure 15D**).

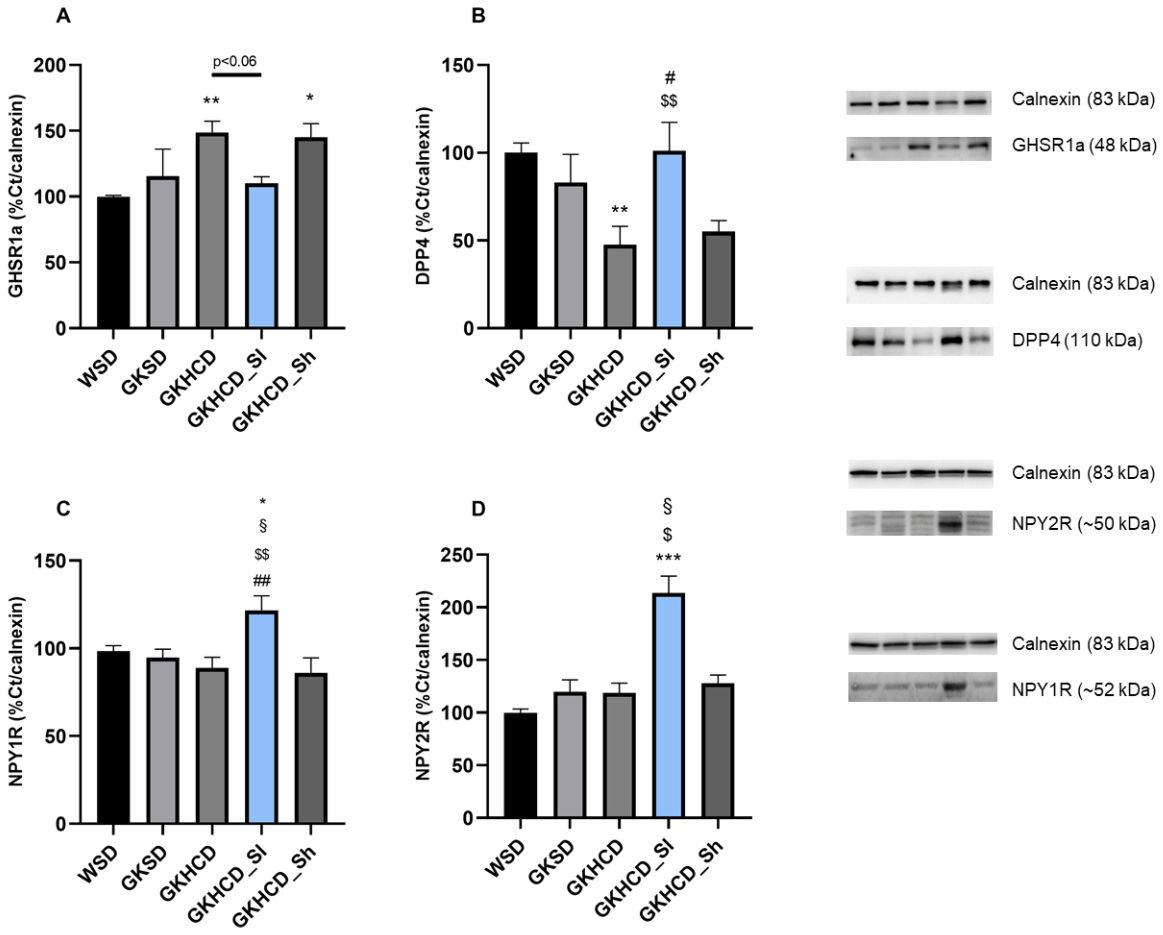

Figure 15 – VSG modulates the ghrelin/NPY axis in the pEAT of type 2 diabetic obese rats. Surgery decreased GHSR1a levels (A), while leading to an augment in DPP4 (B), NPY1R (C) and NPY2R (D). Representative images of western blot proteins of interest and loading controls (calnexin) are shown at the right panel. WSD – Wistar rats standard diet-fed, GKSD – GK rats standard diet-fed, GKHCD – GK rats high caloric diet-fed, GKHCD_Sl - GK rats high caloric diet-fed submitted to VSG and GKHCD_Sh - GK rats high caloric diet-fed submitted to sham surgery. Bars represent mean ± SEM and Kruskal-Wallis comparisons were conducted to compare among the groups. * vs WSD, § vs GKSD, $ vs GKHCD and # vs GKHCD_Sh. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

GHRELIN AND DES-ACYL GHRELIN STIMULATE LIPID ACCUMULATION AND ADIPOGENESIS IN 3T3-L1 CELL LINE

Since ghrelin/NPY axis in pEAT was modulated by VSG, we hypothesized that ghrelin might have an influence of adipose cells, that could explain the marked improvement in AT plasticity and expandability after surgery. In order to understand what would be these potential mechanisms, we recurred to the 3T3-L1 fibroblasts cell line, that are able to differentiate into mature adipocytes, so that we could study the direct effect of both ghrelin and des-acyl ghrelin in adipocytes.

We started by verifying ghrelin or des-acyl ghrelin's effect on 3T3-L1 fibroblasts viability. A 24-hour incubation period with 0.1, 1, 10, 30 and 100 ng/mL of ghrelin (**Figure 16 left**) or des-acyl ghrelin (**Figure 16 right**) was carried out in 3T3-L1 cells, with no alterations of cell viability registered, in comparison to the control group (only supplemented with 10% FBS/DMEM). Treatment concentrations were chosen with regards to the existing literature and having in consideration the plasmatic concentration range of both ghrelin and des-acyl ghrelin [79, 81].

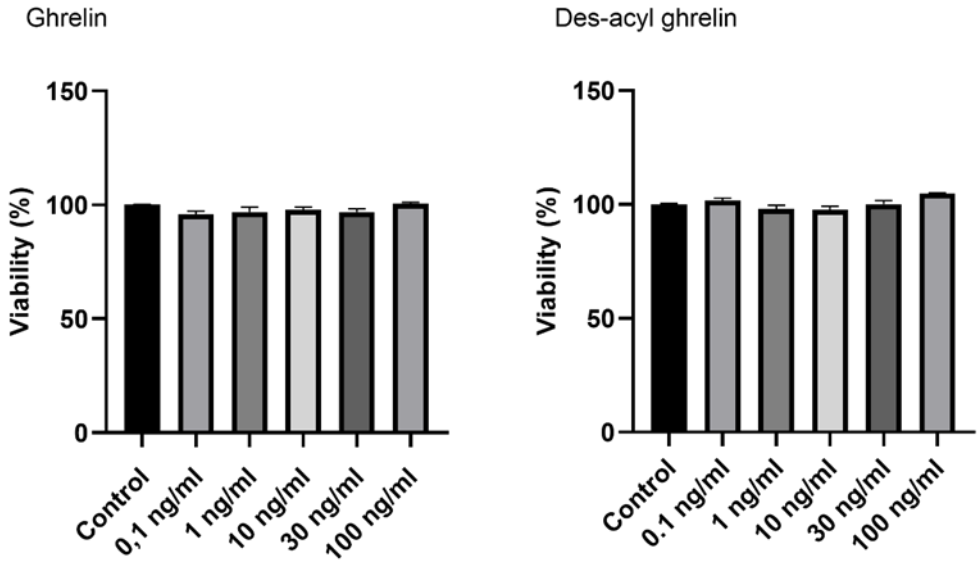

Figure 16 – 3T3-L1 fibroblasts' viability is not affected by ghrelin and des-acyl ghrelin treatment. Bars represent mean ± SEM and Kruskal-Wallis comparisons were conducted to compare among the groups.

Afterwards, we assessed ghrelin and des-acyl ghrelin's ability to induce lipid retention, while stimulating adipogenesis. 3T3-L1 adipocytes were exposed, after a pre-incubation with a differentiation cocktail, to growing concentrations of both ghrelin isoforms (0.1, 1, 10, 30 and 100

ng/mL), during a period of 7 days. The effects on adipogenesis were observed through PPARγ levels, the main adipogenic transcription factor. Both ghrelin (**Figure 17 left**) and des-acyl ghrelin (**Figure 17 right**) led to a significant increase in PPARγ levels in 3T3-L1 cells, at the concentration of 100 ng/mL. In the case of acylated ghrelin, PPARγ levels were significantly increased versus the treatment with 1 ng/mL ($p<0.05$) and treatment with 10 ng/mL ($p<0.05$) (**Figure 17 left**), while for des-acyl ghrelin the significant statistic was in relation to the negative control ($p<0.05$) (**Figure 17 right**).

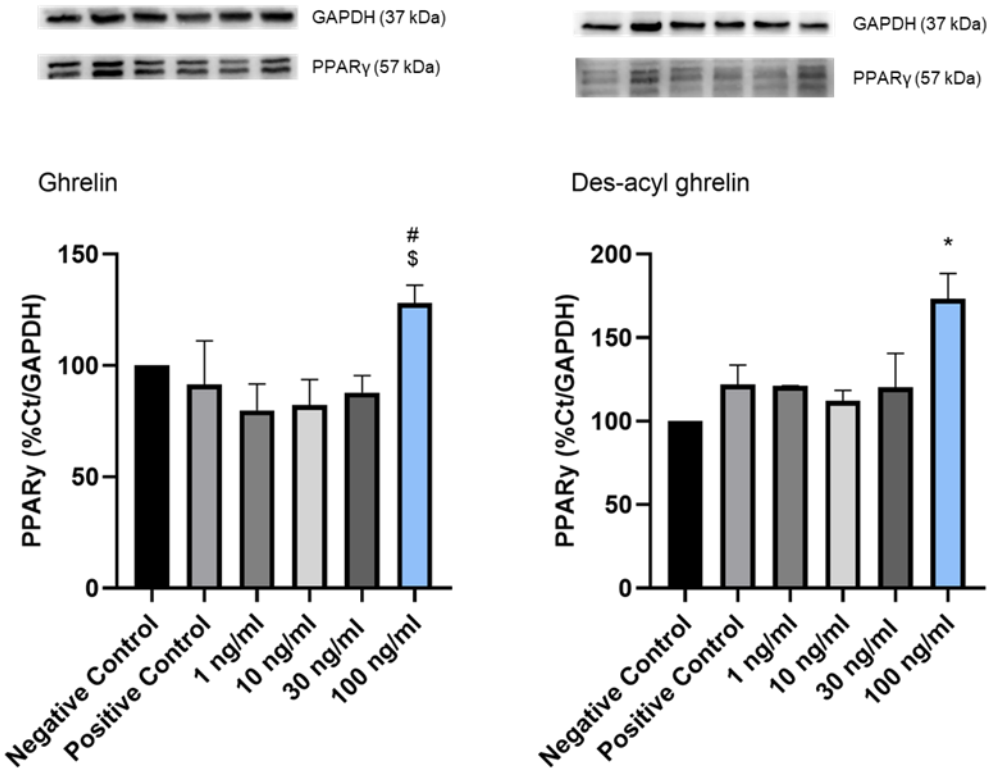

Figure 17 – The effect of ghrelin and des-acyl ghrelin in the levels of a key regulator of adipogenesis (PPARγ). The differentiating adipocytes were treated with growing concentrations of both ghrelin isoforms (1, 10, 30 and 100 ng/mL). Both ghrelin and des-acyl ghrelin (100 ng/mL) stimulate PPARγ levels in 3T3-L1 cell line. Representative images of western blot proteins of interest and loading controls (GAPDH) are shown at the top. Bars represent mean ± SEM and Kruskal-Wallis comparisons were conducted to compare among the groups. * vs Negative Control, # vs 1 ng/mL and $ vs 10 ng/mL. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

In order to evaluate acylated ghrelin and des-acyl ghrelin's effects in lipid accumulation in adipocytes, a staining with Oil Red O was performed. Given the previous results, where we saw an increase in PPARγ at the concentration of 100 ng/mL for both peptide forms, we proceded with that concentration and also with the one right bellow, 30 ng/mL. Furthermore, we studied the effect of inhibiting both GHRS1a and GOAT in lipid accumulation in adipocytes, by treating cells incubated with ghrelin/des-acyl ghrelin with 1 μM of DLS and 1 μM of GO-CoA-Tat, the inhibitors for both GHSR1a and GOAT, respectively.

Pre adipocytes (cells that were not treated with differentiation cocktail) did not show Oil Red O staining in lipid droplets, at day 7 (**Figure 18A and 19A**) and the same was verified in the negative controls (**Figure 18B and 19B**). In the positive controls, lipid accumulation started being visible, with adipocytes acquiring a more rounded shape with lots of fat droplets (**Figure 18C and 19C**). Upon treatment with des-acyl ghrelin (30ng/mL), there was a slight increase in stained lipid droplets (**Figure 18D**), when compared to the negative control, an effect that was maintained even upon GO-CoA-Tat administration (**Figure 18E**). However, the proportion of stained lipid droplets increased in cells treated with 100 ng/mL of des-acyl ghrelin, to a similar extent as the positive control (**Figure 18F**). A slight reduction in stained lipids was found in cells treated with both 100 ng/mL des-acyl ghrelin and GO-CoA-Tat (**Figure 18G**).

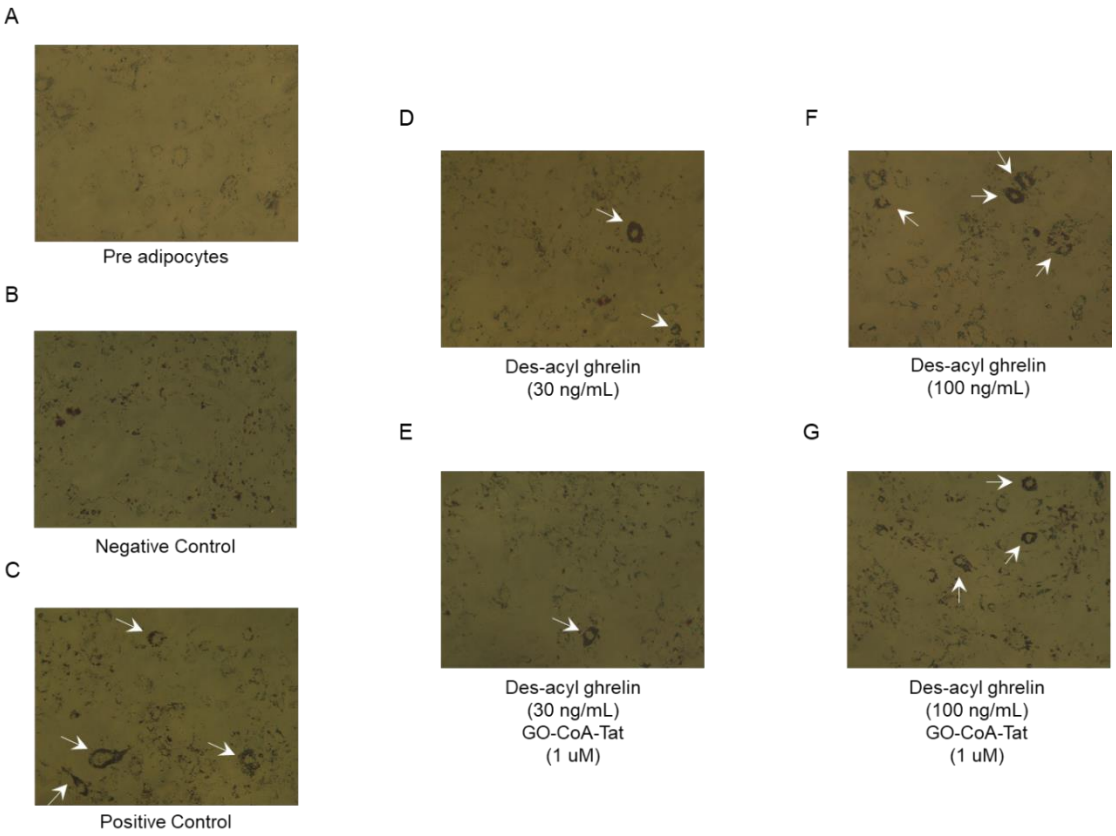

Figure 18 – The effect of des-acyl ghrelin in lipid accumulation in 3T3-L1 adipocytes. Representative images (200x magnification) showing Oil Red O-stained adipocytes in the absence (A, B and C) or presence of des-acyl ghrelin at 30 ng/mL (D) and 100 ng/mL (F). The effect of GOAT inhibitor (GO-CoA-Tat) was also evaluated when incubated with 30 ng/mL and 100 ng/mL des-acyl ghrelin (E and G, respectively).

Treatment with 30 ng/mL of acylated ghrelin induced an increase in stained lipids, in comparison to the negative control (**Figure 19D**), and the same was verified for acyl ghrelin (100 ng/mL) (**Figure 19F**). When cells were treated with both concentrations of acylated ghrelin and 1 μM DLS, GHSR1a inhibitor, (**Figure 19E and 19G**), stained lipids seemed to decrease, in comparison to the administration of the respective concentrations of ghrelin alone. We also confirmed the expression of GHSR1a protein in the 3T3-L1 adipocytes, in mature adipocytes, in the presence or absence of acylated ghrelin (**Figure 20**).

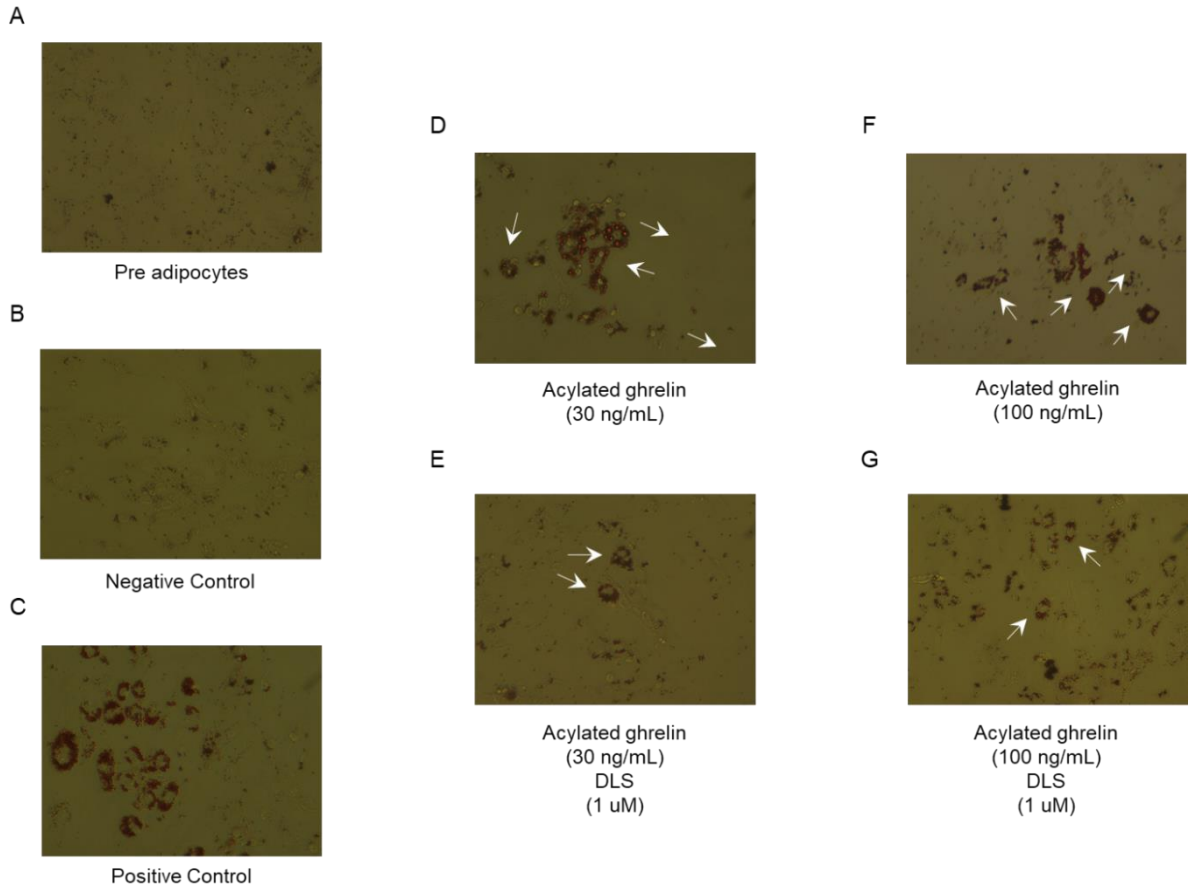

Figure 19 - The effect of acylated ghrelin in lipid accumulation in 3T3-L1 adipocytes. Representative images (200x magnification) showing Oil Red O-stained adipocytes in the absence (A, B and C) or presence of acyl ghrelin at 30 ng/mL (D) and 100 ng/mL (F). The effect of GHSR1a inhibitor (DLS) was also evaluated when incubated with 30 ng/mL and 100 ng/mL acylated ghrelin (E and G, respectively).

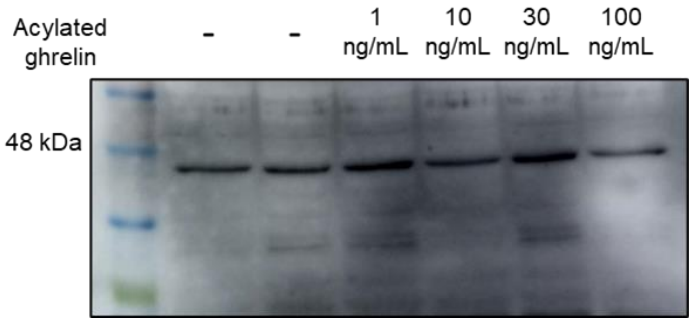

Figure 20 – The GHSR1a protein is expressed in differentiated murine adipocytes from 3T3-L1 cell line.

GHRELIN AND DES-ACYL GHRELIN DO NOT COMPROMISE HUMAN MICROVASCULAR ENDOTHELIAL CELLS VIABILITY

With the purpose of evaluating ghrelin and des-acyl ghrelin involvement in angiogenesis, we selected a human endothelial cell line. Although we did not have the opportunity to study if and how ghrelin/dys-acyl ghrelin regulate sprouting angiogenesis, we assessed cell viability of HMVECs in the presence of growing concentrations of both peptides (1, 10, 30 and 100 ng/mL) after a 24 and 48-hour incubation. We found that neither ghrelin nor des-acyl ghrelin alter cell viability of HMVECs at any of the tested concentration, at both 24 or 48-hour long incubation (**Figure 21**).

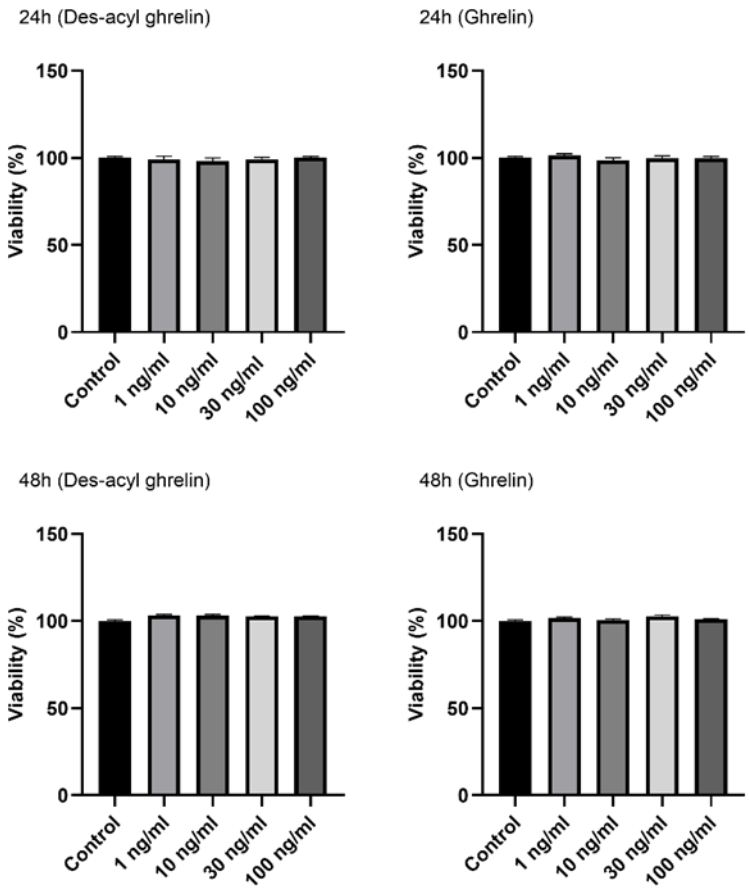

Figure 21 - HMVECs viability upon both ghrelin forms treatment. HMVECs viability is not affected by ghrelin and des-acyl ghrelin treatment (1, 10, 30 and 100 ng/mL). Bars represent mean ± SEM and Kruskal-Wallis comparisons were conducted to compare among the groups.

Chapter 5 - DISCUSSION

Over the past years of ghrelin research, acylated ghrelin has been proposed to induce adiposity and increased body weight indirectly, through the stimulation of orexigenic hypothalamic pathways, that result in food consumption [61]. Nonetheless, some studies have been pointing for an effect in AT, that is at least partially, independent from ghrelin's central actions [77-79]. The endogenous ligand for Y receptors, NPY, whose expression is regulated by GHSR1a signalling, in the hypothalamus, has also been suggested to display direct actions in AT, by promoting adipocytes proliferation, differentiation and plasticity under stress conditions [76].

Furthermore, ghrelin plasmatic levels are deeply dysregulated in the context of obesity and MetS-related T2DM, in both humans and rodents [33, 35, 46]. Metabolic surgery, one of the most popular obesity and T2DM therapeutics, has been demonstrated to allow a normalization in ghrelin's secretion profile, also in rodents and humans [42-44, 46], while restoring AT's health and function in rodents submitted to VSG [48, 50-53].

In this work, we explored how the ghrelin/NPY axis was altered in the human VAT in the context of a growing metabolic dysregulation in obese subjects with either MHO or MUO. Additionally, we investigated how was this axis altered in the VAT of diabetic obese rats, submitted to VSG. Last but not least, we examined whether the different forms of ghrelin were involved in adipocytes' metabolism.

As major findings of this work, we highlight that: 1 – NPY/Y receptors system suffers alterations in VAT expression, in obese individuals with insulin resistance and metabolic dysregulation; 2 – Expression of NPY/Y receptors system correlates with expression of lipid metabolism intermediates, adipokines and vascular function markers, thus highlighting its role in adipose tissue function; 3 – VSG decreases GHSR1a levels in VAT of diabetic obese rats, while upregulating the NPY/Y receptors system; 4 – Ghrelin and des-acyl ghrelin directly stimulate lipid accumulation and adipogenesis *in vitro*.

Recently, several studies have been focusing in describing the precise alterations of plasmatic ghrelin levels in obesity and T2DM and the relationship between ghrelin/NPY in hypothalamus. We then asked what could be happening to this axis in obese patients' VAT, whose function is severely disturbed in MetS and MUO. To our surprise, the *GHSR* gene failed to amplify. In our opinion, this can only mean that the primer was not entirely suitable, since the *GHSR* is already known to be expressed in human VAT [79], and as we have seen, in murine adipocytes. The

gene encoding for GOAT (*MBOAT4*), the enzyme that leads to ghrelin's acylation, was not altered in the several stages of metabolic dysregulation that accompanied obesity. Given that acylated ghrelin levels seem to be increased in human obesity and T2DM [79], one can argue that the VAT is not contributing, at least significantly, for the pool of acylated ghrelin, which may mostly originate in the gastrointestinal system. Indeed, GOAT-mediated ghrelin acylation was shown to depend of fatty acids specifically originated from the dietary pool [66].

In contrary, the NPY receptors were altered in the different groups. More precisely, the expression profile of both *NPY1R* and *NPY5R* were similar, both increasing in the IR Pre-Diabetic group (3) and then declining in the IR Diabetic group (4), in relation to the other IR groups. Thus, it seems that the aggravation of metabolic dysregulation, happening in the pre-diabetic patients, triggers a compensatory effect that upregulates both Y1 and Y5 receptors, that is then lost when T2DM is installed. Intriguingly, the exact opposite appears to occur for *NPY2R*, given that we found a decreased expression right in the IR NG group (2). Thus, we regrouped the subjects in two different groups, IS versus IR, to study the influence of insulin resistance in *NPY2R* VAT expression. Through this analysis, we were able to conclude that insulin resistance specifically downregulates *NPY2R* expression. Furthermore, *NPY* expression showed a tendency to decrease with insulin resistance, while its degrading enzyme, DPP4, showed a stable expression throughout groups. Having in mind that obese T2DM patients present increased NPY plasmatic levels [102], that may exclude the VAT as a meaningful NPY-secreting tissue, at first. However, patients from IR group presented hyperinsulinemia and, given that insulin has an inhibitory effect in hypothalamic NPY [31], the same might be happening in VAT.

The similarity in the expression profiles along groups of *NPY1R* and *NPY5R*, opposing the pattern seen for *NPY2R*, raised the suspicion that the different receptor isoforms could establish distinct interactions in-between them. In fact, we ended up verifying that *NPY1R* and *NPY5R* presented a high positive correlation, while the same happened in-between *NPY2R* and *PPY1R*, suggesting that the Y receptors are paired in their functions.

In order to understand how these alterations in NPY/Y receptor system correlate with anthropometric and components of the MetS in obese patients, we ran a Spearman analysis. We found that both *NPY* and *NPY2R* expression were inversely correlated with weight and BMI, respectively, in the cohort of patients, suggesting that obesity downregulates the *NPY/Y2R* arm

in VAT. Moreover, VAT *NPY* expression showed a positive, despite weak, correlation with HDL levels, that mirror AT metabolic activity [101]. Interestingly, in four-group analysis, such correlation almost reached a significant statistical meaning in the IS NG group (r=0.513, p=0.05), that vanished in the other groups. Altogether, this highlights the importance of VAT-derived NPY in functional AT metabolism, possibly through increased HDL secretion. In the pre-diabetic patients, a negative correlation was found between *NPY*, whose expression tended to be decreased, and HbA1c, what might suggest that the loss on VAT *NPY* expression is somehow related to the increase in HbA1c seen in these patients. Furthermore, patients with insulin resistance (IR group) revealed a negative correlation between *NPY5R* and HbA1c and fasting glycemia, that is inexistent in the IS group, probably meaning that the decreased expression of this particular receptor in the diabetic group might also contribute to the loss of glycaemic homeostasis and increased HbA1c levels.

A very interesting finding was that both *NPY1R* and *NPY5R* were moderately correlated with plasma leptin levels, in a positive fashion, in the IS NG group, an effect that is lost with the onset and development of insulin resistance, when patients usually also develop hyperleptinemia and leptin resistance.

Now that we knew how the NPY/Y system was altered in the VAT of subjects either with MHO or MUO and MetS, and how it was related to some clinical parameters that mirror such metabolic abnormality, we wondered what was the relation between this system and some of the main VAT function markers, in terms of metabolism, plasticity and adipokine secreting behaviour.

The NPY/Y system showed multiple correlations with several markers of AT metabolism, either with lipogenic or lipolytic function, highlighting a relevant role of NPY signalling in the control of adipocytes' metabolism. In line with what was verified before, Y receptors seem to be paired in their actions. In this case, *NPY1R* and *NPY5R* showed stronger correlations with lipogenesis-associated genes, such as *PPARG, CIDEA* and *PLIN1*, whereas *NPY2R* and *PPY1R* were highly correlated with *UCP1 and PYY1R* also having a strong correlation with *PPARA*. Thus, one can conclude that Y receptors regulate both arms of AT metabolism, with *NPY1R* and *NPY5R* promoting anabolism and nutrient storage and *NPY2R* and *PPY1R* favouring catabolism instead. Accordingly, NPY1R and NPY5R are both acknowledged to induce lipid accumulation in 3T3-L1 adipocytes [75]. However, the same authors and others have also shown such effects being

played by NPY2R, in murine cells and mice models [75, 76]. Nonetheless, Chatree *et al.* found that, in humans, VAT *NPY2R* expression was negatively correlated with adipocytes' area, suggesting this receptor action to be anti-adipogenic or stimulator of lipid oxidation [103]. Nonetheless, the action of *NPY2R* on AT metabolism may vary between different species. A role for Y receptors in VAT insulin sensitivity is also supported by our results, since all receptors were positively correlated with the insulin receptor gene, especially the Y1 and Y5 that were decreased in group 4, so was the INSR (data not shown).

Adipokines secretion directly reflet AT health and allow it to regulate peripheral metabolism and to inform on the nutrient availability and metabolic status. Thus, we evaluated the relationship between NPY/Y receptors system and the VAT expression of several crucial adipokines. Both *NPY1R* and *NPY5R* revealed moderate positive correlations with *ADIPOQ*, which is consistent with *PPARG* correlation. In fact, in the IR Diabetic group, that present hypoadiponectinemia, and in none of the other groups, the expression of these receptors is decreased, suggesting a fundamental role of such receptors in AT health. The *LEP* gene was also positively related to *NPY1R* and *NPY5R,* although at a lower extent, while the ligand *NPY* showed a negative correlation, which suggests that the inhibitory effect of leptin in hypothalamic NPY [31] may also happen in the periphery. Considering the aforementioned positive correlation between *NPY1R* and *NPY5R* VAT expression with plasma leptin levels only in the IS NG group (MHO patients), one can now argue that in these individuals, such results can mirror a negative feedback effect, where NPY Y1 and Y5 receptors expression is coupled to increased VAT leptin secretion to then inhibit hypothalamic NPY-induced food intake. In fact, if the Y1 and Y5 receptors promote AT nutrient storage as suggested earlier, it makes sense that such feedback exists to inform brain centres on the current nutritional status. When exploring the relationship between *NPY* and *LEP* in a four-group analysis we saw that in groups 3 (IR Pre Diabetic) and 4 (IR Diabetic), the negative correlation seen in group 2 (IR NG) and 1 (IS NG) (trend) disappears. This may mean that, in dysglicaemic insulin resistant patients, with a higher level of metabolic dysregulation, the antagonistic relation between NPY and leptin is lost, being a probable sign of leptin resistance, and thus explaining why NPY plasma levels are increased in human MUO [102].

We then investigated what was the involvement of NPY/Y receptors system in AT microvasculature. The angiopoietin system (*ANGPT1* and *TEK*) was highly positively correlated

with *NPY1R* and *NPY5R*. However, the marked decreased expression of such receptors in the IR Diabetic group is then accompanied by a reduction on this angiogenesis-regulating system, what may compromise tissue plasticity. A similar behaviour was seen with both *PECAM1* and *VEGFA*, especially with *NPY5R*, which corroborates the decreased AT levels of VEGF seen in obese patients, that harshly decrease with the development of MUO [22, 23]. Despite having low or negligent positive correlations with *VEGFA*, the *NPY2R* and *PPY1R* expression was positively correlated with *FLT1*, that encodes for VEGF receptor 1, thus constituting another arm of the Y receptors system in regulating AT angiogenesis. Finally, *DPP4* negative correlation with *EPAS1*, may indicate NPY's cleaving enzyme as a negative regulator of Y receptors-stimulated VAT angiogenesis, since *EPAS1* encodes for HIF-2α, an important factor of hypoxia-induced angiogenesis.

Overall, the results from the human study shed light on the possible deleterious effects of NPY/Y system dysregulation on VAT health, which parallels with the transition and development of MUO. Despite not having information on the *GHSR*, we now know that its downstream hypothalamic target, the NPY/Y receptors system, appears to be deeply involved in human VAT expandability and angiogenesis regulation. Furthermore, such evidences render this axis as a drug targetable system, in MUO/MetS and evidently T2DM.

Metabolic surgery, especially VSG, is one of the most frequent and efficient therapeutics to combat MetS and T2DM. VSG was shown by us and others to produce a sustained decrease of postprandial ghrelin levels, which may have an impact on NPY/Y receptor system, not only in the hypothalamus but also potentially in other organs, such as the AT [42-44, 46]. Despite not being able to study *GHSR* VAT expression in MHO and MUO patients, we have shown increased levels of GHSR1a in GKHCD and GKHCD_Sh rats, when compared to Wistar controls. If ghrelin really acts as a lipid sensor, as proposed by Tschöp *et al.* [66], it is reasonable to argue that the HCD-derived lipid moiety would increase acylated ghrelin levels in the HCD-fed rats, thus increasing GHSR1a levels precisely in the tissues where those nutrients are stored, such as the pEAT. Interestingly, VSG tended to decrease GHSR1a levels in pEAT, closer to what was seen in the Wistar controls, probably being a consequence of reduced postprandial ghrelin levels. Such reduction of GHSR1a levels is expected to have an impact on NPY/Y receptor system and, in fact, we have shown that both NPY1R and NPY2R are drastically increased in the VAT of the

animals submitted to VSG. Furthermore, our group has previously demonstrated that VSG improves AT vascular health in this model [50], suggesting a possible involvement of NPY1R and NPY2R in angiogenesis amelioration after surgery, a hypothesis that is supported by our results of the human VAT study. Moreover, NPY has been described to induce angiogenesis and tube formation in HUVECs through activation of oligomers of the NPY Y1, Y2 and Y5 receptors [98]. VSG-submitted rats also presented increased levels of pEAT DPP4, that generates NPY species that preferably bind the Y2 and Y5 receptors, what, in this case can reflect a greater activation of Y2 receptor.

So far, we cannot yet comprehend what is the relationship between the GHSR1a activation and NPY/Y receptors VAT levels, being the major limitations of the animal model study the lack of information on VAT NPY levels and on Y5 receptor levels after VSG.

To gain further insight in the mechanisms that ghrelin and des-acyl ghrelin could be stimulating in AT, we performed *in vitro* studies in the 3T3-L1 cell line. We verified that both ghrelin isoforms increase the levels of PPARγ, thus promoting adipogenesis. Furthermore, acylated ghrelin and des-acyl ghrelin induced lipid accumulation in adipocytes. Lipogenic effects of acylated ghrelin were GHSR1a-dependent, while the effects of des-acyl ghrelin seemed independent from GOAT inhibition. Thus, the fact that des-acyl ghrelin also induces lipid accumulation, independently of GOAT, supports the existence of other mechanisms, non-GHSR1a-related, that also mediate ghrelin-induced lipogenesis. These evidences corroborate a role for the ghrelin as a nutrient sensor, directly promoting nutrient storage in fat depots.

Given the close relationship between AT increased nutrient storage and tissue expandability and the importance of the adipo-vascular coupling, we aimed to study if and how ghrelin could directly modulate endothelial cells behaviour and plasticity. However, we could only gather information on HMVECs, confirming that ghrelin did not alter cell viability when cells were treated with growing concentrations of ghrelin. The *in vitro* experiments on both 3T3-L1 and HMVECs cells, despite providing some hints on the putative involvement of ghrelin in regulating AT metabolism and plasticity, still have some limitations. For instance, we have not assessed if ghrelin stimulates NPY secretion from 3T3-L1 adipocytes neither the direct effect of NPY in adipocytes. Moreover, we did not get to study the effects of ghrelin/NPY in endothelial cell angiogenesis.

Chapter 6 – CONCLUSION AND FUTURE PERSPECTIVES

Obesity and MetS prevalence are increasing at a speed rate, as the overall quality of human life improves [4, 7]. Moreover, the emergency on battling MUO and MetS augments, as they both constitute significant risk factors for the onset and development of life-threatening diseases such as T2DM and CVD [2, 3]. Nowadays, the AT is recognized as a determinant multifactorial organ that controls overall metabolism and several authors have been claiming AT dysfunction as one of the most prominent contributors to metabolic dysregulation and obesity-associated MetS [8, 19]. On the other hand, Mets and T2DM patients present marked alterations of gut hormone secretome, that is restored after metabolic surgery procedures, such as the VSG [33, 35, 42, 44, 50]. In particular, ghrelin secretion profile is markedly altered in diabetic obese patients submitted to surgery. The ghrelin/NPY axis is heavily studied in the hypothalamus, but the action of this system in the AT is not yet understood. Thus, we aimed to unravel if the ghrelin/NPY axis was altered in the VAT of obese subjects with several degrees of metabolic dysregulation. To our surprise, the *GHSR* failed to amplify, but others have shown already its expression in the human VAT [79]. Nonetheless, we found that the NPY/Y receptors system expression in VAT is downregulated for the Y1, Y2 and Y5 subtypes, along the transition from MHO to MUO, being correlated with the development of MetS components. We also showed that Y receptors were correlated to several markers of AT function, involved in AT metabolism, plasticity and endocrine behaviour, meaning that this system might regulate several key events in AT, namely vascular adaptation and angiogenesis. We showed that VSG induced an increase in both Y1 and Y2 receptor in the VAT of HCD-fed GK rats, which showed improved microvasculature. Given the increase in Y receptors induced by VSG, while decreasing GHSR1a, it would be interesting to study if the same happens in the VAT from MUO patients after gastrectomy, although obtaining biopsies from human tissue is not always feasible. Open questions still remain on the putative GHSR1a-dependent NPY expression in AT and the next approach should be measuring AT NPY levels in the VSG-submitted animals, that presented reduced VAT GHSR1a. Since our results of the human VAT study suggest that leptin might have an inhibitory effect on peripheral NPY, leptin receptor levels in the VAT of VSG-submitted animals should also be evaluated. Lastly, we demonstrated and corroborated previous findings, that ghrelin and des-acyl ghrelin stimulate lipid accumulation and adipogenesis in murine adipocytes. Given the tight adipo-vascular coupling in healthy AT, it is expected that ghrelin stimulates angiogenesis, thus favouring tissue

expandability. However, we could not study this hypothesis. Still, in the near future, it would be interesting to isolate endothelial cells from the stromovascular fraction of AT to unravel the precise role of both ghrelin forms and also NPY, in AT angiogenesis, by evaluating sprouting angiogenesis ability and the expression of angiogenesis markers. Furthermore, future studies should focus on identifying the cellular source of AT NPY. To do so and to understand if NPY secretion is regulated by ghrelin we could measure NPY levels in the cell medium of 3T3-L1 cells or AT-derived endothelial cells, upon ghrelin treatment.

Notwithstanding, this work allowed to gain awareness on the potential of Y receptors as therapeutic targets for AT dysfunction, in the context of metabolic diseases, such as MetS and T2DM, as well as their probable involvement in regulating AT angiogenesis, plasticity and metabolic and endocrine functions. We also unravelled the importance of VSG as a tool for modulating the ghrelin/NPY axis in the VAT.

BIBLIOGRAPHY

1. Shin, J., Lee, J., Lim, S., Ha, H., Kwon, H., Park, Y., Lee, W., Kang, M., Yim, H., Yoon, K., & Son, H. (2013). Metabolic syndrome as a predictor of type 2 diabetes, and its clinical interpretations and usefulness. *Journal of Diabetes Investigation*, *4*(4), 334–343. https://doi.org/10.1111/jdi.12075
2. Saklayen, M. G. (2018). The Global Epidemic of the Metabolic Syndrome. *Current Hypertension Reports*, *20*(2). https://doi.org/10.1007/s11906-018-0812-z
3. Nsiah, K., Shang, V. O., Boateng, K. A., & Mensah, F. O. (2015). Prevalence of metabolic syndrome in type 2 diabetes mellitus patients. *International Journal of Applied and Basic Medical Research*, *5*(2), 133–138. https://doi.org/10.4103/2229-516X.157170
4. World Health Organization. (2020, March). Obesity and overweight: Fact Sheet. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight, accessed at September 13th 2020
5. International Diabetes Federation. (2019). IDF Diabetes Atlas—9th edition. DiabetesAtlas. https://diabetesatlas.org/en/, accessed at September 13th 2020
6. Gaio, V., Antunes, L., Namorado, S., Barreto, M., Gil, A., Kyslaya, I., Rodrigues, A. P., Santos, A., Bøhler, L., Castilho, E., Vargas, P., do Carmo, I., Nunes, B., & Dias, C. M. (2018). Prevalence of overweight and obesity in Portugal: Results from the First Portuguese Health Examination Survey (INSEF 2015). *Obesity Research & Clinical Practice, 12*(1), 40–50. https://doi.org/10.1016/j.orcp.2017.08.002
7. Observatório Nacional da Diabetes. (n.d.). Retrieved 23 August 2020, from https://www.spd.pt/index.php/observatrio-mainmenu-330, accessed at September 13th 2020
8. Iacobini, C., Pugliese, G., Fantauzzi, C. B., Federici, M., & Menini, S. (2019). Metabolically healthy versus metabolically unhealthy obesity. *Metabolism - Clinical and Experimental, 92*, 51–60. https://doi.org/10.1016/j.metabol.2018.11.009
9. Velho, S., Paccaud, F., Waeber, G., Vollenweider, P., & Marques-Vidal, P. (2010). Metabolically healthy obesity: Different prevalences using different criteria. *European Journal of Clinical Nutrition, 64*(10), 1043–1051. https://doi.org/10.1038/ejcn.2010.114
10. Lemoine, A. Y., Ledoux, S., & Larger, E. (2013). Adipose tissue angiogenesis in obesity. *Thrombosis and Haemostasis, 110*(04), 661–669. https://doi.org/10.1160/TH13-01-0073
11. Stefano, A. B. D., Massihnia, D., Grisafi, F., Castiglia, M., Toia, F., Montesano, L., Russo, A., Moschella, F., & Cordova, A. (2018). Adipose tissue, angiogenesis and angio-MIR under physiological and pathological conditions. *European Journal of Cell Biology, 98*(2–4), 53–64. https://doi.org/10.1016/j.ejcb.2018.11.005
12. Christiaens, V., Lijnen, H.R. (2010). Angiogenesis and development of adipose tissue. *Molecular and Cellular Endocrinology, 318*(1–2), 2–9. https://doi.org/10.1016/j.mce.2009.08.006

13. Matafome, P., & Seica, R. (2017). Function and Dysfunction of Adipose Tissue. In L. Letra & R. Seica (Eds.), *Obesity and Brain Function* (1st ed., pp. 3–31). Springer International Publishing. https://doi.org/10.1007/978-3-319-63260-5
14. Rodrigues, T., Matafome, P., & Seica, R. (2014). A vascular piece in the puzzle of adipose tissue dysfunction: Mechanisms and consequences. *Archives of Physiology and Biochemistry, 120*(1), 1–11. https://doi.org/10.3109/13813455.2013.838971
15. Crewe, C., An, Y. A., & Scherer, P. E. (2017). The ominous triad of adipose tissue dysfunction: Inflammation, fibrosis, and impaired angiogenesis. *The Journal of Clinical Investigation, 127*(1), 74–82. https://doi.org/10.1172/JCI88883
16. Hocking, S. L., Wu, L. E., Guilhaus, M., Chisholm, D. J., & James, D. E. (2010). Intrinsic Depot-Specific Differences in the Secretome of Adipose Tissue, Preadipocytes, and Adipose Tissue–Derived Microvascular Endothelial Cells. *Diabetes, 59*(12), 3008–3016. https://doi.org/10.2337/db10-0483
17. Yiannikouris, F., Gupte, M., Putnam, K., & Cassis, L. (2010). Adipokines and blood pressure control. *Current Opinion in Nephrology and Hypertension, 19*(2), 195–200. https://doi.org/10.1097/MNH.0b013e3283366cd0
18. Kubota, N., Yano, W., Kubota, T., Yamauchi, T., Itoh, S., Kumagai, H., Kozono, H., Takamoto, I., Okamoto, S., Shiuchi, T., Suzuki, R., Satoh, H., Tsuchida, A., Moroi, M., Sugi, K., Noda, T., Ebinuma, H., Ueta, Y., Kondo, T., … Kadowaki, T. (2007). Adiponectin Stimulates AMP-Activated Protein Kinase in the Hypothalamus and Increases Food Intake. *Cell Metabolism, 6*(1), 55–68. https://doi.org/10.1016/j.cmet.2007.06.003
19. Kwon, H., Kim, D., & Kim, J. S. (2017). Body Fat Distribution and the Risk of Incident Metabolic Syndrome: A Longitudinal Cohort Study. *Scientific Reports, 7*(1), 1–8. https://doi.org/10.1038/s41598-017-09723-y
20. Tran, T. T., Yamamoto, Y., Gesta, S., & Kahn, C. R. (2008). Beneficial Effects of Subcutaneous Fat Transplantation on Metabolism. *Cell Metabolism, 7*(5), 410–420. https://doi.org/10.1016/j.cmet.2008.04.004
21. Pasarica, M., Sereda, O. R., Redman, L. M., Albarado, D. C., Hymel, D. T., Roan, L. E., Rood, J. C., Burk, D. H., & Smith, S. R. (2009). Reduced Adipose Tissue Oxygenation in Human Obesity: Evidence for Rarefaction, Macrophage Chemotaxis, and Inflammation Without an Angiogenic Response. *Diabetes, 58*(3), 718–725. https://doi.org/10.2337/db08-1098
22. Trayhurn, P. (2014). Hypoxia and Adipocyte Physiology: Implications for Adipose Tissue Dysfunction in Obesity. *Annual Review of Nutrition, 34*(1), 207–236. https://doi.org/10.1146/annurev-nutr-071812-161156
23. Tinahones, F. J., Coín-Aragüez, L., Mayas, M. D., Garcia-Fuentes, E., Hurtado-del-Pozo, C., Vendrell, J., Cardona, F., Calvo, R., Obregon, M., & El Bekay, R. (2012). Obesity-associated insulin resistance is correlated to adipose tissue vascular endothelial growth factors and metalloproteinase levels. *BMC Physiology, 12*(1), 4. https://doi.org/10.1186/1472-6793-12-4

24. Spencer, M., Yao-Borengasser, A., Unal, R., Rasouli, N., Gurley, C. M., Zhu, B., Peterson, C. A., & Kern, P. A. (2010). Adipose tissue macrophages in insulin-resistant subjects are associated with collagen VI and fibrosis and demonstrate alternative activation. *American Journal of Physiology-Endocrinology and Metabolism, 299*(6), E1016–E1027. https://doi.org/10.1152/ajpendo.00329.2010
25. Bahceci, M., Gokalp, D., Bahceci, S., Tuzcu, A., Atmaca, S., & Arikan, S. (2007). The correlation between adiposity and adiponectin, tumor necrosis factor α, interleukin-6 and high sensitivity C-reactive protein levels. Is adipocyte size associated with inflammation in adults? *Journal of Endocrinological Investigation, 30*(3), 210–214. https://doi.org/10.1007/BF03347427
26. Tam, J., Duda, D. G., Perentes, J. Y., Quadri, R. S., Fukumura, D., & Jain, R. K. (2009). Blockade of VEGFR2 and Not VEGFR1 Can Limit Diet-Induced Fat Tissue Expansion: Role of Local versus Bone Marrow-Derived Endothelial Cells. *PLOS ONE, 4*(3), e4974. https://doi.org/10.1371/journal.pone.0004974
27. Sung, H., Doh, K., Son, J. E., Park, J. G., Bae, Y., Choi, S., Nelson, S. M. L., Cowling, R., Nagy, K., Michael, I. P., Koh, G. Y., Adamson, S. L., Pawson, T., & Nagy, A. (2013). Adipose Vascular Endothelial Growth Factor Regulates Metabolic Homeostasis through Angiogenesis. *Cell Metabolism, 17*(1), 61–72. https://doi.org/10.1016/j.cmet.2012.12.010
28. Stanley, T. L., Zanni, M. V., Johnsen, S., Rasheed, S., Makimura, H., Lee, H., Khor, V. K., Ahima, R. S., & Grinspoon, S. K. (2011). TNF-α Antagonism with Etanercept Decreases Glucose and Increases the Proportion of High Molecular Weight Adiponectin in Obese Subjects with Features of the Metabolic Syndrome. *The Journal of Clinical Endocrinology & Metabolism, 96*(1), E146–E150. https://doi.org/10.1210/jc.2010-1170
29. Yoder, S. M., Kindel, T. L., & Tso, P. (2010). Chapter Eight—Using the Lymph Fistula Rat Model to Study Incretin Secretion. In G. Litwack (Ed.), *Vitamins & Hormones* (Vol. 84, pp. 221–249). Academic Press. https://doi.org/10.1016/B978-0-12-381517-0.00008-4
30. Gribble F. M, Reimann F. (2016) Enteroendocrine Cells: Chemosensors in the Intestinal Epithelium. *Annual Review of Physiology, 78*(1):277-299. doi:10.1146/annurev-physiol-021115-105439
31. Matafome, P., Eickhoff, H., Letra, L., & Seiça, R. (2017). Neuroendocrinology of Adipose Tissue and Gut-Brain Axis. In L. Letra & R. Seica (Eds.), *Obesity and Brain Function* (1st ed., Vol. 19, pp. 49–70). Springer International Publishing. https://doi.org/10.1007/978-3-319-63260-5_3
32. Adam, T. C. M., & Westerterp-Plantenga, M. S. (2005). Glucagon-like peptide-1 release and satiety after a nutrient challenge in normal-weight and obese subjects. *British Journal of Nutrition, 93*(6), 845–851. https://doi.org/10.1079/BJN20041335
33. Zwirska-Korczala, K., Konturek, S. J., Sodowski, M., Wylezol, M., Kuka, D., Sowa, P., Adamczyk-Sowa, M., Kukla, M., Berdowska, A., Rehfeld, J. F., Bielanski, W., & Brzozowski, T. (2007). Basal and postprandial plasma levels of PYY, ghrelin, cholecystokinin, gastrin and insulin in women with moderate and morbid obesity and

metabolic syndrome. *Journal of Physiology and Pharmacology: An Official Journal of the Polish Physiological Society, 58* Suppl 1, 13–35.

34. Carmo-Silva, S., & Cavadas, C. (2017). Hypothalamic Dysfunction in Obesity and Metabolic Disorders. In L. Letra & R. Seica (Eds.), *Obesity and Brain Function* (1st ed., pp. 73–116). Springer International Publishing. https://doi.org/10.1007/978-3-319-63260-5_4
35. Carlson, J. J., Turpin, A. A., Wiebke, G., Hunt, S. C., & Adams, T. D. (2009). Pre- and post- prandial appetite hormone levels in normal weight and severely obese women. *Nutrition & Metabolism, 6*(1), 32. https://doi.org/10.1186/1743-7075-6-32
36. Alexiadou, K., Anyiam, O., & Tan, T. (2019). Cracking the combination: Gut hormones for the treatment of obesity and diabetes. *Journal of Neuroendocrinology, 31*(5), e12664. https://doi.org/10.1111/jne.12664
37. Delporte, C. (2012). Recent Advances in Potential Clinical Application of Ghrelin in Obesity. *Journal of Obesity, 2012*, e535624. https://doi.org/10.1155/2012/535624
38. Fändriks, L. (2017). Roles of the gut in the metabolic syndrome: An overview. *Journal of Internal Medicine, 281*(4), 319–336. https://doi.org/10.1111/joim.12584
39. Welbourn, R., Hollyman, M., Kinsman, R., Dixon, J., Liem, R., Ottosson, J., Ramos, A., Våge, V., Al-Sabah, S., Brown, W., Cohen, R., Walton, P., & Himpens, J. (2019). Bariatric Surgery Worldwide: Baseline Demographic Description and One-Year Outcomes from the Fourth IFSO Global Registry Report 2018. *Obesity Surgery, 29*(3), 782–795. https://doi.org/10.1007/s11695-018-3593-1
40. Nguyen, N. T., & Varela, J. E. (2017). Bariatric surgery for obesity and metabolic disorders: State of the art. *Nature Reviews Gastroenterology & Hepatology, 14*(3), 160–169. https://doi.org/10.1038/nrgastro.2016.170
41. Dimitriadis, G. K., Randeva, M. S., & Miras, A. D. (2017). Potential Hormone Mechanisms of Bariatric Surgery. *Current Obesity Reports, 6*(3), 253–265. https://doi.org/10.1007/s13679-017-0276-5
42. Mans, E., Serra-Prat, M., Palomera, E., Suñol, X., & Clavé, P. (2015). Sleeve gastrectomy effects on hunger, satiation, and gastrointestinal hormone and motility responses after a liquid meal test. *The American Journal of Clinical Nutrition, 102*(3), 540–547. https://doi.org/10.3945/ajcn.114.104307
43. Peterli, R., Steinert, R. E., Woelnerhanssen, B., Peters, T., Christoffel-Courtin, C., Gass, M., Kern, B., von Fluee, M., & Beglinger, C. (2012). Metabolic and Hormonal Changes After Laparoscopic Roux-en-Y Gastric Bypass and Sleeve Gastrectomy: A Randomized, Prospective Trial. *Obesity Surgery, 22*(5), 740–748. https://doi.org/10.1007/s11695-012-0622-3
44. Tsoli, M., Chronaiou, A., Kehagias, I., Kalfarentzos, F., & Alexandrides, T. K. (2013). Hormone changes and diabetes resolution after biliopancreatic diversion and laparoscopic sleeve gastrectomy: A comparative prospective study. *Surgery for Obesity and Related Diseases, 9*(5), 667–677. https://doi.org/10.1016/j.soard.2012.12.006

45. Rosendo-Silva, D., & Matafome, P. (2020). Gut–adipose tissue crosstalk: A bridge to novel therapeutic targets in metabolic syndrome? *Obesity Reviews*, 1-13. https://doi.org/10.1111/obr.13130
46. Eickhoff, H., Louro, T. M., Matafome, P., Vasconcelos, F., Seiça, R., & Castro e Sousa, F. (2015). Amelioration of Glycemic Control by Sleeve Gastrectomy and Gastric Bypass in a Lean Animal Model of Type 2 Diabetes: Restoration of Gut Hormone Profile. *Obesity Surgery, 25*(1), 7–18. https://doi.org/10.1007/s11695-014-1309-8
47. Pournaras, D. J., & le Roux, C. W. (2010). Ghrelin and Metabolic Surgery. *International Journal of Peptides, 2010*. https://doi.org/10.1155/2010/217267
48. Schneck, A., Iannelli, A., Patouraux, S., Rousseau, D., Bonnafous, S., Bailly-Maitre, B., Le Thuc, O., Rovere, C., Panaia-Ferrari, P., Anty, R., Tran, A., Gual, P., & Gugenheim, J. (2014). Effects of sleeve gastrectomy in high fat diet-induced obese mice: Respective role of reduced caloric intake, white adipose tissue inflammation and changes in adipose tissue and ectopic fat depots. *Surgical Endoscopy, 28*(2), 592–602. https://doi.org/10.1007/s00464-013-3211-1
49. Rodríguez, A., Becerril, S., Valentí, V., Moncada, R., Méndez-Giménez, L., Ramírez, B., Lancha, A., Martín, M., Burrell, M. A., Catalán, V., Gómez-Ambrosi, J., & Frühbeck, G. (2012). Short-Term Effects of Sleeve Gastrectomy and Caloric Restriction on Blood Pressure in Diet-Induced Obese Rats. *Obesity Surgery, 22*(9), 1481–1490. https://doi.org/10.1007/s11695-012-0702-4
50. Eickhoff, H., Rodrigues, T., Neves, I., Marques, D., Ribeiro, D., Costa, S., Seiça, R., & Matafome, P. (2019). Effect of Sleeve Gastrectomy on Angiogenesis and Adipose Tissue Health in an Obese Animal Model of Type 2 Diabetes. *Obesity Surgery, 29*(9), 2942–2951. https://doi.org/10.1007/s11695-019-03935-z
51. Jahansouz, C., Xu, H., Hertzel, A. V., Kizy, S., Steen, K. A., Foncea, R., Serrot, F. J., Kvalheim, N., Luthra, G., Ewing, K., Leslie, D. B., Ikramuddin, S., & Bernlohr, D. A. (2018). Partitioning of adipose lipid metabolism by altered expression and function of PPAR isoforms after bariatric surgery. *International Journal of Obesity, 42*(2), 139–146. https://doi.org/10.1038/ijo.2017.197
52. Hoffstedt, J., Andersson, D. P., Hogling, D. E., Theorell, J., Näslund, E., Thorell, A., Ehrlund, A., Rydén, M., & Arner, P. (2016). Long-term Protective Changes in Adipose Tissue After Gastric Bypass. *Diabetes Care*. https://doi.org/10.2337/dc16-1072
53. Trachta, P., Dostálová, I., Haluzíková, D., Kasalický, M., Kaválková, P., Drápalová, J., Urbanová, M., Lacinová, Z., Mráz, M., & Haluzík, M. (2014). Laparoscopic sleeve gastrectomy ameliorates mRNA expression of inflammation-related genes in subcutaneous adipose tissue but not in peripheral monocytes of obese patients. *Molecular and Cellular Endocrinology, 383*(1), 96–102. https://doi.org/10.1016/j.mce.2013.11.013
54. Figueroa-Vega, N., Jordán, B., Pérez-Luque, E. L., Parra-Laporte, L., Garnelo, S., & Malacara, J. M. (2016). Effects of sleeve gastrectomy and rs9930506 FTO variants on

angiopoietin/Tie-2 system in fat expansion and M1 macrophages recruitment in morbidly obese subjects. *Endocrine, 54*(3), 700–713. https://doi.org/10.1007/s12020-016-1070-y

55. Malin, S. K., Bena, J., Abood, B., Pothier, C. E., Bhatt, D. L., Nissen, S., Brethauer, S. A., Schauer, P. R., Kirwan, J. P., & Kashyap, S. R. (2014). Attenuated improvements in adiponectin and fat loss characterize type 2 diabetes non-remission status following bariatric surgery. *Diabetes, Obesity & Metabolism, 16(*12), 1230–1238. https://doi.org/10.1111/dom.12376
56. Tan, T., Behary, P., Tharakan, G., Minnion, J., Al-Najim, W., Albrechtsen, N. J. W., Holst, J. J., & Bloom, S. R. (2017). The Effect of a Subcutaneous Infusion of GLP-1, OXM, and PYY on Energy Intake and Expenditure in Obese Volunteers. *The Journal of Clinical Endocrinology and Metabolism, 102*(7), 2364–2372. https://doi.org/10.1210/jc.2017-00469
57. Boland, B., Mumphrey, M. B., Hao, Z., Gill, B., Townsend, R. L., Yu, S., Münzberg, H., Morrison, C. D., Trevaskis, J. L., & Berthoud, H. (2019). The PYY/Y2R-Deficient Mouse Responds Normally to High-Fat Diet and Gastric Bypass Surgery. *Nutrients, 11*(3). https://doi.org/10.3390/nu11030585
58. Müller, T. D., Nogueiras, R., Andermann, M. L., Andrews, Z. B., Anker, S. D., Argente, J., Batterham, R. L., Benoit, S. C., Bowers, C. Y., Broglio, F., Casanueva, F. F., D'Alessio, D., Depoortere, I., Geliebter, A., Ghigo, E., Cole, P. A., Cowley, M., Cummings, D. E., Dagher, A., … Tschöp, M. H. (2015). Ghrelin. *Molecular Metabolism, 4*(6), 437–460. https://doi.org/10.1016/j.molmet.2015.03.005
59. Serrenho, D., Santos, S. D., & Carvalho, A. L. (2019). The Role of Ghrelin in Regulating Synaptic Function and Plasticity of Feeding-Associated Circuits. *Frontiers in Cellular Neuroscience, 13*. https://doi.org/10.3389/fncel.2019.00205
60. Yin, Y., Li, Y., & Zhang, W. (2014). The Growth Hormone Secretagogue Receptor: Its Intracellular Signaling and Regulation. *International Journal of Molecular Sciences, 15*(3), 4837–4855. https://doi.org/10.3390/ijms15034837
61. Frago, L., & Chowen, J. (2015). Hypothalamic Leptin and Ghrelin Signaling as Targets for Improvement in Metabolic Control. *Current Pharmaceutical Design, 21*(25), 3596–3605. https://doi.org/10.2174/1381612821666150710145428
62. Martins, L., Fernández-Mallo, D., Novelle, M. G., Vázquez, M. J., Tena-Sempere, M., Nogueiras, R., López, M., & Diéguez, C. (2012). Hypothalamic mTOR Signaling Mediates the Orexigenic Action of Ghrelin. *PLOS ONE, 7*(10), e46923. https://doi.org/10.1371/journal.pone.0046923
63. Sun, Y., Wang, P., Zheng, H., & Smith, R. G. (2004). Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *Proceedings of the National Academy of Sciences, 101*(13), 4679–4684. https://doi.org/10.1073/pnas.0305930101
64. Grandt, D., Schimiczek, M., Rascher, W., Feth, F., Shively, J., Lee, T. D., Davis, M. T., Reeve, J. R., & Michel, M. C. (1996). Neuropeptide Y 3–36 is an endogenous ligand

selective for Y2 receptors. *Regulatory Peptides, 67*(1), 33–37. https://doi.org/10.1016/S0167-0115(96)00104-8

65. Nguyen, A. D., Mitchell, N. F., Lin, S., Macia, L., Yulyaningsih, E., Baldock, P. A., Enriquez, R. F., Zhang, L., Shi, Y., Zolotukhin, S., Herzog, H., & Sainsbury, A. (2012). Y1 and Y5 Receptors Are Both Required for the Regulation of Food Intake and Energy Homeostasis in Mice. *PLOS ONE, 7*(6), e40191. https://doi.org/10.1371/journal.pone.0040191
66. Kirchner, H., Gutierrez, J. A., Solenberg, P. J., Pfluger, P. T., Czyzyk, T. A., Willency, J. A., Schurmann, A., Joost, H. G., Jandacek, R., Hale, J. E., Heiman, M. L., & Tschöp, M. H. (2009). GOAT links dietary lipids with the endocrine control of energy balance. *Nature Medicine, 15*(7), 741–745. https://doi.org/10.1038/nm.1997
67. Zhang, W., Chai, B., Li, J., Wang, H., & Mulholland, M. W. (2008). Effect of Des-acyl Ghrelin on Adiposity and Glucose Metabolism. *Endocrinology, 149*(9), 4710–4716. https://doi.org/10.1210/en.2008-0263
68. Blom, W. A. M., Lluch, A., Stafleu, A., Vinoy, S., Holst, J. J., Schaafsma, G., & Hendriks, H. F. J. (2006). Effect of a high-protein breakfast on the postprandial ghrelin response. *The American Journal of Clinical Nutrition, 83*(2), 211–220. https://doi.org/10.1093/ajcn/83.2.211
69. Gao, J., Ghibaudi, L., van Heek, M., & Hwa, J. J. (2002). Characterization of diet-induced obese rats that develop persistent obesity after 6 months of high-fat followed by 1 month of low-fat diet. *Brain Research, 936*(1), 87–90. https://doi.org/10.1016/S0006-8993(02)02493-9
70. Perreault, M., Istrate, N., Wang, L., Nichols, A. J., Tozzo, E., & Stricker-Krongrad, A. (2004). Resistance to the orexigenic effect of ghrelin in dietary-induced obesity in mice: Reversal upon weight loss. *International Journal of Obesity, 28*(7), 879–885. https://doi.org/10.1038/sj.ijo.0802640
71. Geliebter, A., Gluck, M. E., & Hashim, S. A. (2005). Plasma ghrelin concentrations are lower in binge-eating disorder. *The Journal of Nutrition, 135*(5), 1326–1330. https://doi.org/10.1093/jn/135.5.1326
72. McFarlane, M. R., Brown, M. S., Goldstein, J. L., & Zhao, T.-J. (2014). Induced Ablation of Ghrelin Cells in Adult Mice Does Not Decrease Food Intake, Body Weight, or Response to High Fat Diet. *Cell Metabolism, 20*(1), 54–60. https://doi.org/10.1016/j.cmet.2014.04.007
73. Gagnon, J., Baggio, L. L., Drucker, D. J., & Brubaker, P. L. (2015). Ghrelin Is a Novel Regulator of GLP-1 Secretion. *Diabetes, 64*(5), 1513–1521. https://doi.org/10.2337/db14-1176
74. Zhang, C., Wang, L., Wang, R., Liu, Y., Song, L., Yuan, J., Wang, B., & Dong, J. (2018). The Correlation Between Circulating Ghrelin and Insulin Resistance in Obesity: A Meta-Analysis. *Frontiers in Physiology, 9*. https://doi.org/10.3389/fphys.2018.01308
75. Rosmaninho-Salgado, J., Cortez, V., Estrada, M., Santana, M. M., Gonçalves, A.,

Marques, A. P., & Cavadas, C. (2012). Intracellular mechanisms coupled to NPY Y2 and Y5 receptor activation and lipid accumulation in murine adipocytes. *Neuropeptides, 46*(6), 359–366. https://doi.org/10.1016/j.npep.2012.08.006

76. Kuo, L. E., Kitlinska, J. B., Tilan, J. U., Li, L., Baker, S. B., Johnson, M. D., Lee, E. W., Burnett, M. S., Fricke, S. T., Kvetnansky, R., Herzog, H., & Zukowska, Z. (2007). Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. *Nature Medicine, 13*(7), 803–811. https://doi.org/10.1038/nm1611
77. Theander-Carrillo, C., Wiedmer, P., Cettour-Rose, P., Nogueiras, R., Perez-Tilve, D., Pfluger, P., Castaneda, T. R., Muzzin, P., Schürmann, A., Szanto, I., Tschöp, M. H., & Rohner-Jeanrenaud, F. (2006). Ghrelin action in the brain controls adipocyte metabolism. *The Journal of Clinical Investigation, 116*(7), 1983–1993. https://doi.org/10.1172/JCI25811
78. Perez-Tilve, D., Heppner, K., Kirchner, H., Lockie, S. H., Woods, S. C., Smiley, D. L., Tschöp, M., & Pfluger, P. (2011). Ghrelin-induced adiposity is independent of orexigenic effects. *The FASEB Journal, 25*(8), 2814–2822. https://doi.org/10.1096/fj.11-183632
79. Rodríguez, A., Gómez-Ambrosi, J., Catalán, V., Gil, M. J., Becerril, S., Sáinz, N., Silva, C., Salvador, J., Colina, I., & Frühbeck, G. (2009). Acylated and desacyl ghrelin stimulate lipid accumulation in human visceral adipocytes. *International Journal of Obesity, 33*(5), 541–552. https://doi.org/10.1038/ijo.2009.40
80. Liu, J., Lin, H., Cheng, P., Hu, X., & Lu, H. (2009). Effects of ghrelin on the proliferation and differentiation of 3T3-L1 preadipocytes. *Journal of Huazhong University of Science and Technology. [Medical Sciences], 29*(2), 227–230. https://doi.org/10.1007/s11596-009-0218-x
81. Miao, H., Pan, H., Wang, L., Yang, H., Zhu, H., & Gong, F. (2019). Ghrelin Promotes Proliferation and Inhibits Differentiation of 3T3-L1 and Human Primary Preadipocytes. *Frontiers in Physiology, 10*. https://doi.org/10.3389/fphys.2019.01296
82. Lin, L., Saha, P. K., Ma, X., Henshaw, I. O., Shao, L., Chang, B. H. J., Buras, E. D., Tong, Q., Chan, L., McGuinness, O. P., & Sun, Y. (2011). Ablation of ghrelin receptor reduces adiposity and improves insulin sensitivity during aging by regulating fat metabolism in white and brown adipose tissues. *Aging Cell, 10*(6), 996–1010. https://doi.org/10.1111/j.1474-9726.2011.00740.x
83. Rodríguez, A., Gómez-Ambrosi, J., Catalán, V., Rotellar, F., Valentí, V., Silva, C., Mugueta, C., Pulido, M. R., Vázquez, R., Salvador, J., Malagón, M. M., Colina, I., & Frühbeck, G. (2012). The ghrelin O-acyltransferase–ghrelin system reduces TNF-α-induced apoptosis and autophagy in human visceral adipocytes. *Diabetologia, 55*(11), 3038–3050. https://doi.org/10.1007/s00125-012-2671-5
84. Lin, L., Lee, J. H., Buras, E. D., Yu, K., Wang, R., Smith, C. W., Wu, H., Sheikh-Hamad, D., & Sun, Y. (2016). Ghrelin receptor regulates adipose tissue inflammation in aging. *Aging (Albany NY), 8*(1), 178–191.

85. Marques, A. P., Cunha-Santos, J., Leal, H., Sousa-Ferreira, L., Pereira de Almeida, L., Cavadas, C., & Rosmaninho-Salgado, J. (2018). Dipeptidyl peptidase IV (DPP-IV) inhibition prevents fibrosis in adipose tissue of obese mice. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1862*(3), 403–413. https://doi.org/10.1016/j.bbagen.2017.11.012
86. Rodrigues, A. R., Almeida, H., & Gouveia, A. M. (2013). α-MSH signalling via melanocortin 5 receptor promotes lipolysis and impairs re-esterification in adipocytes. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1831*(7), 1267–1275. https://doi.org/10.1016/j.bbalip.2013.04.008
87. Zhao, H., Liu, G., Wang, Q., Ding, L., Cai, H., Jiang, H., & Xin, Z. (2007). Effect of ghrelin on human endothelial cells apoptosis induced by high glucose. *Biochemical and Biophysical Research Communications, 362*(3), 677–681. https://doi.org/10.1016/j.bbrc.2007.08.021
88. Zhu, J., Zheng, C., Chen, J., Luo, J., Su, B., Huang, Y., Su, W., Li, Z., & Cui, T. (2014). Ghrelin protects human umbilical vein endothelial cells against high glucose-induced apoptosis via mTOR/P70S6K signaling pathway. *Peptides, 52*, 23–28. https://doi.org/10.1016/j.peptides.2013.11.015
89. Katare, R., Rawal, S., Munasinghe, P. E., Tsuchimochi, H., Inagaki, T., Fujii, Y., Dixit, P., Umetani, K., Kangawa, K., Shirai, M., & Schwenke, D. O. (2016). Ghrelin Promotes Functional Angiogenesis in a Mouse Model of Critical Limb Ischemia Through Activation of Proangiogenic MicroRNAs. *Endocrinology, 157*(2), 432–445. https://doi.org/10.1210/en.2015-1799
90. Wang, L., Chen, Q., Li, G., & Ke, D. (2015). Ghrelin ameliorates impaired angiogenesis of ischemic myocardium through GHSR1a-mediated AMPK/eNOS signal pathway in diabetic rats. *Peptides, 73*, 77–87. https://doi.org/10.1016/j.peptides.2015.09.004
91. Li, A., Cheng, G., Zhu, G. hui, & Tarnawski, A. S. (2007). Ghrelin stimulates angiogenesis in human microvascular endothelial cells: Implications beyond GH release. *Biochemical and Biophysical Research Communications, 353*(2), 238–243. https://doi.org/10.1016/j.bbrc.2006.11.144
92. Wang, J., He, L., Huwatibieke, B., Liu, L., Lan, H., Zhao, J., Li, Y., & Zhang, W. (2018). Ghrelin Stimulates Endothelial Cells Angiogenesis through Extracellular Regulated Protein Kinases (ERK) Signaling Pathway. *International Journal of Molecular Sciences, 19*(9), 2530. https://doi.org/10.3390/ijms19092530
93. Khazaei, M., Tahergorabi, Z. (2017). Ghrelin did not change coronary angiogenesis in diet-induced obese mice. *Cellular and Molecular Biology (Noisy-Le-Grand, France), 63*(2), 96–99. https://doi.org/10.14715/cmb/2017.63.2.15
94. Tahergorabi, Z., Khazaei, M., & Rashidi, B. (2015). Systemic administration of ghrelin did not restore angiogenesis in hindlimb ischemia in control and diet-induced obese mice. *Bratislavske Lekarske Listy, 116*(1), 35–40. https://doi.org/10.4149/bll_2015_007
95. Tahergorabi, Z., Rashidi, B., & Khazaei, M. (2013). Ghrelin does not modulate

angiogenesis in matrigel plug in normal and diet-induced obese mice. *Journal of Research in Medical Sciences: The Official Journal of Isfahan University of Medical Sciences, 18*(11), 939–942.

96. Wang, L., Li, G., Chen, Q., & Ke, D. (2015). Octanoylated ghrelin attenuates angiogenesis induced by oxLDL in human coronary artery endothelial cells via the GHSR1a-mediated NF-κB pathway. *Metabolism - Clinical and Experimental, 64*(10), 1262–1271. https://doi.org/10.1016/j.metabol.2015.07.008
97. Conconi, M. T., Nico, B., Guidolin, D., Baiguera, S., Spinazzi, R., Rebuffat, P., Malendowicz, L. K., Vacca, A., Carraro, G., Parnigotto, P. P., Nussdorfer, G., & Ribatti, D. (2004). Ghrelin inhibits FGF-2-mediated angiogenesis in vitro and in vivo. *Peptides, 25*(12), 2179–2185. https://doi.org/10.1016/j.peptides.2004.08.011
98. Movafagh, S., Hobson, J. P., Spiegel, S., Kleinman, H. K., & Zukowska, Z. (2006). Neuropeptide Y induces migration, proliferation, and tube formation of endothelial cells bimodally via Y1, Y2, and Y5 receptors. *The FASEB Journal, 20*(11), 1924–1926. https://doi.org/10.1096/fj.05-4770fje
99. Castelan F°, J. de B., Bettiol, J., d'Acampora, A. J., Castelan, J. V. E., Caon de Souza, J., Bressiani, V., & Giroldi, S. B. (2007). Sleeve Gastrectomy Model in Wistar Rats. *Obesity Surgery, 17*(7), 957–961. https://doi.org/10.1007/s11695-007-9150-y
100. Levy, J. C., Matthews, D. R., & Hermans, M. P. (1998). Correct Homeostasis Model Assessment (HOMA) Evaluation Uses the Computer Program. *Diabetes Care, 21*(12), 2191–2192. https://doi.org/10.2337/diacare.21.12.2191
101. Rodrigues, T., Borges, P., Mar, L., Marques, D., Albano, M., Eickhoff, H., Carrêlo, C., Almeida, B., Pires, S., Abrantes, M., Martins, B., Uriarte, C., Botelho, F., Gomes, P., Silva, S., Seiça, R., & Matafome, P. (2020). GLP-1 improves adipose tissue glyoxalase activity and capillarization improving insulin sensitivity in type 2 diabetes. *Pharmacological Research, 161*, 105198. https://doi.org/10.1016/j.phrs.2020.105198
102. Tang, H.-N., Xiao, F., Chen, Y.-R., Zhuang, S.-Q., Guo, Y., Wu, H.-X., & Zhou, H.-D. (2020). Higher Serum Neuropeptide Y Levels Are Associated with Metabolically Unhealthy Obesity in Obese Chinese Adults: A Cross-Sectional Study. *Mediators of Inflammation, 2020*, 9 pages. https://doi.org/10.1155/2020/7903140
103. Chatree, S., Sitticharoon, C., Maikaew, P., Uawithya, P., & Chearskul, S. (2018). Adipose Y5R mRNA is higher in obese than non-obese humans and is correlated with obesity parameters. *Experimental Biology and Medicine (Maywood, N.J.), 243*(9), 786–795. https://doi.org/10.1177/1535370218774889

ANNEX I

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REVIEW

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Gut–adipose tissue crosstalk: A bridge to novel therapeutic targets in metabolic syndrome?

Daniela Rosendo-Silva[1,2] | Paulo Matafome[1,2,3]

[1]Coimbra Institute for Clinical and Biomedical Research (iCBR) and Institute of Physiology, Faculty of Medicine and Center for Innovative Biomedicine and Biotechnology (CIBB), University of Coimbra, Coimbra, Portugal

[2]Clinical Academic Center of Coimbra (CACC), Coimbra, Portugal

[3]Department of Complementary Sciences, Instituto Politécnico de Coimbra, Coimbra Health School (ESTeSC), Coimbra, Portugal

Correspondence
Paulo Matafome, Faculty of Medicine, Pole III of University of Coimbra, Subunit 1, 1st floor, Azinhaga de Santa Comba, Celas, Coimbra 3000-354, Portugal.
Email: paulo.matafome@uc.pt

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Summary

The gut is one of the main endocrine organs in our body, producing hormones acknowledged to play determinant roles in controlling appetite, energy balance and glucose homeostasis. One of the targets of such hormones is the adipose tissue, a major energetic reservoir, which governs overall metabolism through the secretion of adipokines. Disturbances either in nutrient and metabolic sensing and consequent miscommunication between these organs constitute a key driver to the metabolic complications clustered in metabolic syndrome. Thus, it is essential to understand how the disruption of this crosstalk might trigger adipose tissue dysfunction, a strong characteristic of obesity and insulin resistance. The beneficial effects of metabolic surgery in the amelioration of glucose homeostasis and body weight reduction allowed to understand the potential of gut signals modulation as a treatment for metabolic syndrome-related obesity and type 2 diabetes. In this review, we cover the effects of gut hormones in the modulation of adipose tissue metabolic and endocrine functions, as well as their impact in tissue plasticity. Furthermore, we discuss how the modulation of gut secretome, either through surgical procedures or pharmacological approaches, might improve adipose tissue function in obesity and metabolic syndrome.

KEYWORDS
adipose tissue, gut hormones, metabolic surgery, obesity

1 | INTRODUCTION

The gut and the adipose tissue (AT) are among the most active endocrine organs of our organism, and the signals they originate regulate common mechanisms, such as insulin secretion and action, glucose and lipid metabolism, appetite and energy balance. The crosstalk between them may be crucial for the treatment of metabolic disorders, because many gut signals produced during fasting or after a meal apparently act on and regulate several AT functions. Metabolic surgery (MetS) was shown to have a strong impact on AT function, and some of such events are related to the remodelling of gut hormone profile.[1] Moreover, glucagon-like peptide 1 (GLP-1) receptor agonists were shown to act in AT and to result in fat mass and body weight reduction, with possible positive impacts in the metabolic and endocrine function, as well as in the plasticity of the tissue (reviewed in Section 2). Understanding this crosstalk may constitute the foundations of the identification of new therapeutic targets for obesity and metabolic syndrome.

Abbreviations List: ABCG1, ATP-binding cassette sub-family G member 1; AMPK, 5′ AMP-activated kinase; AT, adipose tissue; ATGL, adipose tissue triglyceride lipase; C/EBP, CCAAT-enhancer-binding protein; CCK, cholecystokinin; CVD, cardiovascular disease; EEC, enteroendocrine cell; GHSR1a, growth hormone secretagogue receptor 1 alpha; GIP, glucose-dependent insulinotropic peptide; GLP-1/2, glucagon-like peptide 1/2; GLUT4, glucose transporter 4; GPR, G protein-coupled receptor; LPL, lipoprotein lipase; MetS, metabolic syndrome; OXM, oxyntomodulin; PKA, protein kinase A; PPARγ, peroxisome proliferator-activated receptor gamma; PYY, peptide YY; RYGB, Roux-en-Y gastric bypass; SREBP-1c, sterol regulatory element-binding transcription factor 1c; T2DM, type 2 diabetes mellitus; TAG, triglyceride; TNF-α, tumour necrosis factor alpha; VEGF, vascular endothelial growth factor; VSG, vertical sleeve gastrectomy; WAT, white adipose tissue.

1.1 | Metabolic and endocrine functions of AT

The white adipose tissue (WAT) is mainly composed of adipocytes and the stromovascular fraction, whose crosstalk renders the AT with a high dynamicity and plasticity, conferring it the ability to remodel accordingly to the energetic status and environmental changes.[2]

As an energetic buffer, the AT favours the hydrolysation of triglycerides (TAGs) into fatty acids and glycerol (lipolysis), in order to fulfil the energetic demands, or, on the other hand, after meals, insulin stimulates TAG synthesis through sterol regulatory element-binding transcription factor 1 (SREBP-1c)-induced lipogenesis (reviewed by Matafome and Seica).[3] Lipid anabolism will force tissue expansion, which requires adipogenesis (differentiation of preadipocytes into mature adipocytes) and angiogenesis. This adipo-vascular coupling results from the differentiation of preadipocytes along the vascular wall, being angiogenesis regulated by adipocyte-derived factors and adipogenesis also dependent of capillarization, so that each adipocyte is irrigated by one, or more, capillaries.[2,4] Neo-vascularization is associated with hyperplasia (increased adipocyte number), whereas angiogenesis deficits and dilation from the already existent capillaries lead to hypertrophy (increased adipocyte size).[4] Thus, AT remodelling requires continuous vascular dynamicity and plasticity, avoiding formation of hypoxic regions, usually associated with inflammation, fibrosis and hypertrophic growth.[2] AT angiogenesis will depend on the balance between pro- and anti-angiogenic mediators, such as the vascular endothelial growth factor (VEGF), being hypoxia the main stimuli for gene expression.[2,4] The interaction between pro- and anti-angiogenic factors during the angiogenic process, as well as the alterations occurring during the loss of tissue plasticity, are not currently known.

WAT is by far the largest endocrine organ of the body, with a secretome currently known to include more than 600 factors. The wide panoply of AT products not only regulates its vascular function, but it is also involved in lipid and glucose metabolism, appetite regulation, coagulation, blood pressure, immune function, reproduction, among many other functions.[3,5] Moreover, adipokines, namely adiponectin, also regulate WAT functions, such as glucose uptake.[3] Similarly to gut hormones, one of the classical functions of WAT secretome is regulation of appetite and energy homeostasis in the hypothalamus, mainly due to leptin action.[3] Besides being a very active endocrine tissue, AT is also the target of many hormones and factors, namely, the ones secreted by the gastrointestinal tract.

1.2 | The gut as an endocrine organ

The gut, or gastrointestinal tract, is considered one of the main endocrine organs in the body. The enteroendocrine cells (EECs), located along the epithelium of the gut, secrete signals in response to meal-derived stimuli. More than 30 hormones were currently identified to be secreted by the gut, acting either locally or in several organs such as brain, AT or pancreas (reviewed by Gribble and Reimann).[6]

EECs in the stomach can be divided in enterochromaffin or enterochromaffin-like cells, which produce serotonin and histamine, respectively; D-cells—somatostatin; G-cells—gastrin; and X/A-like cells—ghrelin. The duodenum houses K-, I- and S-cells, which secrete glucose-dependent insulinotropic polypeptide (GIP), cholecystokinin (CCK) and secretin, respectively. M-cells can also be found in this region, secreting ghrelin, somatostatin and motilin. The ileum region is richer in neurotensin-releasing cells (N-cells) and L-cells, the main producers of glucagon-like peptides 1/2 (GLP-1/2) and oxyntomodulin (OXM), also releasing peptide YY (PYY), whose secretion increases in the distal small intestine (reviewed by Gribble et al.).[6,7] Colonic L cells-derived GLP-1 was recently shown to directly improve glucose tolerance, while PYY, from the same cells, was suggested to decrease food intake through a neuropeptide Y2 receptor-dependent mechanism.[8] Besides insulin and glucagon, the pancreas also produce other hormones, such as amylin and pancreatic polypeptide.[9] Available data suggest that different nutrients could exert a stimulatory effect in specific EEC populations. However, it remains to uncover if the effects result from a differential expression of the nutrient-sensing pathways among the EEC cellular types or nutrient absorption scattering along the GI tract. Increasing body of evidence also supports the idea that the same subtype of EECs co-secretes several hormones, as well as the existence of several signals that can modulate the secretion of the same hormone.[7]

Oscillations of lumen nutrients have been suggested as the main trigger for secretion of gut hormones by EECs.[6] The presence of nutrients in the postprandial state mainly favours the release of gastrin, CCK, PYY and pancreatic hormones (insulin and amylin) to promote satiety, as well as GLP-1 and GIP, to also promote insulin-dependent anabolism.[6,9] On the other hand, the absence of nutrients during fasting triggers the secretion of ghrelin, somatostatin and glucagon, as well as serotonin, which gut synthesis and plasma levels are elevated in fasted mice to a similar extent than glucagon.[6,10] Although the acylated form of ghrelin was the only one observed to bind to ghrelin receptor (growth hormone secretagogue receptor 1 alpha, GHSR-1a) and to activate orexigenic neurons, fasting was shown to increase des-acyl ghrelin secretion, rather that the acylated form, questioning the classical relation between fasting and ghrelin-induced appetite.[11]

Regarding glucose-mediated gut hormone release, GLP-1 secretion has been shown to be dependent on K_{ATP} channel closure in proglucagon-expressing GLUTag cells and rats, although others have contested such involvement[6,12–14] Müller et al. have also described the role of sodium/glucose co-transporter 1 in glucose-induced GLP-1 secretion in both rodents and humans, while the sweet taste receptors have been implicated in both glucose-dependent GLP-1 and PYY secretion in humans but did not affect CCK.[15,16] Glucose is also determinant for serotonin release, mainly from colonic enterochromaffin cells, while fructose is important for serotonin secretion from duodenal cells.[17] Fructose was also shown to induce GLP-1 secretion in humans, mice and GLUTag cells.[13] Inversely, intravenous glucose infusions reduced plasma ghrelin levels, in humans.[16]

Long-chain and short-chain fatty acids and monoacylglycerol were shown to trigger both GIP and GLP-1 secretion in K and L cells

in vitro and in vivo, through G-protein coupled receptors (GPRs), GPR40, GPR120 and GPR119 (monoacylglycerol), but the exact mechanisms are still to be addressed (reviewed by Gribble et al.).[6,13,17] In opposition, acute exposure to short-chain fatty acids has no effects in serotonin secretion.[18] Fatty acids trigger CCK secretion through long-chain fatty acids receptors, although the immunoglobulin-like domain containing receptor 1 has also been suggested to be involved in response to lipoproteins and fatty acids.[6] As well, the fatty acid dodecanoic acid was also implicated in ghrelin decrease and increased PYY and GLP-2 secretion.[16,19] Additionally, besides their role on lipids emulsification, bile acids were also shown to lead to GLP-1 secretion from GLUTag cells, through GPR bile acid receptor 1, while having an inhibitory role on CCK release.[20,21]

EECs function is also regulated by amino acids and di-, tri-, oligopeptides (reviewed by Gribble and Reimann).[6] While GIP secretion does not seem to be stimulated upon protein ingestion, GLP-1 secretion from primary intestinal epithelial cells, in response to oligopeptides, was shown to rely on both peptide transporter-1 and calcium-sensing receptor.[13,22] The latter is also suspected to be involved in PYY secretion.[16] Studies have also been pointing out for a probable involvement of umami receptors in CCK secretion in a piglet model, upon activation by branched chain amino acids, mainly L-leucine and L-isoleucine.[23] Similarly, tryptophan is a crucial contributor to gut-derived serotonin synthesis.[6]

Gut signals are the bridge between nutrient sensing in the gastrointestinal tract and a diverse variety of actions that aim to regulate the overall metabolism (appetite regulation, food digestion and nutrient absorption and glucose homeostasis). In the next chapter of this review, we will exclusively address the direct effects of gut hormones in WAT.

2 | CROSSTALK BETWEEN GUT AND AT

Several gut hormones participate in the gut–brain axis and are known to influence the activity of brain centres controlling energy homeostasis, but, in fact, many authors have been suggesting that such effects are not exclusively due to (an)orexigenic activity.[24,25] In what concerns to WAT, such hormones inform on the patterns of food ingestion and nutrient absorption undergoing in the intestine and shape the metabolic activity of fat reservoirs, in order to favour the adequate physiological response to achieve homeostasis. More concretely, these hormones modulate nutrient uptake and storage, tissue plasticity and endocrine behaviour, which reinforces the existence of a dynamic gut–WAT crosstalk (Figure 1).

2.1 | Modulation of adipocyte metabolic function

2.1.1 | Lipogenesis and lipolysis

Intravenous infusion of acylated ghrelin in rodents resulted in increased inguinal and retroperitoneal WAT mass, with down-regulation of ATP-binding cassette sub-family G member 1 (ABCG1) (involved in lipid efflux from cells) and upregulation of SREBP-1c.[26] Stimulation of human adipocytes undergoing differentiation, with both forms of ghrelin, in a range of concentrations that contains those found in subjects with or without obesity and T2DM, resulted in SREBP1c activation, increased expression of lipoprotein lipase (LPL) and lipogenic enzymes and reduced glycerol outflow (Figure 2).[25] Such effects may be the result of a preventive mechanism to maintain energy supplies in WAT during starvation. Amylin, GIP and CCK might exert similar effects as ghrelin, despite their different timings of secretion. Amylin increases insulin effect in 3T3-L1 adipocytes, showing reduced glycerol release and increased fatty acids incorporation.[27] GIP also increases insulin action in inducing fatty acid uptake by epididymal fat pads, but it has no effect when insulin is not present in the incubation medium.[28] Furthermore, acute infusion of healthy men with physiological postprandial concentrations of GIP resulted in increased circulating TAGs hydrolysis and adipocyte re-esterification, also reducing fatty acid outflow, when combined with slight hyperglycaemia and hyperinsulinaemia.[29] Accordingly, GIP-mediated, but

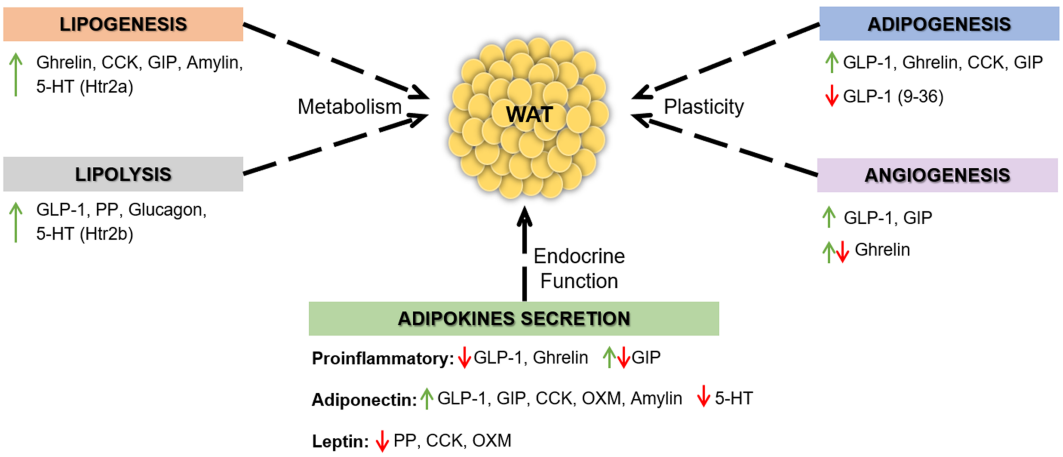

FIGURE 1 The role of gut hormones in regulating several events of adipose tissue function (lipid metabolism, endocrine function and adipogenesis/angiogenesis). GLP-1, glucagon-like peptide 1; GIP, glucose-dependent insulinotropic peptide; CCK, cholecystokinin; OXM, oxyntomodulin; PP, pancreatic polypeptide; PYY, peptide YY; 5-HT, serotonin

not GLP-1, stimulation of LPL gene was observed through pathways involving both Akt, 5′ AMP-activated protein kinase (AMPK) and cAMP response element binding (CREB)-dependent transcription.[30] CCK-8, one of the most abundant CCK forms, was also shown to increase LPL activity in AT, through a CCK-2 receptor-dependent mechanism.[31]

Regarding lipolytic stimuli, GLP-1 (100 nM) was shown to act as a lipolysis-promoting and lipogenesis-inhibitor factor in human AT explants and adipocytes, as well as in 3T3-L1 adipocytes. Upon GLP-1 receptor-dependent activation of cAMP/protein kinase A (PKA), perilipin is hyperphosphorylated, thus inducing fat mobilization and exposure to AT lipases (ATGL) (Figure 2).[32] Noteworthy, GLP-1 opposes insulin effects in terms of lipid metabolism while being insulinotropic and, despite studies on GLP-1 effects in AT use 10- to 100-nM concentration,[33,34] the normal plasmatic concentration of GLP-1, in humans, is in the pM range.[35] Such observation may suggest that these effects might occur through different mechanisms and periods, although the regulation between GLP-1 and insulin effects on lipolysis is still not clear. The same lipolytic effect was verified after administration of glucagon, which stimulated glycerol release in isolated adipocytes from epididymal rat fat pads[36] and in human subcutaneous AT explants, at 35 ng ml^{-1}, whereas plasma levels are around 300–500 pg ml^{-1}.[37] Denervation of the lumbar fat depots from rats showed that glucagon-induced lipolytic activity is not dependent on sympathetic mechanisms.[38] However, both high and basal physiological glucagonemia (1.5 and 0.5 ng $kg^{-1}.min^{-1}$) were unsuccessful in increasing interstitial glycerol in healthy humans' subcutaneous AT, during microdialysis.[39] Pancreatic polypeptide (0–12 nM) also enhances basal and stimulated lipolysis from chicken adipocytes, surpassing the idea that pancreatic polypeptide only acts through the autonomic nervous system.[40] Serotonin acts via Htr2a to inhibit lipolysis, in a 3T3-L1 model, favouring lipogenesis instead. In agreement, AT serotonin synthesis ablation led to a great reduction in visceral AT mass.[41] However, knockout mice models for Htr2b apparently suggest that serotonin directly stimulates lipolysis in adipocytes during fasting through this receptor, suggesting a dual role for serotonin in regulating lipid mobilization in AT, according to the energetic status.[10] GLP-2, whose specific receptor was shown to be expressed in mice visceral AT, does not seem to have a relevant effect in terms of AT metabolism.[42]

2.1.2 | Insulin sensitivity and glucose uptake

The implication of GLP-1 in ameliorating insulin sensitivity in the AT of rodents and humans with type 2 diabetes mellitus (T2DM) and obesity is widely recognized, although the mechanisms are not fully clarified. In fact, $Glp1r^{-/-}$ mice revealed an abnormal glucose homeostasis that was independent of weight and food intake,[43] and GLP-1 was shown to resolve insulin resistance in endoplasmic reticulum-stressed 3T3-L1 adipocytes.[33] Gao et al. elucidated the role of GLP-1 signalling in AT insulin sensitivity, which complied essentially an upregulation of insulin receptor subunit β and substrate 1, as well as glucose transporter 4 (GLUT4) (Figure 2).[34] However, in 3T3-L1 adipocytes, GLP-1

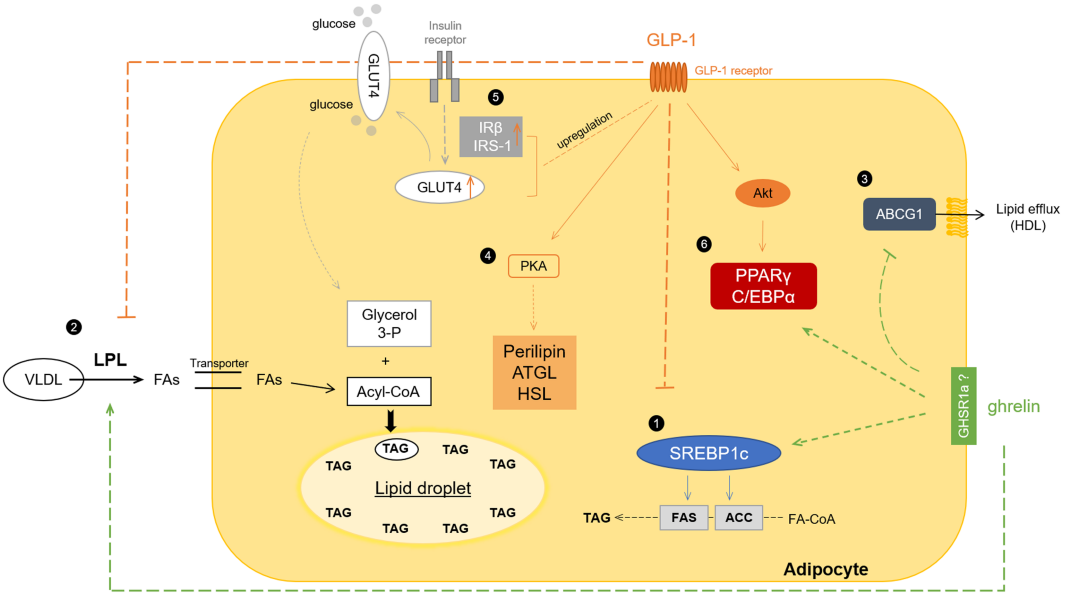

FIGURE 2 Distinct effects of GLP-1 and ghrelin in regulating lipid metabolism. Ghrelin favours all the mechanisms governing lipid storage, such as lipogenesis (1), lipid uptake (2) and inhibition of lipid efflux (3). In opposition, non-cleaved GLP-1 stimulates lipolysis releasing fatty acids for mitochondrial oxidation (4), which may partially account for GLP-1 induced weight loss, and increases insulin sensitivity (5). Both GLP-1 and ghrelin seem to stimulate adipogenesis (6). GLP-1, glucagon-like peptide 1; GHSR1a, growth hormone secretagogue receptor 1 alpha; SREBP-1c, sterol regulatory element-binding transcription factor 1c; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; FAs, fatty acids; TAG, triglyceride; VLDL, very low-density lipoprotein; LPL, lipoprotein lipase; ABCG1, ATP-binding cassette sub-family G member 1; HDL, high-density lipoprotein; PKA, protein kinase A; ATGL, adipose tissue triglyceride lipase; HSL, hormone-sensitive lipase; GLUT4, glucose transporter 4; IRβ, beta insulin-receptor subunit; IRS-1, insulin receptor substrate 1

administration, in a usual concentration for in vitro studies (100 nM), did not elicited an increase in insulin-dependent glucose uptake, while its analogue, exedin-4, was shown to induce such event, at the same concentration.[44] GLP-1 (9-36), the metabolite originated upon GLP-1 cleavage by dipeptidyl peptidase IV, that was once thought to be metabolically irrelevant, reduces postprandial blood glucose levels in healthy individuals independently of gastric emptying and insulin action, despite authors have shown a lower magnitude of the effect than the one elicited by the intact form of GLP-1.[45] Indeed, GLP-1 (9-36) (10 nM) was described to abolish glucose uptake in differentiated human subcutaneous AT stem cells.[46] Overall, such results suggest that AT insulin-mediated glucose uptake might be differentially regulated by different GLP-1 isoforms and may not exclusively rely on GLP-1 receptor signalling, highlighting the complexity of this peptide involvement in insulin sensitivity.

Regarding other peptides, both short-term amylin and GIP infusion, of close to physiological doses, in rats and humans, respectively, increase insulin-induced glucose uptake in AT, which is in accordance with their lipogenesis-stimulating actions.[47,48] Moreover, diet-induced male obese mice given an intraperitoneal injection of OXM display an increase in glucose uptake by WAT. However, it was not addressed the possible involvement of glucagon or GLP-1 receptors.[49] On the other hand, genetic and pharmacological ablation of gut-derived serotonin synthesis, in either chow or high fat fed mice, results in improved glucose tolerance and increase in WAT glucose uptake, suggesting that serotonin might jeopardize insulin sensitivity in AT, thuss contributing to insulin resistance.[50,51]

In summary, the precise role of gut hormones in regulating lipid and glucose uptake and metabolism, as well as their interplay and mutual interactions in AT, is still far from being completely understood. However, evidences claim that GIP may be involved in potentiating insulin-mediated glucose uptake and lipogenesis, while ghrelin (both forms) is apparently involved in lipid retention and accumulation in the adipocytes. In opposition, native GLP-1 drives lipolysis stimulation, possibly supplying fatty acid oxidation, and seems to increase insulin sensitivity.

2.2 | Modulation of adipocyte endocrine function

Adipokines secretion allows AT to regulate peripheral metabolism and to inform on the nutrient availability and metabolic status. Gut hormones are able to modulate the secretory profile of adipokines, thus having the power to control peripheral metabolism through AT manipulation.

Regarding adiponectin secretion, it was shown to be triggered by amylin (0.1 nM—within the physiological range) administration to 3T3-L1 adipocytes, an effect also seen after GLP-1 (100 nM) administration to human mature adipocytes.[27,32] Furthermore, adiponectin secretion was shown to be stimulated by OXM (400 nmol) in humans and by CCK (10 μg kg^{-1}) in rat subcutaneous and visceral AT in an insulin-dependent manner.[52,53] Amylin co-administration with leptin ex vivo to human AT explants was shown to have an additive effect in leptin signalling, without increasing ObR expression. Because amylin signals through a GPR, thus resulting in the activation of different pathways, this gut hormone might be, in turn, increasing leptin sensitivity and ObR-dependent signalling.[54] Leptin levels were decreased after chronic CCK administration in rats and after a subcutaneous administration of OXM (400 nmol) in humans, which might be a consequence of AT mass reduction, an effect that OXM has been described to provoke.[31,53] WAT leptin expression was, as well, reduced by peripheric pancreatic polypeptide administration to food-deprived mice, but not in non-deprived ones, an effect shown to involve, at least partially, the sympathetic enervation.[55]

PYY serum levels were shown to correlate with visfatin expression in subcutaneous AT,[56] a crucial adipokine for glucose metabolism and mitochondrial function.[3] Given the fact that both serum PYY and visfatin levels are negatively correlated with weight gain,[56] such evidences may put forward a protective/favouring action in increasing subcutaneous/visceral AT visfatin expression, possibly mediated by PYY.

Although studies in cultured adipocytes have suggested the opposite,[57] in vivo studies have shown the power of GIP receptor signalling in attenuating inflammation, because both GIP or a long-lasting analogue led to a decrease in the inflammatory profile of visceral AT secretome, while increasing adiponectin secretion.[58,59] Such contradictory evidences might be associated with differences inherent to the sensitivity of the model itself to GIP concentrations. As well, adenovirus-associated GLP-1 also led to a reduction in nuclear factor kappa B-mediated interleukin-6, TNF-α and monocyte chemoattractant protein-1 production in the visceral AT of *ob/ob* mice.[60] Furthermore, Rodríguez et al. found out that acylated and des-acyl ghrelin, in the pM range, could play an anti-inflammatory role by decreasing TNF-α-induced apoptosis in human adipocytes, through inhibition of caspase-8 and caspase-3.[61] Inhibition of gut serotonin production led, in mice, to a reduction in WAT inflammation,[51] suggesting that peripheral elevation of serotonin levels might contribute to a pro-inflammatory environment in AT. Moreover, activation of serotonin cascades signalling in AT leads to a decrease in adiponectin expression, possibly explaining why insulin sensitivity improves upon inhibition of serotonin synthesis.[41,51]

2.3 | Modulation of AT plasticity: Adipogenesis and angiogenesis

Adipogenesis requires a proper interaction between the pre-adipocytes and the stromal vascular fraction in order to guide cell migration. The adipo-vascular coupling, mainly regulated by fibroblast and vascular growth signals, allows a healthy expansion and retraction of the tissue according to the energetic balance of the organism.[2]

Ghrelin directly modulates AT behaviour, independently of its food intake-promoting action in the arcuate nucleus. In fact, it acts as an adipogenic factor through the GHSR1a, because, along with its SREBP1c-stimulating effects, it activates peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding

protein alpha (C/EBPα) in human visceral adipocytes, from 0.1 to 1000 pM (Figure 2).[25] Nevertheless, des-acyl ghrelin (GHSR1a-independent) also induced adipogenesis, possibly suggesting non-GHSR1a-related mechanisms.[25,26] The hormones CCK, GIP and GLP-1 also contribute to AT expansion, because they all induce an increase in PPARγ, either in vivo or in vitro.[52,58,62] Mice under a high fat regimen plus a long-lasting GIP analogue treatment displayed increased adipocyte hypertrophy, when compared with diet alone and had higher expression of lipid droplet proteins.[58] On the other hand, GLP-1 led to an increase in lipid droplet number rather than size, in 3T3-L1 and in diabetic *ob/ob* mice, meaning that it mainly stimulates adipocyte hyperplasia.[60,62] Such effect is likely to derive from Akt-dependent upregulation of C/EBPβ/γ and PPARγ as well as an increase in cell proliferation (Figure 2).[62] Conversely, truncated GLP-1 (9-36) (10 nM) inhibits cell growth and differentiation and promotes apoptosis in human subcutaneous AT stem cells.[46] These effects might be mediated through non-GLP-1 receptor-related mechanisms since the (9–36) peptide has low affinity for the classical receptor, but until the date, there are no evidences of its binding site.[46] Given the different consequences of hypertrophy and hyperplasia for lipidic metabolism profile and insulin sensitivity, such GLP-1 effects are currently thought to underlie a favourable WAT remodelling, although the correct balance between all the factors is probably the best approach to induce a physiological tissue expansion.[62,63]

The involvement of gut hormones in shaping endothelial cells function (angiogenesis and vasodilatory response) has been addressed in several models. Among them, we highlight GLP-1 and GIP that have been strongly implicated in the amelioration of endothelial dysfunction in several models.[64,65] The involvement of GLP-1 and its metabolites, especially GLP-1 (9-36), in the improvement of vasodilatory response of endothelial cells is reported, in detail, by Li et al.[66] GIP infusion during a hyperglycaemia/hyperinsulinaemia clamp (mimicking postprandial levels) resulted, in healthy, but not in men with obesity, in increased blood flow in subcutaneous AT that was accompanied by capillary recruitment. Such effect was not elicited by insulin alone, which is also in accordance with GIP stimulation of insulin-induced glucose uptake and fatty acids storage.[67] The precise mechanism underlying such effect is still unknown, but it might involve nitric oxide-dependent mechanisms, because GIP was shown to induce nitric oxide raise in bovine aortic endothelial cells.[68] Moreover, it is known that nitric oxide stimulates LPL activity in cultured pre-adipocytes.[69] Thus, GIP-induced free fatty acids uptake described above can be the result of the vascular change elicited by this incretin in postprandial-like situations, triggering nitric oxide release and a consequent stimulation of adipocyte LPL. Unlike GIP, GLP-1 infusion in healthy men also raised AT blood flow, but at fasting glucose and insulin levels, and to a similar extent as the one seen after meals. The involvement of the sympathetic nervous system was excluded due to unaltered plasma levels of catecholamines, but the infusion of 1.5 pmol kg^{-1} min^{-1} resulted in slightly supraphysiological GLP-1 levels, raising concerns regarding the normal physiological response.[70]

Recently, a study suggested that ghrelin could also employ a preponderant role in such matter, due to its angiogenesis-induction effects in endothelial cells from both human umbilical vein and mice bone marrow cells. Knocking-out GHSR1a resulted in decreased AT mass and vascular network.[71] However, GHRS1a deletion was not conditional, meaning that, instead of local inability of acylated ghrelin-GHSR1a to induce angiogenesis, hypothalamic ablation of the receptor could result in decreased food intake and a consequent loss of fat mass. Matrigel plug injection in subcutaneous AT, in the presence or absence of acylated ghrelin, showed no effects in AT angiogenic response, in mice, either healthy or with obesity.[72] The effect of ghrelin in other models, such as the chick chorioallantoic membrane or human dermal microvascular endothelial cells, is also not consensual.[72] These evidences may suggest that ghrelin regulates angiogenesis according to the cell type, thus exerting a pro- or anti-angiogenic action. The existing studies use a myriad of peptides concentrations and different incubation times, which might lead to different responses, even when in the same cellular type.

3 | DISEASE-ASSOCIATED ALTERATION IN GUT SIGNALS SECRETION: MODULATION OF GUT HORMONES AS A STRATEGY TO IMPROVE AT FUCTION

Gut hormones secretion and bioavailability during pre- or post-meal periods differ naturally among individuals, and gut endocrinology may be changed by simple dietary habits or complex pathologies. Indeed, the MetS has been shown to carry a pronounced dysregulation of gut humoral signals.[73] Despite deregulation of mechanisms governed by the gut-hypothalamic axis is a major driven force to obesity, AT dysfunction is, as well, tightly linked to the development of MetS (reviewed by Matafome and Seica).[3] Although AT dysfunction is a major risk factor for MetS and MetS-affected patients present alterations in gut hormones secretion patterns, the causality remains to be determined: Are such events parallel in the development of MetS or the unbalanced gut hormonal milieu, caused by dietary habits, underlies AT malfunction? In the next sections, we will discuss how gut hormones secretion is changed in MetS and T2DM and how can we modulate it to improve AT function and MetS features (Figure 3).

3.1 | Changes of gut hormone profile in metabolic syndrome and type 2 diabetes

When compared with normal-weight controls, the anorexigenic hormone GLP-1 has been reported to be significantly decreased in overweight and obese subjects after a test meal, even though pre-prandial GLP-1 levels were similar in both.[35] Similar observations on GLP-1 levels were reported in Goto-Kakizaki rats, which also revealed a reduction in postprandial secretion of PYY.[74] Furthermore, Zwirska-Korczala et al. described weaker postprandial PYY response and lower fasting and postprandial CCK plasma levels in women with obesity and MetS.[75] In T2DM patients, postprandial GIP levels were not observed to suffer the expected raise, as observed in non-diabetic

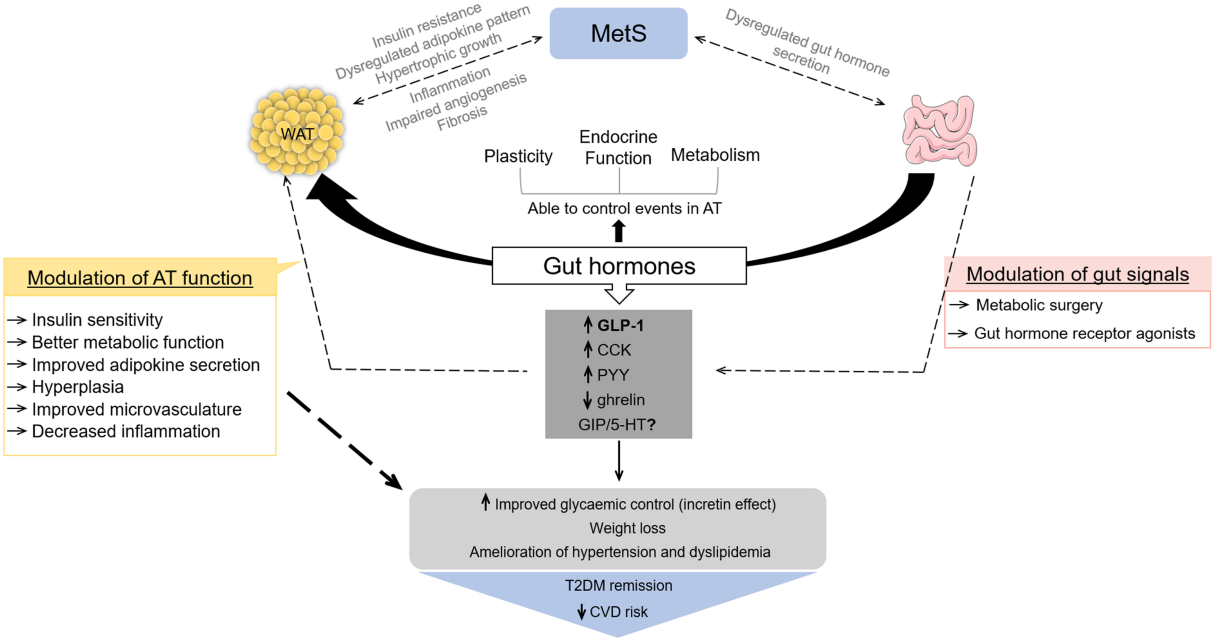

FIGURE 3 An integrated view on the gut–adipose tissue crosstalk. Dysfunctional adipose tissue and dysregulated gut hormone profile are both features of metabolic syndrome or type 2 diabetes. Given that gut hormones are known to regulate several aspects of adipose tissue function, restoration of gut hormone profile through bariatric surgery or the use of analogues may be a promising strategy in the amelioration of metabolic syndrome-associated adipose tissue dysfunction. MetS, metabolic syndrome; AT, adipose tissue; GLP-1, glucagon-like peptide 1; CCK, cholecystokinin; PYY, peptide YY; GIP, glucose-dependent insulinotropic peptide; 5-HT-serotonin; T2DM, type 2 diabetes mellitus; CVD, cardiovascular disease

individuals, suggesting post-secretion degradation of GIP to be a crucial contributor to the loss of incretin effect.[76]

Lower fasting total ghrelin plasma levels were reported in subjects with obesity, in comparison with normal-weight controls, but the progressive decline observed after meal ingestion found in healthy individuals is absent in those affected by obesity, and the same is verified in rodents.[74,77] Indeed, such results highlight the relation between obesity/MetS and the failure in reducing ghrelin levels after meal ingestion, giving rise to a hypothesis claiming that high-fat diets could induce ghrelin resistance.[78]

Both fasting and postprandial glucagon levels were shown to be increased in the context of obesity and T2DM.[79] Serotonin levels are increased with human obesity, before and after intraduodenal glucose infusion,[80] the same being verified in mice,[51] while, in contrary, GLP-2 postprandial levels are apparently decreased.[81]

3.2 | The role of MetS in modulating gut hormone profile: Development of mimetics

Initially implemented as a strategy to diminish food intake, through reduction of gastric volume, and/or promotion of nutrient malabsorption,[82] soon it was noticed that MetS could alter gut hormones secretion. The altered secretion profile of enteroendocrine signals is currently one of the most accepted events to explain T2DM remission that happens few days after surgery, even before significant weight loss.[83] Amelioration of glycaemic control is independent from weight loss and, thus, may be related, not only to the central actions of these hormones but also to a robust increase in the incretin effect and direct effects in other organs.

Nowadays, vertical sleeve gastrectomy (VSG) and Roux-en-Y gastric bypass (RYGB) are the most frequently performed surgical approaches for obesity and T2DM.[82] The anorexigenic hormones GLP-1, CCK and PYY suffer major increases after meal consumption in RYGB and VSG-submitted patients.[84,85] Evidences regarding postoperative GLP-2 secretion also support a significant increase in plasma that appears to be sustainable in time. Pancreatic polypeptide plasma levels were consistent among controls and surgery-submitted individuals, not revealing significant alterations.[84,85] On the other hand, circulating serotonin levels are increased 1-year post gastrectomy, but this increase was shown to be more pronounced in patients who regained weight, when in comparison with weight stable subjects, in a 2-year follow-up study.[86] Some other hormones remain either inconsistent or uncharacterized. For instance, results on GIP levels are incoherent after RYGB, either increasing or decreasing, and no significant changes have been observed after VSG, although a recent metanalysis revealed a trend to its decrease.[85,87] After RYGB, OXM levels are raised upon an oral glucose tolerance test in humans, but its secretion profile remains unclear in patients submitted to VSG.[88] Postoperative postprandial ghrelin levels are decreased in a vast number of studies in humans and rodents, even in long-term follow-up, whereas fasting levels seem to be target of controversy. However, the existent studies do not usually discriminate between total or acylated ghrelin levels.[74,89] To what concerns to VSG, the

diminished ghrelin secretion might be due to the resection of the stomach's gastric fundus, which can explain the more pronounced effect than RYGB.[90] Somatostatin, the hormone responsible for the attenuation in GLP-1, GIP and CCK secretion, remains uncharacterized in patients submitted to metabolic surgery.[84,88]

The effect of RYGB-induced nutrients rerouting in ECCs population is still not entirely clear. RYGB induced an increase in L-, K- and I-cells populations due to thickening of the mucosa, without major alterations in cell density and expression of *PYY*, *GCG*, *GIP* and *CCK*.[91,92] Strikingly, VSG neither change intestinal mucosa's area nor GIP secretion but was described to augment GLP-1 positive cells without affecting L-cell transcriptome.[93,94] Therefore, the debate is still open on whether and how surgical approaches modulate EECs abundance and diversity, which may also arise from slight differences in surgical techniques or from post-surgery dietary habits. One possible explanation relies on the intestinotrophic peptide GLP-2, postulated to have a possible involvement in crypt stem cells proliferation, thus constituting a probable contributor for the increase in L-cells population.[95] On the other hand, increased rates of gastric emptying and nutrient flow into the intestines after surgery might acutely result in enhanced nutrient-sensing and GLP-1 secretion, which could chronically favour an increase in L-cell population.[96] However, the exact mechanisms of GLP-1 regulation are still obscure. A recent study has shown that metformin stimulates GLP-1 secretion directly from human gut epithelial tissue and increases GLP-1 plasma concentration, suggesting other mechanisms involved in L-cell regulation.[97] Still, the common finding that both RYGB and VSG elicit increases in plasmatic GLP-1 highlight a critical role of early GLP-1 involvement in the amelioration of hyperglycaemia.

Pharmacological GLP-1-based therapies preserve the incretin ability through a less invasive way and surpass GLP-1 short half-life.[98] Liraglutide (modified GLP-1 combined with palmitic acid), exenatide (synthetic of the dipeptidyl peptidase IV-resistant exendin-4), dulaglutide (GLP-1 analogue combined with each Fc domain of an immunoglobulin G) and semaglutide (acylated GLP-1 peptide with an amino acid modification at position 8) constitute the most up to date, reliable incretin mimetics used in the treatment of T2DM.[98] They all share long-term lowering effects in fasting plasma glucose and glycated haemoglobin, with liraglutide and semaglutide inducing the most drastic results.[99,100] However, the frequent side effects and minimal weight loss often seen after their administration led to development of triple agonists—unimolecular combination of GLP-1/GIP/glucagon receptors agonists or GOP (GLP-1/OXM/PYY) hormonal infusion.[100] Merging GLP-1/GIP's incretin activity with the thermogenic activity of glucagon resulted in synergistic and more powerful effects in glucose homeostasis and cholesterol and body weight reduction in diet-induced obesity and streptozotocin-treated mice.[100,101] The GOP infusion, developed by Tan et al., led to a marked decrease in food intake and postprandial blood glucose levels in individuals with obesity. However, fasting glucose and insulin were not changed, probably because the individuals were not diabetic, and the time frame of the study (3 days with no follow-up) did not allowed body weight monitoring.[102]

3.3 | Consequences of gut hormone remodelling for AT function

Not neglecting the obvious importance of the increased hypothalamic anorexigenic activity inherent to the above described therapeutic approaches, one cannot exclude the putative direct effects of gut hormones in metabolic effector organs, such as the AT. Some studies in rodents have been claiming that surgery-induced beneficial effects cannot be exclusively attributed to the decreased caloric intake.[103] Indeed, it can be speculated that a shift into a more catabolic profile of AT could underlie the surgery and pharmacotherapy-induced improvement in glucose homeostasis and body weight observed in VSG and RYGB-submitted rodents and humans. Beyond reducing visceral AT weight, VSG led to an increase in vascular differentiation and angiogenesis in obese T2DM rats, which might improve microvasculature. The also observed increased levels of AMPK, that point to a raise in fatty acids oxidation, reflect a metabolic shift in AT that may underlie the amelioration of triglyceridaemia and cholesterolaemia and weight loss also found in operated animals.[1] In humans, VSG, however, decreases adipogenesis-involved markers in subcutaneous AT, and the same was found in post-RYGB subjects.[104] However, biopsies were done 1 week after the procedures, and another recent study has shown that reshaping of AT morphology and hyperplasia of subcutaneous AT adipocytes was only seen in the patients 5 years after the gastric bypass.[105] Post-operative human subcutaneous AT also reveals a marked decrease in angiopoietin 1/2 and tie expression, which suggests a decrease in angiogenic capacity. Macrophage recruitment to the subcutaneous AT and expression of pro-inflammatory mediators also decrease after VSG.[106]

In subcutaneous AT, MetS induces a huge increase in lipid oxidation, suggesting that, after the procedure, the organism adopts a more energetic dissipation-prone status rather than favouring its storage.[104] Surgery induces a decrease in leptin and a raise in adiponectin levels, and the latter highly correlates with enhanced postoperative insulin sensitivity.[107] In fact, 1 year after RYGB, insulin downstream signals and GLUT4 were increased in AT of patients with obesity and T2DM.[108] Overall, these results reinforce the crucial role of AT for the surgery-induced glucose homeostasis.

The involvement of gut hormones in the post-surgery AT remodelling remains an incognita. Nonetheless, GLP-1-mediated effects constitute the most explored explanation on driving AT metabolic shift, increased insulin sensitivity and amelioration on tissue's microvasculature and inflammation after surgery, considering its effects in AT under normal conditions (described in chapter 2). However, this is mere speculation, because no studies until the date addressed such question. D'Alessio et al. showed that GLP-1 receptor blockage in gastric bypass-submitted subjects decreased glucose-stimulated insulin secretion, highlighting a pivotal role for GLP-1 in glucose homeostasis.[109] In order to understand if GIP was also contributing to the incretin effect after RYGB, patients were given a dipeptidyl peptidase IV inhibitor (sitagliptin) alone or in combination with GLP-1 receptor antagonist. It turned out that sitagliptin was unable to lower postprandial glucose levels upon GLP-1 receptor

inhibition, corroborating higher GLP-1's engagement in glucose homeostasis.[110] Nevertheless, whole-body GLP-1 receptor knockout mice submitted to MetS have been opposing such involvement of GLP-1, because ablation of the receptor did not alter the magnitude of the responses after VSG regarding body weight and glucose homeostasis.[111] A recent study where Y2 receptor deficient mice were submitted to RYGB also revealed no alterations in such parameters, in comparison with RYGB-submitted wild-type mice, discarding possible involvement of PYY_{3-36}-Y2 signalling.[112] Despite constituting a powerful scientific tool, one cannot exclude the possibility of distinct roles of gut hormone receptors in each tissue, which may not be discerned in non-conditional knockout, as well as evolutionary development of compensatory mechanisms in these animal models that might be masquerading the results. Thus, the utilization of gut hormones receptor agonists/antagonists might be helpful in dissecting their precise involvement. In fact, the GLP-1 receptor agonists exenatide and liraglutide lead to an overall fat mass reduction but specially of visceral AT depots in mice and humans with T2DM.[113–115] Such effects can be the result of AMPK activation, which is in accordance with the increase in both fatty acids uptake and oxidation and adipocyte 'beiging' observed with both agonists.[113,114] Exenatide-treated rodents also show increased expression of omentin-1 in visceral AT, adiponectin in both visceral and subcutaneous AT, as well as decreased leptin expression, which was associated with improved insulin sensitivity.[116] On the other hand, liraglutide administration (0.6 mg for 2 weeks and 1.2 mg for the following 14 weeks) did not change *ADIPOQ* and *LEP* expression in human subcutaneous AT, despite weight loss after 4 months was far from diet-induced weight reduction, where adiponectin expression tended to augment.[115] In terms of AT vascular function, both liraglutide and exenatide were described to improve the ex vivo anti-contractile capability of mice perivascular and human subcutaneous AT explants through a GLP-1 receptor-dependent AMPK activation and nitric oxide production.[117,118] Diet-induced obese mice receiving exenatide during 4 weeks also had improved AT angiogenesis that ended up to alleviate hypoxia-induced inflammation.[119] Altogether, these evidences emphasize the efficacy in GLP-1 receptor agonists not only in ameliorating AT metabolic behaviour but also to promote a well-controlled relationship between vascularization and immune function, allowing a healthy AT expansion/retraction. Having that said, Pastel et al., in a randomized control trial with T2DM-affected subjects with overweight/obesity, found that the liraglutide-treated patients (0.6 mg for 2 weeks and 1.2 mg for the following 14 weeks) registered an increase in inflammation and fibrosis markers in subcutaneous AT, when compared with a dietary restriction arm. Despite at baseline all subjects were under metformin treatment, in the liraglutide group, some dropped or had its dosage reduced during the course of the study, what may have had impact in the results.[115] Information on dulaglutide and semaglutide effects in AT are still scarce, due to their recent approval. Nonetheless, both agonists induced a significant reduction in epicardial AT mass in subjects with obesity and T2DM.[120] A substudy of the SUSTAIN 8 trial also reported a total fat mass decrease upon semaglutide administration accompanied by a marginal reduction in visceral AT depots. It is to note that the trial lacked an appropriate placebo, and because the individuals were already under metformin treatment, these effects may not be fully attributable to semaglutide.[121]

As for triple agonists, the existing literature has been mainly focusing in monitoring systemic parameters, thus lacking information on tissue-specific action, and, besides that, no clinical trial results are yet available. Nevertheless, it seems that the reduction in AT mass is the primary result of GLP-1 and glucagon effects.[101]

4 | CONCLUSIONS AND FUTURE PERSPECTIVES

Gut hormones are acknowledged to play a major role in the regulation of overall metabolism. In response to luminal nutrient stimuli, the EECs release signals that act not only via the gut-brain axis. Despite dysregulation of the gut-hypothalamic axis is a major driven force to obesity and MetS, the AT constitutes the other arm that can participate in this crosstalk. Thus, given the escalating incidence of such diseases, there is a strong urge in truly understanding the behaviour and biology of AT. As highlighted above, gut hormones are able to regulate several events in AT in physiological conditions, such as metabolism, endocrine behaviour and plasticity. In fact, the modulation of gut signals elicited by the therapeutic approaches used in MetS results in numerous adaptations in AT function and remodelling that might contribute, at least in part, to the resolution of T2DM and decreased risk of CVD (Figure 3).

Determining the precise role of gut hormones, not only in AT but also in other affected tissues, constitutes an important strategy to understand the effects of MetS and gut signals-based therapies in metabolic control. As equally relevant is the development of tools that allow the study of subcutaneous and visceral AT depots, to better understand how they differentially behave in response to therapy, although obtaining visceral AT biopsies from humans is not always feasible. Despite several details of the results of MetS are still to explain, the current information is already sufficient to highlight the importance of gut hormones as powerful drug targets for MetS-associated diseases. Thus, further studies should continue addressing the beneficial effects of gut hormones modulation, what can turn out to be a difficult challenge, especially when is still not completely clear how is their secretion altered in the disease context.

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CONFLICT OF INTEREST

No conflict of interest was declared.

ORCID

Daniela Rosendo-Silva https://orcid.org/0000-0002-4276-5920
Paulo Matafome https://orcid.org/0000-0002-3422-290X

REFERENCES

1. Eickhoff H, Rodrigues T, Neves I, et al. Effect of sleeve gastrectomy on angiogenesis and adipose tissue health in an obese animal model of type 2 diabetes. *Obes Surg*. 2019;29(9):2942-2951. https://doi.org/10.1007/s11695-019-03935-z
2. Lemoine AY, Ledoux S, Larger E. Adipose tissue angiogenesis in obesity. *Thromb Haemost*. 2013;110(04):661-669. https://doi.org/10.1160/TH13-01-0073
3. Matafome P, Seica R. Function and dysfunction of adipose tissue. In: Letra L, Seica R, eds. *Obesity and Brain Function*. Advances in Neurobiology. 1st ed. Cham: Springer International Publishing; 2017:3-31 doi:10.1007/978-3-319-63260-5.
4. Matafome P, Rodrigues T, Seica R. Glycation and hypoxia: two key factors for adipose tissue dysfunction. *Curr Med Chem*. 2015;22(20):2417-2437. https://doi.org/10.2174/0929867322666150209155633
5. Yiannikouris F, Gupte M, Putnam K, Cassis L. Adipokines and blood pressure control. *Curr Opin Nephrol Hypertens*. 2010;19(2):195-200. https://doi.org/10.1097/MNH.0b013e3283366cd0
6. Gribble FM, Reimann F. Enteroendocrine cells: chemosensors in the intestinal epithelium. *Annu Rev Physiol*. 2016;78(1):277-299. https://doi.org/10.1146/annurev-physiol-021115-105439
7. Gribble FM, Reimann F, Roberts GP. Gastrointestinal hormones. In: Said HM, ed. *Physiology of the Gastrointestinal Tract*. 6th ed. Vol.1 London: Academic Press; 2018:31-54.
8. Lewis JE, Miedzybrodzka EL, Foreman RE, et al. Selective stimulation of colonic L cells improves metabolic outcomes in mice. *Diabetologia* Published Online. 2020;63(7):1396-1407. https://doi.org/10.1007/s00125-020-05149-w
9. Boswell T. Food intake: behavioral endocrinology. In: Choe JC, ed. *Encyclopedia of Animal Behavior*. 2nd ed. Vol.1 Oxford: Academic Press; 2019:533-538 doi:10.1016/B978-0-12-809633-8.01054-2.
10. Sumara G, Sumara O, Kim JK, Karsenty G. Gut-derived serotonin is a multifunctional determinant to fasting adaptation. *Cell Metab*. 2012;16(5):588-600. https://doi.org/10.1016/j.cmet.2012.09.014
11. Kirchner H, Gutierrez JA, Solenberg PJ, et al. GOAT links dietary lipids with the endocrine control of energy balance. *Nat Med*. 2009;15(7):741-745. https://doi.org/10.1038/nm.1997
12. Kuhre RE, Frost CR, Svendsen B, Holst JJ. Molecular mechanisms of glucose-stimulated GLP-1 secretion from perfused rat small intestine. *Diabetes*. 2015;64(2):370-382. https://doi.org/10.2337/db14-0807
13. Müller TD, Finan B, Bloom SR, et al. Glucagon-like peptide 1 (GLP-1). *Mol Metab*. 2019;30:72-130. https://doi.org/10.1016/j.molmet.2019.09.010
14. Sun EW, de Fontgalland D, Rabbitt P, et al. Mechanisms controlling glucose-induced GLP-1 secretion in human Small intestine. *Diabetes*. 2017;66(8):2144-2149. https://doi.org/10.2337/db17-0058
15. Gerspach AC, Steinert RE, Schönenberger L, Graber-Maier A, Beglinger C. The role of the gut sweet taste receptor in regulating GLP-1, PYY, and CCK release in humans. *Am J Physiol Endocrinol Metab*. 2011;301(2):E317-E325. https://doi.org/10.1152/ajpendo.00077.2011
16. Steinert RE, Feinle-Bisset C, Asarian L, Horowitz M, Beglinger C, Geary N. Ghrelin, CCK, GLP-1, and PYY(3–36): secretory controls and physiological roles in eating and glycemia in health, obesity, and after RYGB. *Physiol Rev*. 2017;97(1):411-463. https://doi.org/10.1152/physrev.00031.2014
17. Sankoda A, Harada N, Kato T, et al. Free fatty acid receptors, G protein-coupled receptor 120 and G protein-coupled receptor 40, are essential for oil-induced gastric inhibitory polypeptide secretion. *J Diabetes Investig*. 2019;10(6):1430-1437. https://doi.org/10.1111/jdi.13059
18. Martin AM, Lumsden AL, Young RL, Jessup CF, Spencer NJ, Keating DJ. Regional differences in nutrient-induced secretion of gut serotonin. *Physiol Rep*. 2017;5(6):e13199. https://doi.org/10.14814/phy2.13199
19. Feltrin KL, Patterson M, Ghatei MA, et al. Effect of fatty acid chain length on suppression of ghrelin and stimulation of PYY, GLP-2 and PP secretion in healthy men. *Peptides*. 2006;27(7):1638-1643. https://doi.org/10.1016/j.peptides.2006.01.023
20. Parker HE, Wallis K, le Roux CW, Wong KY, Reimann F, Gribble FM. Molecular mechanisms underlying bile acid-stimulated glucagon-like peptide-1 secretion. *Br J Pharmacol*. 2012;165(2):414-423. https://doi.org/10.1111/j.1476-5381.2011.01561.x
21. Koide M, Okabayashi Y, Otsuki M. Role of endogenous bile on basal and postprandial CCK release in humans. *Dig Dis Sci*. 1993;38(7):1284-1290. https://doi.org/10.1007/BF01296080
22. Santos-Hernández M, Miralles B, Amigo L, Recio I. Intestinal signaling of proteins and digestion-derived products relevant to satiety. *J Agric Food Chem*. 2018;66(39):10123-10131. https://doi.org/10.1021/acs.jafc.8b02355
23. Tian M, Heng J, Song H, et al. Branched chain amino acids stimulate gut satiety hormone cholecystokinin secretion through activation of the umami taste receptor T1R1/T1R3 using an in vitro porcine jejunum model. *Food Funct*. 2019;10(6):3356-3367. https://doi.org/10.1039/C9FO00228F
24. Perez-Tilve D, Heppner K, Kirchner H, et al. Ghrelin-induced adiposity is independent of orexigenic effects. *FASEB J*. 2011;25(8):2814-2822. https://doi.org/10.1096/fj.11-183632
25. Rodríguez A, Gómez-Ambrosi J, Catalán V, et al. Acylated and desacyl ghrelin stimulate lipid accumulation in human visceral adipocytes. *Int J Obes (Lond)*. 2009;33(5):541-552. https://doi.org/10.1038/ijo.2009.40
26. Davies JS, Kotokorpi P, Eccles SR, et al. Ghrelin induces abdominal obesity via GHS-R-dependent lipid retention. *Mol Endocrinol*. 2009;23(6):914-924. https://doi.org/10.1210/me.2008-0432
27. Miegueu P, St-Pierre DH, Munkonda MN, Lapointe M, Cianflone K. Amylin stimulates fatty acid esterification in 3T3-L1 adipocytes. *Mol Cell Endocrinol*. 2013;366(1):99-107. https://doi.org/10.1016/j.mce.2012.12.008
28. Beck B, Max J. Gastric inhibitory polypeptide enhancement of the insulin effect on fatty acid incorporation into adipose tissue in the rat. *Regul Pept*. 1983;7(1):3-8. https://doi.org/10.1016/0167-0115(83)90276-8
29. Asmar M, Simonsen L, Madsbad S, Stallknecht B, Holst JJ, Bülow J. Glucose-dependent Insulinotropic polypeptide may enhance fatty acid re-esterification in subcutaneous abdominal adipose tissue in lean humans. *Diabetes*. 2010;59(9):2160-2163. https://doi.org/10.2337/db10-0098
30. Kim S, Nian C, McIntosh CHS. Glucose-dependent insulinotropic polypeptide (GIP) increases human adipocyte lipoprotein lipase (LPL) expression through cyclic AMP (cAMP) response element binding protein (CREB) and cAMP-responsive CREB coactivator 2 (TORC2) mediated trans-activation of the LPL gene. *J Lipid Res* Published online. 2010;51(11):3145–3157. https://doi.org/10.1194/jlr.M006841
31. Plaza A, Merino B, Cano V, et al. Cholecystokinin is involved in triglyceride fatty acid uptake by rat adipose tissue. *J Endocrinol*. 2018;236(3):137-150. https://doi.org/10.1530/JOE-17-0580
32. Bekay RE, Coín-Aragüez L, Fernández-García D, et al. Effects of glucagon-like peptide-1 on the differentiation and metabolism of human adipocytes. *Br J Pharmacol*. 2016;173(11):1820-1834. https://doi.org/10.1111/bph.13481
33. Jiang Y, Wang Z, Ma B, et al. GLP-1 improves adipocyte insulin sensitivity following induction of endoplasmic reticulum stress. *Front Pharmacol*. 2018;9:1168. https://doi.org/10.3389/fphar.2018.01168
34. Gao H, Wang X, Zhang Z, et al. GLP-1 amplifies insulin signaling by up-regulation of IRβ, IRS-1 and Glut4 in 3T3-L1 adipocytes.

Endocrine. 2007;32(1):90-95. https://doi.org/10.1007/s12020-007-9011-4
35. Adam TCM, Westerterp-Plantenga MS. Glucagon-like peptide-1 release and satiety after a nutrient challenge in normal-weight and obese subjects. *Br J Nutr*. 2005;93(6):845-851. https://doi.org/10.1079/BJN20041335
36. Jolly SR, Lombardo YB, Lech JJ, Menahan LA. Effect of aging and cellularity on lipolysis in isolated mouse fat cells. *J Lipid Res*. 1980;21(1):44-52.
37. Richter WO, Robl H, Schwandt P. Human glucagon and vasoactive intestinal polypeptide (VIP) stimulate free fatty acid release from human adipose tissue in vitro. *Peptides*. 1989;10(2):333-335. https://doi.org/10.1016/0196-9781(89)90039-9
38. Lefebvre P, Luyckx A, Bacq ZM. Effects of denervation on the metabolism and the response to glucagon of white adipose tissue of rats. *Horm Metab Res*. 1973;5(4):245-250. https://doi.org/10.1055/s-0028-1093959
39. Gravholt CH, Møller N, Jensen MD, Christiansen JS, Schmitz O. Physiological levels of glucagon do not influence lipolysis in abdominal adipose tissue as assessed by microdialysis. *J Clin Endocrinol Metab*. 2001;86(5):2085-2089. https://doi.org/10.1210/jcem.86.5.7460
40. Oscar TP. Enhanced lipolysis from broiler adipocytes pretreated with pancreatic polypeptide. *J Anim Sci*. 1993;71(10):2639-2644. https://doi.org/10.2527/1993.71102639x
41. Oh C-M, Namkung J, Go Y, et al. Regulation of systemic energy homeostasis by serotonin in adipose tissues. *Nat Commun*. 2015;6:1-12. https://doi.org/10.1038/ncomms7794
42. Beaudry JL, Drucker DJ. Proglucagon-derived peptides, glucose-dependent insulinotropic polypeptide, and dipeptidyl peptidase-4-mechanisms of action in adipose tissue. *Endocrinology*. 2020;161(1):bqz029. https://doi.org/10.1210/endocr/bqz029
43. Scrocchi LA, Brown TJ, Maclusky N, et al. Glucose intolerance but normal satiety in mice with a null mutation in the glucagon–like peptide 1 receptor gene. *Nat Med*. 1996;2(11):1254-1258. https://doi.org/10.1038/nm1196-1254
44. Idris I, Patiag D, Gray S, Donnelly R. Exendin-4 increases insulin sensitivity via a PI-3-kinase-dependent mechanism: contrasting effects of GLP-1. *Biochem Pharmacol*. 2002;63(5):993-996. https://doi.org/10.1016/S0006-2952(01)00924-8
45. Meier JJ, Gethmann A, Nauck MA, et al. The glucagon-like peptide-1 metabolite GLP-1-(9–36) amide reduces postprandial glycemia independently of gastric emptying and insulin secretion in humans. *Am J Physiol Endocrinol Metab*. 2006;290(6):E1118-E1123. https://doi.org/10.1152/ajpendo.00576.2005
46. Cantini G, Di Franco A, Mannucci E, Luconi M. Is cleaved glucagon-like peptide 1 really inactive? Effects of GLP-1(9-36) on human adipose stem cells. *Mol Cell Endocrinol*. 2016;439:10-15. https://doi.org/10.1016/j.mce.2016.10.013
47. Moreno P, Acitores A, Gutiérrez-Rojas I, et al. Amylin effect in extrapancreatic tissues participating in glucose homeostasis, in normal, insulin-resistant and type 2 diabetic state. *Peptides*. 2011;32(10):2077-2085. https://doi.org/10.1016/j.peptides.2011.09.007
48. Asmar M, Arngrim N, Simonsen L, et al. The blunted effect of glucose-dependent insulinotropic polypeptide in subcutaneous abdominal adipose tissue in obese subjects is partly reversed by weight loss. *Nutr Diabetes*. 2016;6(5):e208-e208. https://doi.org/10.1038/nutd.2016.15
49. Parlevliet ET, Heijboer AC, Schröder-van der Elst JP, et al. Oxyntomodulin ameliorates glucose intolerance in mice fed a high-fat diet. *Am J Physiol Endocrinol Metab*. 2008;294(1):E142-E147. https://doi.org/10.1152/ajpendo.00576.2007
50. Martin AM, Yabut JM, Choo JM, et al. The gut microbiome regulates host glucose homeostasis via peripheral serotonin. *PNAS*. 2019;116(40):19802-19804. https://doi.org/10.1073/pnas.1909311116
51. Crane JD, Palanivel R, Mottillo EP, et al. Inhibiting peripheral serotonin synthesis reduces obesity and metabolic dysfunction by promoting brown adipose tissue thermogenesis. *Nat Med*. 2015;21(2):166-172. https://doi.org/10.1038/nm.3766
52. Plaza A, Merino B, Olmo ND, Ruiz-Gayo M. The cholecystokinin receptor agonist, CCK-8, induces adiponectin production in rat white adipose tissue. *Br J Pharmacol*. 2019;176(15):2678-2690. https://doi.org/10.1111/bph.14690
53. Wynne K, Park AJ, Small CJ, et al. Subcutaneous Oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, Controlled Trial. *Diabetes*. 2005;54(8):2390-2395. https://doi.org/10.2337/diabetes.54.8.2390
54. Moon H, Chamberland JP, Diakopoulos KN, et al. Leptin and amylin act in an additive manner to activate overlapping signaling pathways in peripheral tissues: in vitro and ex vivo studies in humans. *Diabetes Care*. 2011;34(1):132-138. https://doi.org/10.2337/dc10-0518
55. Asakawa A, Inui A, Yuzuriha H, et al. Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. *Gastroenterology*. 2003;124(5):1325-1336. https://doi.org/10.1016/S0016-5085(03)00216-6
56. Sitticharoon C, Nway NC, Chatree S, Churintaraphan M, Boonpuan P, Maikaew P. Interactions between adiponectin, visfatin, and omentin in subcutaneous and visceral adipose tissues and serum, and correlations with clinical and peripheral metabolic factors. *Peptides*. 2014;62:164-175. https://doi.org/10.1016/j.peptides.2014.10.006
57. Chen S, Okahara F, Osaki N, Shimotoyodome A. Increased GIP signaling induces adipose inflammation via a HIF-1α-dependent pathway and impairs insulin sensitivity in mice. *Am J Physiol Endocrinol Metab*. 2014;308(5):E414-E425. https://doi.org/10.1152/ajpendo.00418.2014
58. Varol C, Zvibel I, Spektor L, et al. Long-acting glucose-dependent insulinotropic polypeptide ameliorates obesity-induced adipose tissue inflammation. *J Immunol* Published Online. 2014;193(8):4002-4009. https://doi.org/10.4049/jimmunol.1401149
59. Ben-Shlomo S, Zvibel I, Varol C, et al. Role of glucose-dependent insulinotropic polypeptide in adipose tissue inflammation of dipeptidylpeptidase 4-deficient rats. *Obesity*. 2013;21(11):2331-2341. https://doi.org/10.1002/oby.20340
60. Lee Y-S, Park M-S, Choung J-S, et al. Glucagon-like peptide-1 inhibits adipose tissue macrophage infiltration and inflammation in an obese mouse model of diabetes. *Diabetologia*. 2012;55(9):2456-2468. https://doi.org/10.1007/s00125-012-2592-3
61. Rodríguez A, Gómez-Ambrosi J, Catalán V, et al. The ghrelin O-acyltransferase–ghrelin system reduces TNF-α-induced apoptosis and autophagy in human visceral adipocytes. *Diabetologia*. 2012;55(11):3038-3050. https://doi.org/10.1007/s00125-012-2671-5
62. Yang J, Ren J, Song J, et al. Glucagon-like peptide 1 regulates adipogenesis in 3T3-L1 preadipocytes. *Int J Mol Med*. 2013;31(6):1429-1435. https://doi.org/10.3892/ijmm.2013.1350
63. Vishvanath L, Gupta RK. Contribution of adipogenesis to healthy adipose tissue expansion in obesity. *J Clin Invest*. 2019;129(10):4022-4031. https://doi.org/10.1172/JCI129191
64. Cai X, She M, Xu M, et al. GLP-1 treatment protects endothelial cells from oxidative stress-induced autophagy and endothelial dysfunction. *Int J Biol Sci*. 2018;14(12):1696-1708. https://doi.org/10.7150/ijbs.27774
65. Ojima A, Matsui T, Maeda S, Takeuchi M, Yamagishi S. Glucose-dependent Insulinotropic polypeptide (GIP) inhibits signaling pathways of advanced glycation end products (AGEs) in endothelial cells via its antioxidative properties. *Horm Metab Res*. 2012;44(7):501-505. https://doi.org/10.1055/s-0032-1312595
66. Li J, Zheng J, Wang S, Lau HK, Fathi A, Wang Q. Cardiovascular benefits of native GLP-1 and its metabolites: an indicator for GLP-

1-therapy strategies. *Front Physiol*. 2017;8:15. https://doi.org/10.3389/fphys.2017.00015
67. Asmar M, Asmar A, Simonsen L, Dela F, Holst JJ, Bülow J. GIP-induced vasodilation in human adipose tissue involves capillary recruitment. *Endocr Connect*. 2019;8(6):806-813. https://doi.org/10.1530/EC-19-0144
68. Savage PJ, Harvey AP, Robinson E, Grieve DJ. Investigation of endothelial nitric oxide (NO) signalling in response to the incretin hormone glucose-dependent insulinotropic polypeptide (GIP). *BMC Proc*. 2012;6(4):O38. https://doi.org/10.1186/1753-6561-6-S4-O38
69. Yan H, Aziz E, Shillabeer G, et al. Nitric oxide promotes differentiation of rat white preadipocytes in culture. *J Lipid Res*. 2002;43(12):2123-2129. https://doi.org/10.1194/jlr.M200305-JLR200
70. Asmar A, Asmar M, Simonsen L, et al. Glucagon-like peptide-1 elicits vasodilation in adipose tissue and skeletal muscle in healthy men. *Physiol Rep*. 2017;5(3):e13073. https://doi.org/10.14814/phy2.13073
71. Wang J, He L, Huwatibieke B, et al. Ghrelin stimulates endothelial cells angiogenesis through extracellular regulated protein kinases (ERK) signaling pathway. *Int J Mol Sci*. 2018;19(9):2530. https://doi.org/10.3390/ijms19092530
72. Tahergorabi Z, Rashidi B, Khazaei M. Ghrelin does not modulate angiogenesis in matrigel plug in normal and diet-induced obese mice. *J Res Med Sci*. 2013;18(11):939-942.
73. Armani A, Berry A, Cirulli F, Caprio M. Molecular mechanisms underlying metabolic syndrome: the expanding role of the adipocyte. *FASEB J*. 2017;31(10):4240-4255. https://doi.org/10.1096/fj.201601125RRR
74. Eickhoff H, Louro TM, Matafome PN, Vasconcelos F, Seiça RM, Castro e Sousa F. Amelioration of glycemic control by sleeve gastrectomy and gastric bypass in a lean animal model of type 2 diabetes: restoration of gut hormone profile. *Obes Surg*. 2015;25(1):7-18. https://doi.org/10.1007/s11695-014-1309-8
75. Zwirska-Korczala K, Konturek SJ, Sodowski M, et al. Basal and postprandial plasma levels of PYY, ghrelin, cholecystokinin, gastrin and insulin in women with moderate and morbid obesity and metabolic syndrome. *J Physiol Pharmacol*. 2007;58(Suppl 1):13-35.
76. Calanna S, Christensen M, Holst JJ, et al. Secretion of glucose-dependent Insulinotropic polypeptide in patients with type 2 diabetes: systematic review and meta-analysis of clinical studies. *Diabetes Care*. 2013;36(10):3346-3352. https://doi.org/10.2337/dc13-0465
77. Carlson JJ, Turpin AA, Wiebke G, Hunt SC, Adams TD. Pre- and post- prandial appetite hormone levels in normal weight and severely obese women. *Nutr Metab*. 2009;6(1):32. https://doi.org/10.1186/1743-7075-6-32
78. Carmo-Silva S, Cavadas C. Hypothalamic dysfunction in obesity and metabolic disorders. In: Letra L, Seica R, eds. *Obesity and Brain Function*. Advances in Neurobiology. 1st ed. Vol.19 Cham, Switzerland: Springer International Publishing; 2017:73-116 doi:10.1007/978-3-319-63260-5_4.
79. Manell H, Staaf J, Manukyan L, et al. Altered plasma levels of glucagon, GLP-1 and glicentin during OGTT in adolescents with obesity and type 2 diabetes. *J Clin Endocrinol Metab*. 2016;101(3):1181-1189. https://doi.org/10.1210/jc.2015-3885
80. Young RL, Lumsden AL, Martin AM, et al. Augmented capacity for peripheral serotonin release in human obesity. *Int J Obes (Lond)*. 2018;42(11):1880-1889. https://doi.org/10.1038/s41366-018-0047-8
81. Cazzo E, Pareja JC, Chaim EA, Coy CSR, Magro DO. Comparison of the levels of C-reactive protein, GLP-1 and GLP-2 among individuals with diabetes, morbid obesity and healthy controls: an exploratory study. *Arq Gastroenterol*. 2018;55(1):72-77. https://doi.org/10.1590/s0004-2803.201800000-14
82. Fändriks L. Roles of the gut in the metabolic syndrome: an overview. *J Intern Med*. 2017;281(4):319-336. https://doi.org/10.1111/joim.12584
83. Holst JJ, Madsbad S, Bojsen-Møller KN, et al. Mechanisms in bariatric surgery: gut hormones, diabetes resolution, and weight loss. *Surg Obes Relat Dis*. 2018;14(5):708-714. https://doi.org/10.1016/j.soard.2018.03.003
84. le Roux CW, Aylwin SJB, Batterham RL, et al. Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters. *Ann Surg*. 2006;243(1):108-114. https://doi.org/10.1097/01.sla.0000183349.16877.84
85. Dimitriadis GK, Randeva MS, Miras AD. Potential hormone mechanisms of bariatric surgery. *Curr Obes Rep*. 2017;6(3):253-265. https://doi.org/10.1007/s13679-017-0276-5
86. Demerdash HM, Sabry AA, Arida EA. Role of serotonin hormone in weight regain after sleeve gastrectomy. *Scand J Clin Lab Invest*. 2018;78(1–2):68-73. https://doi.org/10.1080/00365513.2017.1413714
87. McCarty TR, Jirapinyo P, Thompson CC. Effect of sleeve gastrectomy on ghrelin, GLP-1, PYY, and GIP gut hormones: a systematic review and meta-analysis. *Ann Surg* Published online. 2019;272(1):72-80. https://doi.org/10.1097/SLA.0000000000003614
88. Meek CL, Lewis HB, Reimann F, Gribble FM, Park AJ. The effect of bariatric surgery on gastrointestinal and pancreatic peptide hormones. *Peptides*. 2016;77:28-37. https://doi.org/10.1016/j.peptides.2015.08.013
89. Peterli R, Steinert RE, Woelnerhanssen B, et al. Metabolic and hormonal changes after laparoscopic Roux-en-Y gastric bypass and sleeve gastrectomy: a randomized, Prospective Trial. *Obes Surg*. 2012;22(5):740-748. https://doi.org/10.1007/s11695-012-0622-3
90. Huang R, Ding X, Fu H, Cai Q. Potential mechanisms of sleeve gastrectomy for reducing weight and improving metabolism in patients with obesity. *Surg Obes Relat Dis*. 2019;15(10):1861-1871. https://doi.org/10.1016/j.soard.2019.06.022
91. Rhee NA, Wahlgren CD, Pedersen J, et al. Effect of Roux-en-Y gastric bypass on the distribution and hormone expression of small-intestinal enteroendocrine cells in obese patients with type 2 diabetes. *Diabetologia*. 2015;58(10):2254-2258. https://doi.org/10.1007/s00125-015-3696-3
92. Hansen CF, Bueter M, Theis N, et al. Hypertrophy dependent doubling of L-cells in Roux-en-Y gastric bypass operated rats. *PLoS ONE*. 2013;8(6):e65696. https://doi.org/10.1371/journal.pone.0065696
93. Cavin J, Couvelard A, Lebtahi R, et al. Differences in alimentary glucose 1 absorption and intestinal disposal of blood glucose following Roux-en-Y gastric bypass vs sleeve Gastrectomy. *Gastroenterology*. 2015;150(2):454-464. https://doi.org/10.1053/j.gastro.2015.10.009
94. Rollins KA, Opitz L, Arnold M, Simon E, Neubauer H, Wolfrum S. The L cell transcriptome is unaffected by vertical sleeve gastrectomy but highly dependent upon position within the gastrointestinal tract. *Peptides*. 2019;113:22-34. https://doi.org/10.1016/j.peptides.2019.01.001
95. Rowland KJ, Brubaker PL. Life in the crypt: a role for glucagon-like peptide-2? *Mol Cell Endocrinol*. 2008;288(1–2):63-70. https://doi.org/10.1016/j.mce.2008.02.014
96. Chambers AP, Smith EP, Begg DP, et al. Regulation of gastric emptying rate and its role in nutrient-induced GLP-1 secretion in rats after vertical sleeve gastrectomy. *Am J Physiol Endocrinol Metab*. 2013;306(4):E424-E432. https://doi.org/10.1152/ajpendo.00469.2013
97. Bahne E, Sun EWL, Young RL, et al. Metformin-induced glucagon-like peptide-1 secretion contributes to the actions of metformin in type 2 diabetes. *JCI Insight*. 2018;3(23):e93936. https://doi.org/10.1172/jci.insight.93936
98. Sharma D, Verma S, Vaidya S, Kalia K, Tiwari V. Recent updates on GLP-1 agonists: current advancements & challenges. *Biomed*

Pharmacother. 2018;108:952-962. https://doi.org/10.1016/j.biopha.2018.08.088
99. Heimbürger SM, Brønden A, Johansen NJ, Dejgaard TF, Vilsbøll T, Knop FK. The efficacy and safety of exenatide once weekly in patients with type 2 diabetes. *Expert Opin Pharmacother*. 2019;20(5):501-510. https://doi.org/10.1080/14656566.2019.1571040
100. Alexiadou K, Anyiam O, Tan T. Cracking the combination: gut hormones for the treatment of obesity and diabetes. *J Neuroendocrinol*. 2019;31(5):e12664. https://doi.org/10.1111/jne.12664
101. Finan B, Yang B, Ottaway N, et al. A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents. *Nat Med*. 2015;21(1):27-36. https://doi.org/10.1038/nm.3761
102. Tan T, Behary P, Tharakan G, et al. The effect of a subcutaneous infusion of GLP-1, OXM, and PYY on energy intake and expenditure in obese volunteers. *J Clin Endocrinol Metab*. 2017;102(7):2364-2372. https://doi.org/10.1210/jc.2017-00469
103. Schneck A, Iannelli A, Patouraux S, et al. Effects of sleeve gastrectomy in high fat diet-induced obese mice: respective role of reduced caloric intake, white adipose tissue inflammation and changes in adipose tissue and ectopic fat depots. *Surg Endosc*. 2014;28(2):592-602. https://doi.org/10.1007/s00464-013-3211-1
104. Jahansouz C, Xu H, Hertzel AV, et al. Partitioning of adipose lipid metabolism by altered expression and function of PPAR isoforms after bariatric surgery. *Int J Obes (Lond)*. 2018;42(2):139-146. https://doi.org/10.1038/ijo.2017.197
105. Hoffstedt J, Andersson DP, Hogling DE, et al. Long-Term Protective Changes in Adipose Tissue after Gastric Bypass. *Diabetes Care* Published Online. 2016;40(1):77-84. https://doi.org/10.2337/dc16-1072
106. Figueroa-Vega N, Jordán B, Pérez-Luque EL, Parra-Laporte L, Garnelo S, Malacara JM. Effects of sleeve gastrectomy and rs9930506 FTO variants on angiopoietin/Tie-2 system in fat expansion and M1 macrophages recruitment in morbidly obese subjects. *Endocrine*. 2016;54(3):700-713. https://doi.org/10.1007/s12020-016-1070-y
107. Faraj M, Havel PJ, Phélis S, Blank D, Sniderman AD, Cianflone K. Plasma acylation-stimulating protein, adiponectin, leptin, and ghrelin before and after weight loss induced by gastric bypass surgery in morbidly obese subjects. *J Clin Endocrinol Metab*. 2003;88(4):1594-1602. https://doi.org/10.1210/jc.2002-021309
108. Albers PH, Bojsen-Møller KN, Dirksen C, et al. Enhanced insulin signaling in human skeletal muscle and adipose tissue following gastric bypass surgery. *Am J Physiol Regul Integr Comp Physiol*. 2015;309(5):R510-R524. https://doi.org/10.1152/ajpregu.00228.2014
109. Salehi M, Prigeon RL, D'Alessio DA. Gastric bypass surgery enhances glucagon-like peptide 1–stimulated postprandial insulin secretion in humans. *Diabetes*. 2011;60(9):2308-2314. https://doi.org/10.2337/db11-0203
110. Svane MS, Bojsen-Møller KN, Nielsen S, et al. Effects of endogenous GLP-1 and GIP on glucose tolerance after Roux-en-Y gastric bypass surgery. *Am J Physiol Endocrinol Metab*. 2016;310(7):E505-E514. https://doi.org/10.1152/ajpendo.00471.2015
111. Wilson-Pérez HE, Chambers AP, Ryan KK, et al. Vertical sleeve Gastrectomy is effective in two genetic mouse models of glucagon-like peptide 1 receptor deficiency. *Diabetes*. 2013;62(7):2380-2385. https://doi.org/10.2337/db12-1498
112. Boland B, Mumphrey MB, Hao Z, et al. The PYY/Y2R-deficient mouse responds normally to high-fat diet and gastric bypass surgery. *Nutrients*. 2019;11(3):585. https://doi.org/10.3390/nu11030585
113. Shao Y, Yuan G, Zhang J, Guo X. Liraglutide reduces lipogenetic signals in visceral adipose of db/db mice with AMPK activation and Akt suppression. *Drug Des Devel Ther*. 2015;2015(9):1177-1184. https://doi.org/10.2147/DDDT.S79175
114. Zhou J, Poudel A, Chandramani-Shivalingappa P, Xu B, Welchko R, Li L. Liraglutide induces beige fat development and promotes mitochondrial function in diet induced obesity mice partially through AMPK-SIRT-1-PGC1-α cell signaling pathway. *Endocrine*. 2019;64(2):271-283. https://doi.org/10.1007/s12020-018-1826-7
115. Pastel E, McCulloch LJ, Ward R, et al. GLP-1 analogue-induced weight loss does not improve obesity-induced AT dysfunction. *Clin Sci*. 2017;131(5):343-353. https://doi.org/10.1042/CS20160803
116. Feng W-H, Yuan X-W, Tong G-Y, et al. Correlated increase of omentin-1 and adiponectin by exenatide, avandamet and dietary change in diet-induced obese rats. *Folia Biol*. 2013;59(6):217-224.
117. Han F, Hou N, Liu Y, et al. Liraglutide improves vascular dysfunction by regulating a cAMP-independent PKA-AMPK pathway in perivascular adipose tissue in obese mice. *Biomed Pharmacother*. 2019;120:109537. https://doi.org/10.1016/j.biopha.2019.109537
118. Koska J, Sands M, Burciu C, et al. Exenatide protects against glucose- and lipid-induced endothelial dysfunction: evidence for direct vasodilation effect of GLP-1 receptor agonists in humans. *Diabetes*. 2015;64(7):2624-2635. https://doi.org/10.2337/db14-0976
119. Xian Y, Chen Z, Deng H, et al. Exenatide mitigates inflammation and hypoxia along with improved angiogenesis in obese fat tissue. *J Endocrinol*. 2019;242(2):79-89. https://doi.org/10.1530/JOE-18-0639
120. Iacobellis G, Villasante Fricke AC. Effects of semaglutide versus dulaglutide on epicardial fat thickness in subjects with type 2 diabetes and obesity. *J Endocr Soc*. 2020;4(4):bvz042. https://doi.org/10.1210/jendso/bvz042
121. McCrimmon RJ, Catarig A-M, Frias JP, et al. Effects of once-weekly semaglutide vs once-daily canagliflozin on body composition in type 2 diabetes: a substudy of the SUSTAIN 8 randomised controlled clinical trial. *Diabetologia*. 2020;63(3):473-485. https://doi.org/10.1007/s00125-019-05065-8

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