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XXXIII CICLO DEL DOTTORATO DI RICERCA IN BIOMEDICINA MOLECOLARE

MODULATION OF ADIPOSE TISSUE METABOLISM BY UNACYLATED GHRELIN AND ITS RELEVANCE IN OBESITY

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ABSTRACT

Adipose tissue is a multi-functional organ with metabolic implications far beyond its role in energy storage, therefore its relevance in obesity and consequent metabolic dysregulation has been largely investigated. Ghrelin is a gastric hormone whose circulating levels are predominantly in the unacylated form (UnAG). While the acylated form was first characterized for its important effects on energy balance, UnAG has recently shown to be independently involved in several metabolic functions, including a role as positive modulator of oxidative stress, inflammation and insulin sensitivity in skeletal muscle. However, to date, very little is known about UnAG effects in adipose tissue, another important metabolic organ.

The current study proposes to investigate UnAG implications in rodents adipose tissue metabolism by testing 1) 4-day of UnAG sustained exogenous administration on healthy rats and 2) transgenic systemic overexpression of UnAG in mice treated with standard or high-fat diet (HFD). Complexively, our results showed that in healthy rodents UnAG does not modify mitochondrial activity, antioxidant systems and inflammatory state but it decreases insulin sensitivity in both models of UnAG administration or overexpression. Interestingly though, in obese mice UnAG systemic overexpression, the hormone was found to prevent alterations of mitochondrial function and dynamics, redox state and inflammatory response. Consistently, UnAG also improved insulin sensitivity of obese mice, and finally prevented HFD-related adipocytes enlargement with lower actin remodeling.

In support of these *in vivo* results, clinical analyses in humans showed that UnAG circulating levels decrease in elderly overweight-obese individuals compared to lean and are predictive of 5-year mucle mass independently from BMI. Consistently, in a general population cohort fat-to-mass ratio was negatively correlated to UnAG levels, independently from BMI and other metabolic variables.

Globally, our findings in animal and human studies suggest UnAG as a novel modulator of adipose tissue metabolism with important implications in obesity pathogenesis, suggesting UnAG as a potential candidate for further studies aiming to contrast obesity and obesity-associated metabolic complications.

SYNOPSIS

Obesity is a medical condition alarmingly spreading worldwide. It is characterized by positive energy balance and fat accumulation, with consequent dangerous physiological alterations and increased health risk. Adipose tissue is the organ specialized in storing excess of energy assumed from food intake in form of lipid drops in adipocytes, and it plays a pivotal role in obesity-derived metabolic dysregulation. Adverse consequences of obesity affect adipose tissue-specific but also whole body metabolic alterations, including development of insulin resistance and type 2 diabetes (T2D), activation of inflammatory cascade, reduction of lipid turnover and increase of ectopic fat accumulation in several organs, particularly in the liver and skeletal muscle.

In many ways, mitochondria are also involved in the obesity-induced pathogenesis of adipose tissue. Core of energy production and thus of metabolic processes, mitochondrial unbalance in energy generation and expenditure that occurs in obesity, soon impair mitochondrial functionality. Dysfunctional mitochondria are characterized by exceeding reactive oxygen species production and oxidative stress and consequent increase of superoxide dismutase levels, which together represent one of the risk factors for insulin sensitivity impairment. Moreover, mitochondrial dynamics are crucial for adipocytes maintenance, determined by continuous events of fusion and fission, orchestrated by GTPases activity. In addition, as an endocrine organ, WAT secretes various humoral factors which, in altered metabolic condition such as obesity, may shift from anti-inflammatory to pro-inflammatory cytokines, thus contributing to systemic inflammation.

A disruption of mitochondrial functionality and a pro-inflammatory profile contribute to impair cells insulin sensitivity. Insulin is essential for glucose disposal in the skeletal muscle, but it plays an opposite role in white adipose tissue (WAT) where it suppresses lipolysis and is required for lipogenesis and triglycerides storage. Insulin resistance in adipocytes translates in unrestricted lipolysis and increased plasma level of non-esterified fatty acids (NEFAs). These components eventually modulate WAT metabolism which, in obesity, is reflected in increased lipid storage with consequent increased cell size and tissue expansion guided by adipocytes remodeling.

Ghrelin is a gastrointestinal hormone involved in several metabolic processes, first discovered for its orexigenic effect. Ghrelin circulates in two forms, acylated (AG) which accounts for ~10% of total circulating ghrelin, and predominant circulating unacylated (UnAG) form. Although at first AG was the most considered form because of its role in appetite stimulation and growth hormone (GH) release through its hormone segretagogue-receptor (GHS-R), lately studies gave the evidence

that UnAG was not bioinactive and its metabolic potential started emerging. UnAG does not show any affinity for GHS-R, however it shows independent metabolic activities at different levels. It was reported that UnAG is an independent regulator of glucose and lipid metabolism, as well as it seems involved in antioxidant mechanisms. Additional data from our group supported the role of UnAG in the stimulation of muscle anabolism by increasing insulin signaling and lowering oxidative stress and inflammation. Moreover, as a study conducted in our lab already showed, UnAG correlates and predicts insulin sensitivity in human.

However, UnAG implications in adipose tissue and consequent effects on obesity-related altered metabolism have not been completely understood yet.

The current study proposes to investigate UnAG metabolic effects on white adipose tissue (WAT) first in a Wistar rat model of 4-day UnAG subcutaneous administration, and then in a constitutive UnAG overexpressing transgenic mice model. In this latter model, UnAG effects were also evaluated in obesity-induced treatment with high-fat diet (HFD). Neither UnAG sustained administration or UnAG overexpression modified caloric intake, body weight or NEFA plasma levels, while HFD, as expected, increased caloric intake and body weight, inducing systemic and muscle insulin resistance.

Mitochondrial functionality was first tested through biochemical assays in order to evaluate Citrate Synthase and Cytochrome C Oxidase activity, two important enzymes respectively implicated in citric acid cycle and electron transport chain, essential reactions for cell energy production from macronutrients. In physiological condition, 4-days UnAG administration as well as constitutive UnAG overexpression did not seem to cause any alteration of rodents white adipose tissue as no differences were found in these animals compared to the control group. However, CS action strongly decreased in obese mice compared to wild-type, but UnAG overexpression showed a recover of CS activity.

To further elucidate UnAG on mitochondria, fusion and fission regulators were tested in order to assess mitochondrial dynamics, important to preserve mitochondrial functionality in stress condition; fusion allows to mix mitochondrial content, thus attenuating oxidative stress products, while fission creates new mitochondria and can indirectly induce cellular apoptosis in cases of exceeding stress. Mitofusin1 (Mfn1) and OPA1 are the main regulator of mitochondrial fusion, while Drp1 is involved in mitochondrial fission. Drp1 was increased in HFD mice compared to control, however UnAG overexpression seemed to reduce Drp1 levels toward normal status in HFD mice. At the same time, HFD decreases OPA1 and Mfn1 in comparison to standard diet, but

UnAG increases Mfn1 protein levels with a similar trend for OPA1, therefore UnAG constitutive overexpression reduces fission and increases fusion in mitochondria, thus reversing fat diet effects already described in literature. These data are consistent with the finding that decreased mitochondrial fusion in adipocytes associates with increased fat deposition in WAT, since mitochondrial fusion promotes glucose utilization and lipogenesis.

Disrupted mitochondrial homeostasis, soon translates in unbalanced redox state. Oxidative stress within the adipose tissue was tested by measure of total and oxidized glutathione, and results on 4-day UnAG administration showed a decreased GSSG/tot(GSSG+GSH) ratio, thus suggesting decreased adipocytes oxidative stress. However, the same treatment did not affect the antioxidant defense system in terms of dismutase levels. In support of these findings, transgenic mice with constitutive expression of UnAG with standard diet showed unchanged levels of antioxidant enzymes superoxide dismutase as well. Importantly though, UnAG systemic overexpression reversed altered superoxide dismutase levels of HFD-fed mice.

Consistently, neither UnAG sustained administration nor UnAG constitutive overexpression in healthy rodents modified TNF-α levels, a major marker of inflammation status. Interestingly, UnAG prevented the increase of TNF-α levels in obese mice undergoing HFD.

Consistent with the evidence that mitochondrial function and oxidative stress together with inflammation status affect insulin sensitivity, the latter was first assessed in rodents adipose tissue by protein blotting of AKT and GSK3β levels, two main actors of insulin signaling cascade. These markers were unanimously decreased in UnAG administered rats compared to wild-type; xMap high throughput analysis of insulin signaling pathway confirmed western blot results, showing that 4-days UnAG treatment induces a general negative modulation of insulin signaling in WAT, including mTOR and downstream mediators. Constitutive UnAG overexpression in transgenic mice showed a similar trend of AKT and GSK-3β decreased levels, suggesting that long-term UnAG effect may be potentially compensated by homeostatic processes. These results are in contrast with previous data published from our group of UnAG effect on insulin sensitivity on skeletal muscle, suggesting that UnAG potentially induces glucose disposal in myocytes while inhibiting fat storage in adipocytes.

Importantly, in HFD-treated mice UnAG overexpression was associated with improved insulin action compared to HFD-fed wild-type, despite no impact on caloric intake or body weight, which was similar to wild-type obese animals. Epididymal fat amount as well, resulted comparable between wild-type and UnAG overexpressing transgenic mice, however intriguingly UnAG

decreased fat-to-muscle mass ratio, suggesting a potential role in the modulation of body composition.

In addition, histological analyses supported the hypothesis that HFD induces an enlargement of adipocytes size, consistent with increased lipid accumulation in these cells. Transgenic obese mice importantly showed contained fat cells size compared to obese wild-type mice. These findings were supported by the analysis of the main factors involved in adipocytes remodeling, including Arp2, Cofilin1 and Profilin2, which showed that UnAG contrasts adipocytes size remodeling caused by lipid storage induced-HFD.

Taken together, these results suggest that UnAG effects in adipose tissue sustain inhibition of lipogenesis and fat accumulation induced by HFD, preventing dysfunctional metabolic processes of the WAT caused by obesity including mitochondrial functionality and dynamics, oxidative stress, inflammation and adipocytes expansion, and possibly addressing energy flux toward fuel disposing organs, including skeletal muscle.

Finally, in the current study UnAG was also considered in association to body composition in terms of muscle and fat mass in humans. A group of 2500 individuals from two municipalities of the North-East of Italy were randomly recruited to take part in the MoMa epidemiological study, aimed to investigate correlation between metabolic syndrome and related factors. Among these, a cohort of 450 elderly individuals (Age>65y; M/F: 179/271) was examined and divided in two groups: lean, and overweight and obese. As previously described in literature, our results confirmed that UnAG and AG levels are reduced in overweight and obese subjects compared to lean ones. Association studies then supported that, although both UnAG and AG negatively correlate to BMI in elderly subjects, the association resulted much stronger for UnAG compared to AG. Interestingly, association analysis were repeated on a subgroup of this cohort which participated to a 5-year follow-up, showing that UnAG but not AG predicted 5-year muscle mass even after adjustment for confounding variables, suggesting a role of UnAG as a predictor of 5-year muscle mass in a cohort of elderly people of the North-East of Italy.

Further analysis showed that UnAG but not AG correlates with decreased fat/muscle mass ratio, independently on BMI and other confounding variables. These findings suggest that, in agreement with the effects seen in the adipose tissue in rodents, UnAG may play a direct role on its metabolism with potential clinical relevance in obesity.

In conclusion, the current results indicate UnAG as a strong modulator of adipose tissue metabolism. In particular, we have shown that UnAG impacts on multiple adipocyte mechanisms,

including insulin signalling, mitochondrial function and dynamics and remodeling, ultimately leading to morphological tissue changes. These findings are synergistic to recent discovered effects of the hormone on skeletal muscle, and further indicate UnAG as an important player in obesity related metabolic complications.

Contents

1. INTRODUCTION

1.1 ADIPOSE TISSUE

1.1.1 Definition

Accumulation of excessive energy has always been a necessity for all the animal species: adipose tissue (AT) represents the organ that overcomes this request. Its origin is mainly mesodermal, although its distribution is various and heterogeneous depending on the species [1].

Excessive calories, in form of triglycerides, flow through the blood to finally be stored in lipid droplets in specialized cells known as adipocytes [2], which are the functional units of the adipose tissue. However, adipose tissue hosts many other cells, including preadipocytes, macrophages, lymphocytes, neutrophils and endothelial cells, and its composition varies depending on fat location and characteristics [1].

The idea of adipose tissue as a passive organ only designated to store lipids has been long passed, and its relevance as a major regulator of nutritional homeostasis and energy balance has become clear. Moreover, it also exerts a mechanical protective role by surrounding many organs [3].

Particularly in mammals, adipose tissue differentiates in white adipose tissue (WAT), which is considered the main site of energy storage in form of triglycerides, and brown adipose tissue (BAT), which utilizes lipid oxidation to produce heat and to regulate thermogenesis against hypothermia [4].

The WAT is variously distributed in the human body, however it is mainly categorized in subcutaneous adipose tissue and visceral adipose tissue, despite there is a minor presence of fat accumulation also in other areas including pericardial and perivascular, periarticular, intramuscular, retro-orbital and bone marrow. Interestingly, the different WAT depots associate with different metabolic aspects [4].

Although all the metabolic activities and interactions of the adipose tissue make it an essential component of the articulate machine that the organism represents, it is common to think at adipose tissue as a negative compound, mainly because of its associations with metabolic disorders and, particularly, obesity. In fact, increase in body weight is often related to increased risk of cardiovascular complications and obesity, even though this risk relates more to how fat is distributed in the body rather than to its total amount, since it is well known that abdominal obesity presents a major health risk compared to peripheral and gluteofemoral obesity [5]. Fat

distribution depends on many factors, including sex, genetic predisposition, age, health state and lifestyle [3].

1.1.2 Subtypes: visceral versus subcutaneous adipose tissue

The main classification of WAT distinguishes visceral (VAT) from subcutaneous adipose tissue (SAT), where the former is known to be involved in metabolic disorders while the latter has shown interesting positive associations in the metabolic homeostasis [5].

The two VAT and SAT can be compared in terms of cell composition: first, it is important to notice that also adipocytes conformation changes as much as they absorb free fatty acids (FFAs) and triglycerides (TGs), thus getting bigger. Larger adipocyes stuffed with lipid droplets tend to become dysfunctional and resistant to the insulin-effect. Small and insulin-sensitive adipocytes are usually typical of SAT, while VAT content is mostly composed of large adipocytes [6]. Although adipocytes remain the main cellular component of the adipose tissue, also preadipocytes, inflammatory cells, immune cells, fibroblasts take part to the complex system of this organ, which is implicated in interactions with the surrounding connective, vascular and neural tissues [4]. All of these components may contribute to differentiate distinct fat compounds. Particularly, the inflammatory system constitutes a discriminatory factor in the different regulation and functionality of VAT and SAT: in fact, VAT shows a prevalence of the inflammatory cells and in particular macrophages compared to SAT, thus contributing to the impaired tissue functionality typical of visceral-fat accumulation [7]. Adipocytes are able to synthesize pro-inflammatory and anti-inflammatory proteins, hence asserting their active role in modulating metabolic alterations [8].

Visceral and subcutaneous adipose tissue are in turn sub-classified based on the depots location, and even different districts of the same class of adipose tissue may present different metabolisms [9], thus giving an idea of the complexity and faint contribution of this organ which is, to date, still not well determined.

Inside the abdomen, visceral adipose tissue is recognizable in different deposits, particularly classified as omental, mesenteric and retroperitoneal [4]. However, it is important to notice that there is not a tight correlation between human and rodent depots, for example the omentum contains the main visceral fat in humans while in rodents it is barely present [10], even though an evolutionary conservation of regional fat remains clear.

However, determining how the different depots vary in terms of adipocytes behavior, secretion of metabolites and reception and elaboration of external signals remain incompletely understood. Macotela et al. in 2012 studied how pre-adipocytes with different origin showing depots

correlated-specific gene expression profile, kept different behaviors even after prolonged isolation and under the same environmental stimuli, highlighting the specificity that adipocytes can acquire [11].

1.1.3 Cellular heterogeneity of adipose tissue

Adipocytes are the main cells representing the adipose tissue, but it would be reductive to classify adipocytes as a unique cell type. Variations in adipocytes characteristics are expressed in comparison between WAT and BAT, but also within the same fat compartment. WAT adipocytes present a single vacuole for fat accumulation, while many vacuoles and highly mitochondria number characterize brown adipocytes [12].

As already mentioned, adipose tissue has a cell heterogeneity characterized by the abundance of adipocytes that share adipose spot with other cell types. The percentage of adipocytes into a specific fat depot is variable, thus contributing to the characterization of different adipose tissue compartments [13]. The remaining space is employed by stromatic cells: stem cells, preadipocytes, macrophages, but also neutrophils and lymphocytes, in a proportion that depends on fat location and on its inflammatory state [14].

1.1.3.1 Adipocytes

The characteristic cell of adipose tissue, the adipocyte, results from a process of maturation of its precursor, the preadipocyte, after a process of proliferation and differentiation that is differently regulated depending on adipose location [15].

In a review of 2014, Ràfols describes the transition from preadipocytes to mature adipocytes as a four-step process, where the transcription factors PPARγ and C/EBP family members are key player [4]. First, cells proliferate until they reach confluence, thus implicating contact inhibition and *proliferation arrest*. Secondly, hormonal signals induce a *clonal expansion* followed by a step of *early differentiation* in which adipocytes start expressing their distinctive genes and asserting the characteristic role of energy storage by collecting lipids. Particularly in this initial step of differentiation, C/EBPβ factor results crucial. Eventually, in a last stage of *final differentiation*, PPARγ and C/EBPα steer the activation of transcription genes cascade that allow to complete adipocyte maturation (Figure 1) [4].

Preadipocytes maturate in a variable time that depends on tissue location and on its receiving stimuli, particularly by pro-inflammatory cytokines. This allows to distinguish preadipocytes with fast differentiation from preadipocytes with slow differentiation. Visceral adipose tissue tends to

be mainly constituted by the subtype with slow proliferation, while subcutaneous adipose tissue shows more equilibrate distribution of the two subtypes [15], supporting the idea of adipose tissue as a very complex and plastic organ.

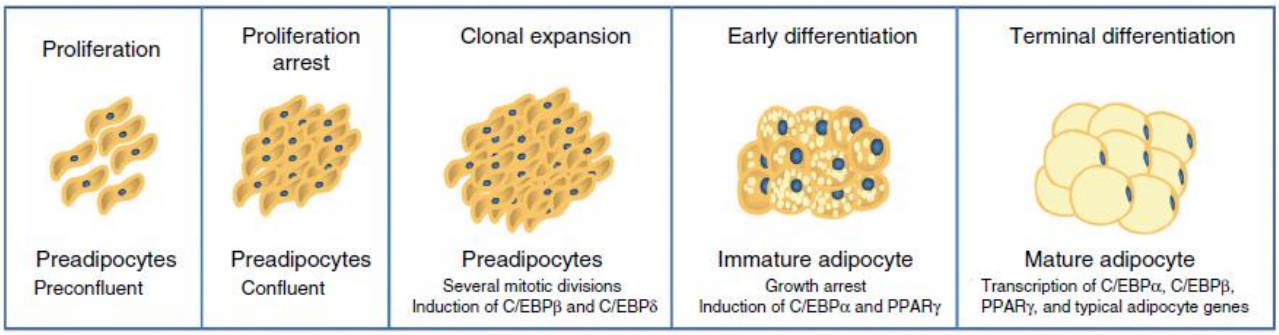


Figure 1. *Steps determining the transition from preadipocyte to mature adipocyte* [4].

1.1.3.2 Macrophages, neutrophils and lymphocytes

The stromatic cells composing and enriching the adipose tissue are a fundamental factor that influences metabolism and functionality of this endocrine organ.

Interestingly, it has been verified that macrophages amount increases when increasing WAT size. Thus, obese subjects show a major presence of macrophages compared to lean subjects [16]. Macrophages relevance relates with their association to inflammatory state typical of obesity, mainly because of their ability to secrete pro-inflammatory cytokines [7].

However, it is important to better characterize macrophages origin and potential functions. It is well known that monocytes are precursors of macrophages [17], circulating cells revoked from the blood stream in response to signals like chemoattractant protein-1 (MCP-1) produced by adipocytes [18]. Other cytokines such as colony stimulating factor-1 (CSF-1) are secreted by adipocytes to promote monocytes transition to macrophages [16]. At the same time, many studies reported macrophages to be originated from preadipocytes of WAT, in fact the two cell types share some features, particularly the cytokines produced and the phagocytic capacity [18,19].

Lymphocytes contribute to the heterogeneity of adipose tissue as well, in a variable amount that depends on the anatomical fat location and on its metabolic state. Obesity correlates with a chronic inflammatory condition characterized by an increase of the pro-inflammatory component and withdrawal of lymphocytes and macrophages infiltration. The crosstalk between the immune system and adipose cells occurs because of cytokines and adipokines that constitute the language of these cells-interactions [20]. Macrophages are commonly activated by T helper lymphocytes through the IFNγ factor, leading toward a pro-inflammatory response; these are the so-called type

1 macrophages (M1). However, different signals released by adipocytes including IL-4 and IL-13, address macrophages toward an anti-inflammatory profile (M2) [21]. Studies confirmed that obese animals present increased portion of M1 macrophages, while lean animals WAT is enriched in M2 cells. This condition also correlates with fat insulin-sensitivity, which results decreased in WAT of obese individuals with M1 infiltrated cells [22,23].

However, in early stages of WAT development, neutrophils are the first immune cells infiltrating the adipose tissue as a response to initial inflammation, thus inducing a tendency of immune cell types and particularly macrophages into anti-inflammatory feedback. As long as the inflammation persists, the immune system in the adipose tissue shifts supporting the pro-inflammatory response [24].

1.1.4 Adipose tissue metabolism

1.1.4.1 Core of adipose tissue metabolism: mitochondria

Mitochondria represent the energetic and thus the metabolic center of the cells. The importance of this cellular organelle is related to the many processes in which it takes part: primary the energetic balance, but also autophagy, apoptosis and inflammation [25].

Energy production, carbohydrates and lipid metabolism mostly take place in the mitochondrion [26]. On the other hand, adipose tissue is surely one of the major organ responsible for energy homeostasis [27]. Lipogenesis, lipolysis and β-oxidation are central in regulating adipocytes metabolism [28], therefore the tight correlation that connects adipose tissue to mitochondrial activity appears linear [29].

Mitochondria in adipocytes are not only involved in their usual and pivotal role of energy producer, but they are also implicated in adipocyte-specific functions, like lipid metabolism, adipogenesis and thermogenesis in the brown adipose tissue [30].

Mitochondria amount in white adipocytes is variable, from low number in WAT to high number in BAT, while it is various in the beige adipose tissue, and the morphology can change as well, although adipocytes mitochondrion tend to an elongated form [31].

White adipose tissue mitochondria are important not only because of their lipogenic function, but they also carry out a protective role in xenobiotics degradation[31].

1.1.4.1.1 Functional mitochondria in adipose tissue

The physiological role that mitochondria play in adipocytes results in many ways, schematized in Figure 2. Mitochondria take part in adipocytes differentiation, because the PPARγ coactivator 1α

(PGC1α), a key factor in differentiation phase, is directly involved in mitochondrial biogenesis [27]. Moreover, the activity of mitochondria during differentiation has been proved by changes in oxygen consumption from preadipocytes to mature adipocytes [32].

In the adipose tissue, center of lipid metabolism, mitochondria are crucial in lipid homeostasis. Mitochondria in adipocytes are localized close to lipid droplets, because of the interaction between the two organelles, and to facilitate lipid transfer and metabolism [33].

Some studies also linked mitochondrial activity to glucose homeostasis and insulin sensitivity in adipocytes: dysfunctional mitochondria entail a reduction of glucose uptake and thus decreased insulin sensitivity [34]. Moreover, improvement of mitochondrial activity induced by PGC1α, also rescued insulin resistance provoked by high level of glucose and fatty acids [35].

Finally, mitochondria are also involved in WAT browning and thermogenesis, the latter representing the main role of brown adipocytes. Uncoupling protein 1 (UCP1), a protein of mitochondrial inner membrane, plays a role in contrasting ATP production thus supporting heat production for energy dissipation [36]. White adipocytes can be converted to brown adipocytes as a result of β-adrenergic stimuli that promote UCP1 expression in the mitochondria [12].

1.1.4.1.2 Dysfunctional mitochondria in adipose tissue

The consequences of dysfunctional mitochondria in adipocytes are severe and affect the homeostasis at whole-body level. Metabolic disorders, in particular obesity and insulin resistance have been related to impaired mitochondrial activity [37]. There are several reasons that may cause this dysfunctionality: reactive oxygen species (ROS) are a product of mitochondrial respiration and constitute a signal in many processes. Its effects depend mainly on ROS concentrations: at low level, ROS mediates insulin signal transduction [38], but it becomes toxic in exceeding levels, entailing a stress condition that promotes inflammatory profile activation [39]. Several studies also indicated how ROS production in high levels leads to impaired glucose uptake and thus insulin resistance [34,40,41]. Besides, excess of ROS in adipocyte hinders its proliferation and differentiation [42]. To guarantee mitochondrial proper functioning, some processes need to be preserved: fusion and fission allow mitochondrial maintenance and ROS production dilution [43]. Biogenesis and mitochondrial degradation, also known as mitophagy, are pivotal as well in the homeostasis of mitochondrial dynamics [44]. Defective or damaged mitochondria are eliminated through Unc-51 like autophagy activating kinase 1 (ULK1) or inhibition of mTORC1, through PTEN-induced kinase 1 (PINK1) – Parkin pathway or alternatively through the ubiquitin-line light chain 3 (LC3) protein activation [44,45,46]. Mitochondrial turnover is critical for a correct homeostasis maintenance of these

organelles. Fat accumulation and insulin resistance might have a negative impact in regulating these mechanisms, validating the strong and mutual support of these components in a healthy balanced condition.

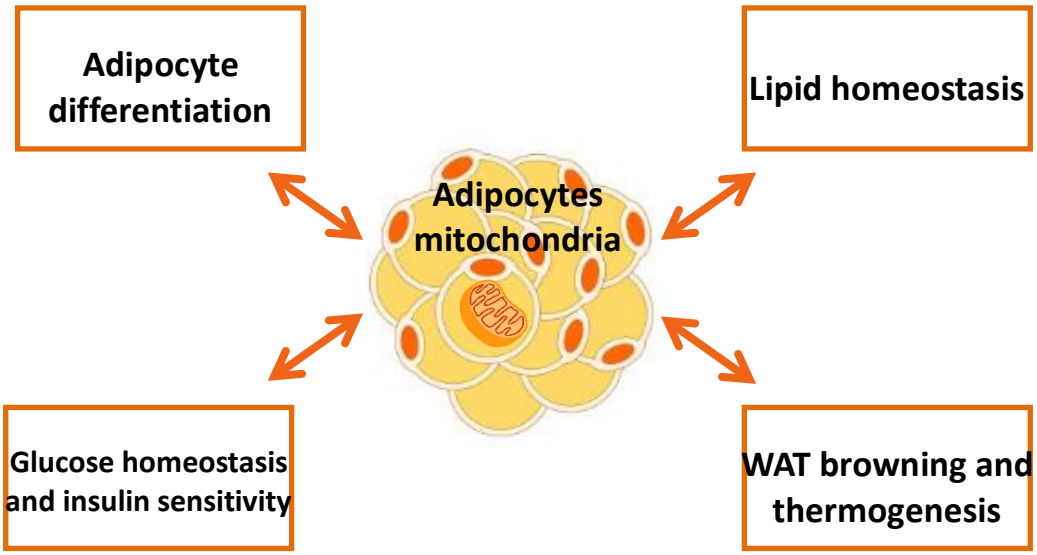


Figure 2. *Main roles of mitochondria in adipocytes*.

1.1.4.2 Insulin signaling in adipose tissue

Insulin effect is pivotal on carbohydrates and lipids metabolism, regulating the transition of glucose into muscle and fat tissues by promoting GLUT4 translocation [47]. Insulin stimulates fatty acids synthesis particularly in adipose tissue and liver. Insulin signaling affects whole body homeostasis, however it particularly targets the three most metabolically active organs: skeletal muscle, liver and, of course, adipose tissue. Insulin exerts its effect by binding its specific insulin receptor (IR), thus activating phosphotyrosine-binding scaffold proteins and with consequent modulation of different downstream effects [48]. Briefly, outcomes of insulin cascade can be summarized in metabolic and mitogenic signals. Mitogenic pathway expects activation of mitogenic-activated protein kinases [49–52]. Although the signaling cascade is conserved in the different cell types, the physiological response remains more tissue-specific [53].

Skeletal muscle is pivotal in energy consumption, therefore insulin is strongly required to allow glucose uptake and glycogen synthesis, in order to store energy in myocytes for later utilization. Studies on human population in healthy and type 2 diabetic conditions showed that insulin-induced glucose disposal accounts mostly (~75%) for glycogen synthesis [54,55], even though glucose oxidation maintains an independent central role [56–58], thus framing muscle insulin activity responsible of glucose utilization and storage.

However, insulin release from pancreatic cells primarily reaches the liver through the portal vein, therefore hepatic cells are exposed to higher insulin concentrations. Here, insulin mediates synthesis of the three macromolecules: carbohydrates by promoting glycogen synthesis, lipids by stimulating *de novo* lipogenesis and protein anabolism. Moreover, insulin strongly decreases glucose production [59].

Finally, insulin is also essential in WAT physiology, where it mainly acts in suppressing lipolysis [60,61]. Importantly, insulin-mediated triglycerides anabolism in adipocytes also implicates the decrease of nonesterified fatty acids plasma levels [62,63].

Commonly, insulin exerts its action by binding the insulin receptor (INSR) expressed by insulin-sensitive cells. This bond between insulin and its receptor activates the insulin receptor substrate 1 (IRS-1) that promotes the signaling cascades: the phosphatidylinositol 3-kinase – protein kinase B (PI3K-AKT) pathway which provides most of insulin effects, and the Ras-mitogen-activated protein kinase (MAPK) (Figure 3). PI3K phosphorylates phosphatidylinoitol diphosphate (PIP_2) thus convering it in phosphatidylinositol triphosphate (PIP_3), which activates phosphoinositide-dependent protein kinase 1 (PDK1). PDK1 in turn, on one side recruits and phosphorylates AKT thus promoting glucose transport (GLUT4) translocation on the cell membrane and glucose uptake; on the other, PDK1 also phosphorylates and activates ribosomal protein 70 kDa S6 kinase1 (p70S6k), which induces cell growth and survival and directly inhibits IRS-1 through phosphorylation, thus providing a negative feedback that reduces AKT activity [64].

In adipocyte, myocytes and hepatocytes AKT phosphorylation induces glycogen synthesis by phosphorylating glycogen synthase kinase 3 (GSK-3 in its two subunits, α and β) with consequent dephosphorilation and activation of glycogen synthase, and finally decreases gluconeogenesis and lipolysis [65,66]. On the other hand, insulin has a growth-effect which is mediated by MAPK activation [65], and in AT it is able to stimulate transcription factors such as PPARγ and SREBP-1c, thus regulating adipocytes maturation [67]. SREBP-1c induction promotes adipogenesis and lipogenesis [68].

Mammalian target of rapamycin (mTOR) activity is also involved in insulin pathway, and often has implication in obesity and insulin resistance. mTOR has two multi-protein complexes: mTOR Complex1 (mTOTC1), triggered by RAS homolog enriched in brain (Rheb), phosphorylates p70S6k which in turn phosphorylates thus inactivating insulin/PI3K pathway. Moreover, mTORC1 prevents initiation factor 4E binding protein (4E-BPs) association to eukaryotic initiation factor (eIF) thus preventing activation of factors involved in cell growth and survival and, particularly in adipocytes, adipogenesis [69].

mTOR Complex2 (mTORC2) phosphorylates AKT on S473, importantly for its activation. Among AKT downstream substrates, tuberous sclerosis protein 2 (TSC2) is phosphorylated and dissociated from the heterodimer with TSC1, thus releasing Rheb and indirectly activating mTORC1.

Insulin increases fatty acids synthesis thus increasing Acetyl-CoA carboxylase (ACC) phosphorilation and activation, and at the same time it stimulates triglyceride synthesis by esterification of glycerol phosphate [70].

Moreover, insulin inhibits WAT lipolysis through phosphodiesterase 3B (PDE3B) which attenuates hormone-sensitive lipase phosphorilation [71].

At the same time, AT plays a role in regulating insulin signaling through the secretion of metabolites that characterize the endocrine action of the adipose tissue, while insulin levels influence adipocytes production of hormones and adipokines [72,73]. Many conditions can impair insulin sensitivity, thus leading to insulin resistance excessive fat may promote this alteration [74]. Accumulation of lipids, a characteristic feature of obesity, has been often associated to insurgence of insulin resistance, and it may occur by promoting adipocytes production of pro-inflammatory adipokines and macrophages infiltration [75].

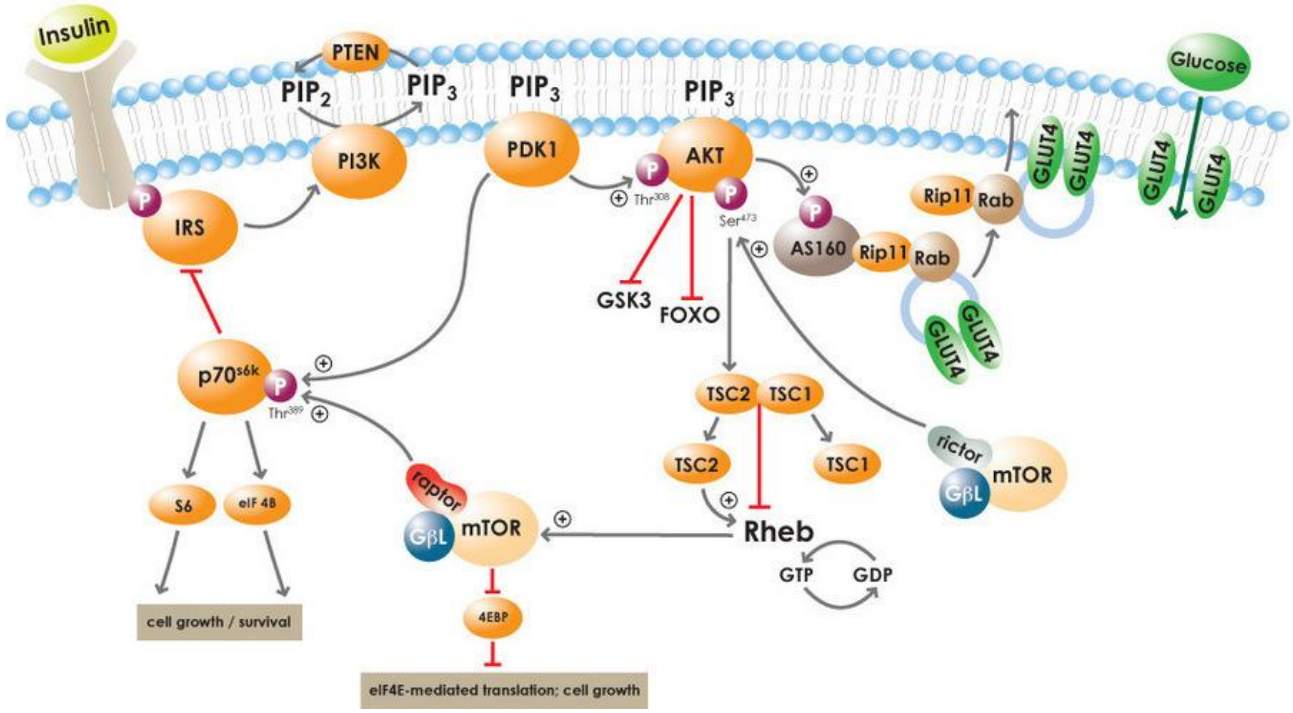


Figure 3. *Insulin signaling pathways mediate glucose uptake, protein translation and cell growth. Image adapted from Cayman Chemical.*

1.1.4.3 Adipocytes metabolism: lipogenesis, lipolysis, β-oxidation

The differences of distinct WAT districts are strongly linked to their different regulation of adipocytes metabolism in terms of lipogenesis and lipolysis.

1.1.4.3.1 *De Novo* Lipogenesis

Fat accumulation in adipose tissue derives from the storage of circulating triglycerides and *de novo* lipogenesis (DNL). Fatty acids acquired by diet are processed in the intestine and liver where they are converted in triglycerides and packed into chylomicrons and very low-density lipoproteins (vLDL), thus constituting the circulating triglycerides. Later, these lipoproteins reach the adipose tissue, where triglycerides enter the adipocyte after a conversion to non-esterified fatty acid (NEFA). This reaction is stimulated by insulin-activated action of lipoprotein lipase [76].

On the other side, DNL regulates the conversion of excessive carbohydrates to fatty acids, to be stored in adipocytes for future demand of energy. Insulin is fundamental in regulating cells glucose uptake: glucose acquired from adipocytes is involved in a series of reactions that lead to the generation of fatty acids, summarized and outlined in Figure 4 [77]. First, the glucose undergoes the reactions of glycolysis and Krebs cycle, crucial processes to generate essential metabolic intermediates. Particularly, the citrate produced in the mitochondria during Krebs cycle (also known as citric acid cycle) is a key regulator of DNL. The synthetized citrate is transported to the cytosol where it is subjected to a series of enzymatic reaction to be finally transformed in fatty acids: first, the ATP-citrate lyase (ACLY) converts citrate to acetyl-CoA, while a second enzyme, acetyl-CoA carboxylases 1 (ACC1) transforms this acetyl-CoA in malonyl-CoA. Then, the latter is converted to palmitate - the first fatty acid - by fatty acid synthase (FASN). Thus, palmitate can be processed to obtain other complex fatty acids [77]. In this process, the transcription factors Sterol Response Element Binding Protein 1c (SREBP-1c) and particularly in adipocytes Carbohydrate Response Element Binding Protein (ChREBP), are involved in the regulation of central factors of the lipogenic pathway [78].

Fatty acids represent a fundamental component of each cell, if just considering that it is the principle element of cell membrane, thus every cell type is able to carry DNL out. However, metabolic tissues including liver, skeletal muscle and, of course, adipose tissue, remain the master regulators of this process, where it is important to regulate metabolic homeostasis [79]. When FAs exceed in a high-carbohydrate diet-induced DNL, it may become dangerous particularly for the liver and skeletal muscle, thus favoring ectopic fat formation and decreasing insulin sensitivity [80].

Adipose tissue remains the main site of fat synthesis, where fatty acids are important precursors of molecules act to prevent insulin resistance and inflammation [81].

Therefore, DNL is strongly diet-correlated: it is inhibited in high-fat diets, while it is significantly activated when food high in carbohydrate is taken in [82]. DNL is as well inhibited during fasting, when insulin is low in the blood while glucagon increases, thus increasing cAMP levels in the cells and inhibiting AMP-activated protein kinase (AMPK) and cAMP-dependent protein kinase (PKA) activities [83,84].

DNL is considered primary in lipid homeostasis, and its dysregulation has been found in metabolic diseases including insulin resistance and diabetes, hyperlipidemia and hyperglycemia and even in cancer [85,86].

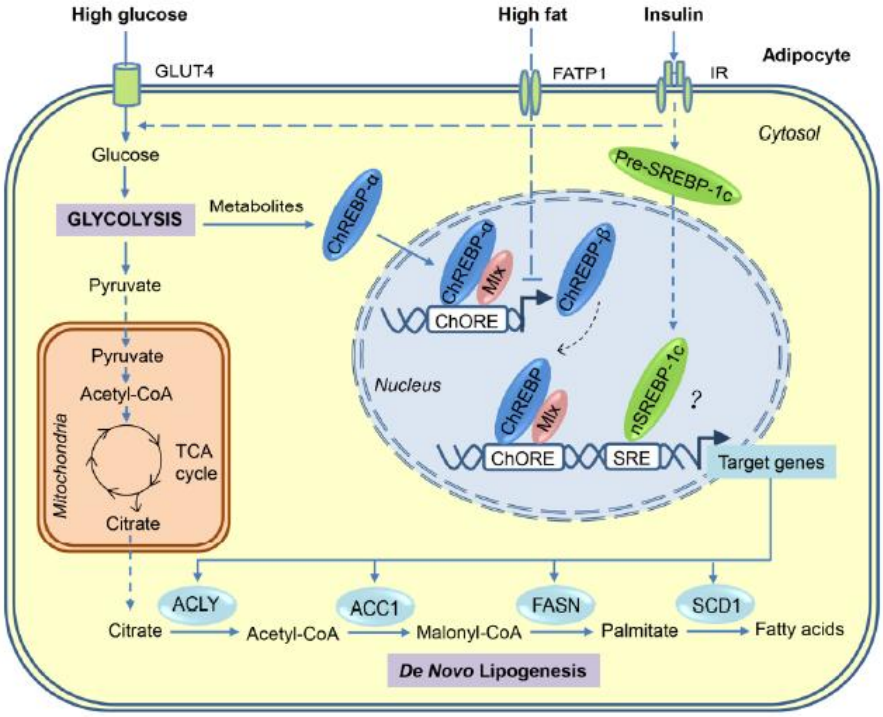


Figure 4. *De novo lipogenesis in adipocytes in different diet-conditions* [77].

1.1.4.3.2 Lipolysis

The stored lipids accumulated in the adipose tissue are mobilized in response to peripheral tissues energy request. This catalytic process is represented by lipolysis, which allows release of FFA from the adipose tissue to the blood. Fat stocks consist in triacylglycerol (TAG) that in times of need are hydrolyzed in few steps to finally obtain FFA and glycerol in a process that involves some molecules. Adipose triglyceride lipase enzyme (ATGL) converts TAG in diacylglycerol (DAG) by releasing a FFA, and in a second moment DAG can be hydrolyzed in two steps by the monoacylglycerol lipase (MGL) that releases monoacylglycerol (MAG), or in one step by the

hormone sensitive lipase (HSL), which performs the release of two FFA and one glycerol [87]. The lipolytic enzymes have a pivotal role in FFA turnover, thus preventing ectopic fat accumulation that may lead to critical consequences, especially when affecting important metabolic organs such as liver thus increasing steatosis risk [88], but also in the muscle where lipids may accumulate in between fibers [89].

The peripheral tissues then utilize the so mobilized lipids, where fatty acids undergo to β-oxidation reaction. A good balance between lipogenesis, lipolysis and β-oxidation is a key factor for the healthy metabolism of the adipose tissue.

1.1.4.3.3 β-oxidation

The FFAs released by the adipocytes reach the target cells where they are processed to be transformed in available fuel. With β-oxidation, triglycerides are hydrolyzed to produce energy in the mitochondria, and it occurs in periods of starvation and energy demand. This conversion (Figure 5) starts in the cytosol, where triglycerides in form of FFA are first transformed in Acyl-CoA, thus allowing the transfer through the external membrane of the mitochondrion. This passage is mediated by the activity of carnitine palmitoyl transferase-1 (CPT1) that catalyzes the addition of a molecule of carnitine to the acyl-CoA, thus forming Acyl-Carnitine in the mitochondria intermembrane space. The so-formed acyl-carnitine enter the mitochondrial matrix favored by the carnitine acyltransferase (CACT), to be then reconverted in acyl-CoA plus carnitine by the carnitine palmitoyl transferase-2 (CPT2). Here, the acyl-CoA is finally ready to undergo to β-oxidation cycle. The hydrolysis of acyl-CoA releases molecules of Acetyl-CoA that enter the Krebs cycle thus leading to ATP production (Figure 5) [87].

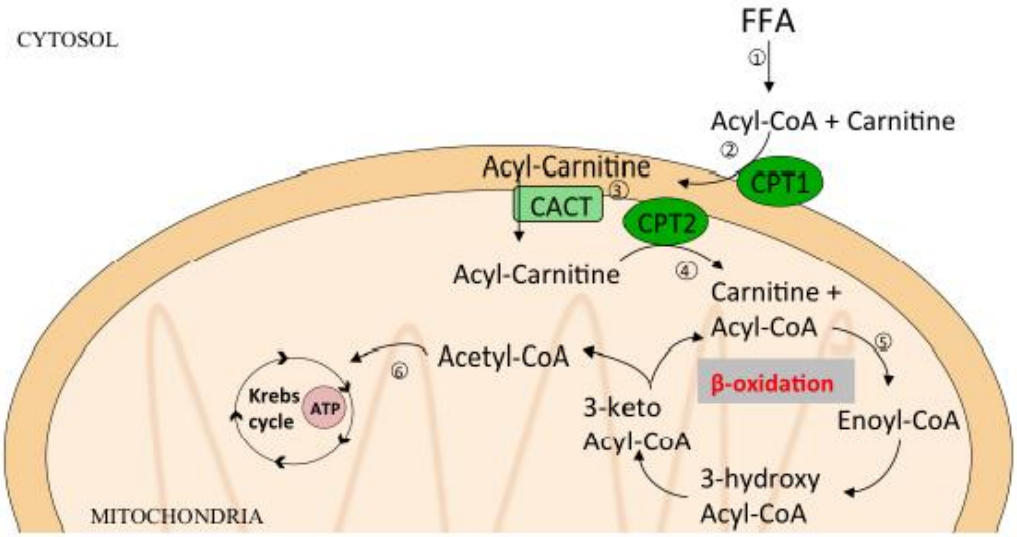


Figure 5. *Representation of lipid oxidation* [87].

β-oxidation is, as well as all the metabolic pathways, subjected to hormonal regulation. Accordingly to lipolysis and lipogenesis coordination, insulin, glucagon and catecholamine are crucial in modulating substrates availability for β-oxidation, but also proper functionality of these mechanisms affect the cell response to hormonal stimuli. Increased glucose uptake decreases FA utilization, as well as excess of triglycerides damages mitochondrial activity, thus impairing its metabolism and eventually impairing insulin response [90].

1.1.5 Adipose tissue: an endocrine organ

Since the mid-1980s, many studies started evaluating the ability of white adipose tissue to secrete metabolites. These findings turned the consideration of the adipose tissue from a static organ only able to store extra lipids, to a dynamic structure capable of affecting several mechanisms, particularly metabolic homeostasis, inflammation and alterations of physiological state, thus gaining a role in many metabolic diseases [91].

The first important hormone only secreted by adipocytes was leptin, discovered in 1994 [92], followed by the discover of adiponectin, another adipose tissue-specific hormone [93]. To date, over 600 molecules have been identified to be produced from adipose tissue, among which are included adipokines, fatty acids, lipoprotein and pro- and anti-inflammatory cytokines, thus underlying the strong influence of this organ in the system physiology [94].

The WAT signaling efficiently works in both incoming and outgoing ways, thus interconnecting with other tissues in a complex crosstalk. This communication involves mainly liver, brain and muscle but also pancreas, reproductive organs and the vascular system [95].

Physiologically, WAT in conditions of excessive food intake acts absorbing lipids in order to prevent lipotoxicity thus preserving the right functionality of other organs. This is allowed by the high sensitivity of WAT to hormones signals, first and foremost insulin [96].

The secretory profile of adipose tissue represents its functional state: “healthy” adipocytes actively secrete adiponectin, leptin, fatty acids and other adipokines, while a compromised adipose tissue exceed in secretion of pro-inflammatory metabolites and molecules that reduce insulin-sensitivity [81]. In fact, many factors produced by adipocytes are proved to be involved in glucose and lipid homeostasis, thus remaining pivotal in metabolic regulation [81].

1.1.5.1 Adiponectin

The adipokine par excellence adipose tissue-specific is the adiponectin, a 30kDa protein. The importance of adiponectin derives from its beneficial activity: it is in fact a promoter of insulin

sensitivity, and it enhances the correct expansion of adipose tissue, thus preventing ectopic fat accumulation [97,98].

Adiponectin is a multimeric protein, and it mostly circulates in form of high molecular weight dodecamers [99], and it results decreased in serum of human and rodents with metabolic alterations such as obesity, type 2 diabetes and insulin-resistance, where reduced adiponectin may be considered also causative [100]. A model of obese rodents overexpressing adiponectin, showed prevention of liver ectopic fat accumulation when assuming a fat-enriched diet [96].

The main target tissues of adiponectin are muscle and liver, where it acts through receptors recognition. On muscle cells, AdipoR1 receptor binds adiponectin and activates AMPK pathway, thus promoting glucose uptake and fatty acids metabolism. In the liver as well, AdipoR1 and AdipoR2 in presence of adiponectin block new synthesis of glucose in hepatic cells [101]. This hepatic decreased lipogenesis induced is coupled with the stimulation of β-oxidation through AMPK activity that triggers PPARα functionality [102].

The increased insulin sensitivity induced by adiponectin is also promoted locally in the adipose tissue, where adiponectin stimulates glucose uptake [98].

The beneficial effects of adiponectin have also been showed in cellular metabolism in terms of enhanced mitochondrial functionality and reduced oxidative stress, as well as decreased production of pro-inflammatory cytokines, thus ameliorating insulin sensitivity and cellular homeostasis [103].

1.1.5.2 Leptin

The first adipose-specific hormone discovered over 25 years ago [92], leptin, became a turning point in the consideration of this not-so passive tissue. Leptin showed a significant activity in regulating food intake and energy consumption in mammals, by carrying its action in the hypothalamus [104]. *In vitro* studies showed how leptin effects enhance fatty acids utilization and oxidation, thus decreasing lipogenesis [105].

Leptin directly targets the liver, where it has a catabolic action stimulating fatty acids utilization and inhibiting lipogenesis by phosphorylating ACC-1 [106,107]. In the skeletal muscle as well, there is an abundant expression of leptin receptors: here leptin activates AMPK and stimulates fatty acids oxidation [108]. Thus, leptin prevents lipid accumulation in these organs.

However, leptin receptors are also present on the adipocytes membrane: many *in vitro* studies showed that leptin has an inhibitory role in insulin-receptor activation and in consequent glucose uptake in adipocytes [109,110]. This suggests a contrasting activity if compared to adiponectin: the

two hormones are differently regulated based on different organism metabolic state. In the adipocytes, during fasting and fat mass reduction, adiponectin production increases thus promoting GLUT4 expression (and glucose uptake) and lipogenesis, while inhibiting lipolysis. Leptin instead is produced and released when fat mass increases, to stimulate lipid utilization and prevent further fat accumulation [111] (Figure 6).

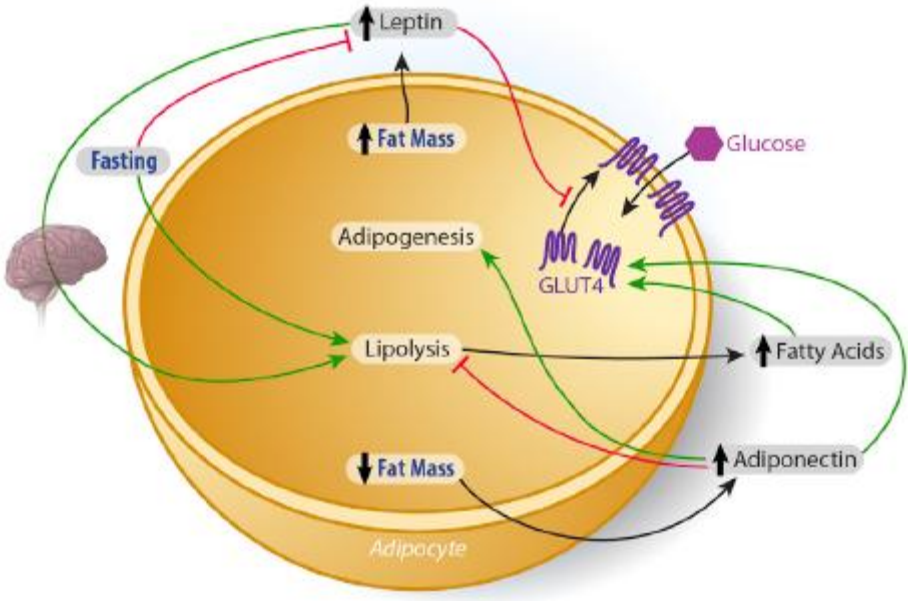


Figure 6. *Endocrine regulation of adiponectin and leptin in adipocyte* [111].

1.1.5.3 Other endocrine signals from adipocytes

Several other factors are secreted by adipocytes to mediate metabolism. Resistin is a polypeptide mainly produced by visceral fat in mice, but also by macrophages infiltrated in human adipose tissue and other cell types [112,113]. Resistin has shown to promote insulin resistance and decrease cellular glucose uptake through a mechanism that includes SOCS3 pathway [114].

Apelin is also an adipocytes-secreted hormone that regulates the cardiovascular system [115]; omentin acts instead to improve insulin sensitivity, but it is produced from non-adipose cells of fat depots, while vaspin induces insulin sensitivity as well but it is a product of adipocytes secretion [116].

Many signal mediators released from adipocytes are also constituted by lipids that play an important role in metabolism regulation and in the adipose tissue communication with other tissues. One main class is the fatty acids ester of hydroxyl fatty acids, commonly known as FAHFAs [117]. These lipids are found to increase insulin sensitivity thus ameliorating metabolic profile in mice [117]. The FAHFAs isomer of palmitic acid-9-hydroxy-stearic-acids (PAHSAs) has particularly caught

attention since it showed a consistent concentration in human serum that positively correlated with insulin sensitivity, while it has been found reduced in insulin-resistant subjects [117]. PHASAs are increased in fasting mice white adipose tissue, and they act increasing GLUT4 expression on adipocytes membranes and enhancing glucose uptake [117]. Moreover, another study showed that PHASA injection improves insulin sensitivity in mice treated with high-fat diet [118].

These factors give interesting evidence of how complex the adipose tissue is and how its deeper analysis and examination may be crucial in countering metabolic syndrome.

1.1.5.4 Inflammatory cytokines

The inflammatory response is an important component of WAT, characterized by the pro- and anti-inflammatory cytokines secreted by adipocytes, called adipokines, and/or other immune cells drown in the adipose tissue, particularly macrophages. The various adiopkines secreted by adipose tissue play a crucial role in whole-body metabolism, with evident implications in the pathogenesis inflammation and obesity, diabetes, atherosclerosis and liver disease [119]. Adipose tissue secretion of pro-inflammatory cytokines and infiltration of immune cells surely represent a hallmark for obesity and diabetes development [120]. Macrophages infiltration associates with the metabolic state, in fact obese and insulin resistant animal models present amplified inflammatory response induced by increased macrophages presence in the adipose tissue [16]. Both subsets of M1 and M2 macrophages take part to the inflammatory response, where M1 produces mainly TNF and IL-6 and spreads the oxidative stress state by releasing reactive oxygen species (ROS) [21], while M2 acts to contrast inflammation, thus secreting the anti-inflammatory cytokine IL-10 and decreasing pro-inflammatory mediators transcription [23]. The systemic inflammation is a characteristic obesity-related metabolic alteration, and plays a pivotal role in the pathogenesis of insulin resistance and diabetes, with evidence in humans and rodent models [121,122].

Some specific adipokines are IL-6 and plasminogen activator inhibitor 1 (PAI-1), relevant because of their ability to activate the immune response [123]. PAI1 is mostly expressed in visceral adipocytes and its regulation directly correlates with obesity [124], as well as most of pro-inflammatory adipokines are positively linked to high caloric diet, metabolic and cardiovascular diseases [123].

TNF-α. Tumor necrosis factor-alpha (TNF-α) is a multi-functional pro-inflammatory cytokine implicated in many biological functions, including immunity, cell proliferation and differentiation, energy metabolism and apoptosis. TNF-α was one of the first discovered cytokine secreted by adipose tissue and macrophages, and was found increased in adipose tissue of diabetic and obese

models, raising soon the idea that TNF-α is a mediator of obesity-related insulin resitance [125]. However, its action also affects other tissues.

TNF-α is important in adipocytes metabolism: *in vitro* studies showed that TNF-α alters glucose homeostasis, promotes lipolysis and inhibits lipogenesis and differentiation in cultured adipocytes [126–128]. Moreover, lipolysis promoted by TNF-α enhances FFAs release and increases hepatic glucose production [129].

IL-6. Interleukin-6 is a cytokine involved in regulation of inflammation, immune response, hematopoiesis and mechanisms of defense [130]. This cytokine is mainly secreted by WAT, liver and skeletal muscle, although IL-6 fraction from WAT also depends on macrophages infiltrated in the adipose tissue [16]. As already decribed for TNF-α, IL-6 also affects lipid metabolism, stimulating lipolysis, FFA concentration and promoting fat oxidation [131].

IL-1. Among the major pro-inflammatory cytokines, there is IL-1, characterized by two subtypes, IL-1α and IL-1β. These two cytokines in part share the same activity, but they also act differently.

Moreover, IL-1 also acts synergistically whith other cytokines, particularly TNF-α, thus responding to the same stimuli and supporting each other's activity [132].

Resistin is an adipokine produced by adipocytes but also by macrophages and monocytes [133], with clear pro-inflammatory role. Resistin is able to promote TNF and IL-6 expression in humans [134], and its implication in development of insulin resistance was also proved in mice models, where resistin stimulates suppressor of cytokine signaling 3 (SOCS3) that downregulates insulin signaling [114]. A strong association of resistin to obesity was also showed in resistin-deficiency model, which presented a strong increment of obesity but increase of insulin signaling as well [135].

RBP4 is primary secreted by the liver, where it is important for the transport of vitamine A [136], however it is also secreted by adipocytes and macrophages, especially from the visceral fat depots [137]. RBP4 is indeed correlated to the state of obesity and insulin resistance, and it contributes to reducing insulin sensitivity by inhibiting insulin receptors activity [138].

Lipocalin 2 is an adipokine implicated in transport of lipophilic small elements, it is highly expressed in the adipose tissue, and thus it is abundant in obese individuals [139], and is activated by another inflammatory induced factor, nuclear factor-kB (NF-kB) [140].

IL-18 is another adipose tissue-produced pro-inflammatory cytokine. Although its levels are increased in obese individuals [141], its role seems controversial since mice models lacking IL-18 correlate to metabolic syndrome such as insulin resistance, obesity, hyperglycemia [142], underlying IL-18 complex implication in the inflammatory condition.

CCL2 is expressed in adipocytes, particularly in lack of glucose [143], and its levels are high in obese mice and humans [144]. CCL2 is involved in inflammation of the adipose tissue and it stimulates macrophages recruitment, thus reducing insulin signaling [143].

CXCL5 as well is important when considering the inflammatory profile of adipose tissue. It is secreted also by macrophages and, consistent with the other pro-inflammatory adipokines, it is highly expressed in obese subjects and promotes insulin resistance [145]. Its expression is supported by other pro-inflammatory cytokines, including TNF and NF-kB [145].

Dysregulation of pro-inflammatory adipokines is a hallmark of metabolic dysfunctions that turn to be supported by adipokines-induced inflammatory state. The control of adipose tissue inflammation might represent a support in containing obesity, insulin resistance and all the pathologies linked to these physiological alterations.

1.1.6 Adipose tissue, obesity and metabolic syndrome

The adipose tissue is one of the most implicated organ in metabolic dysfunctions [146]. Alterations of white adipose tissue physiology strictly correlate to development of a cluster of risks factors, typical of metabolic syndrome (MetS). These metabolic risks generally coexist, thus worsening patients clinical picture, and are represented by declared central obesity, hypertension, dyslipidemia (indicated by high level of triglycerides and low level of high-density lipoprotein, HDL), and impaired glucose metabolism [147]. On the other hand, MetS relates to a major risk in developing insulin resistance, chronic inflammation and oxidative stress [148].

Obesity and insulin resistance surely represent the most common and widespread metabolic dysfunction and are often combined, describing one of the major nowadays public health issue, thus predisposing to other metabolic complications and cancer [149].

1.1.6.1 Metabolic syndrome and obesity

MetS is defined by a set of biochemical, physiological, clinical and metabolic factors related to each other in a complex system and associated to increased risk of cardiometabolic disease and type 2 diabetes mellitus (T2DM) [150,151]. In MetS patients, genetic and environmental factors interplay, all contributing to the worsening of patients clinical profile and eventually causing mortality [152]. The high incidence of metabolic syndrome worldwide reflects the importance of this clinical challenge [152]. Although the concept of MetS started in 1921 [153], it has evolved through the years, and to date the World Health Organization (WHO) [154], the European Group for the study of Insulin Resistance (EGIR) [155], the National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATP III) [156], the American Association of Clinical Endocrinologists (AACE) [157] and the International Diabetes Federation (IDF) [158] have established the criteria that describe MetS, mostly including increased visceral adiposity, dyslipidemia, hypertension and hyperglycaemia [152,159] (Table 1).

Obesity results as a major component of MetS patients [160], and all the reported MetS criteria are associated to obesity [152,161], although the molecular mechanisms regulating these processes are not completely understood. Surely, increase of abdominal fat with consequent release of inflammatory cytokines and FFAs, support a systemic metabolic disruption which is associated to development of obesity and related comorbidities [162].

Table 1. *MetS criteria for the different healthcare organization. Table is adapted from Okafor article.* [159]

Clinical measure		WHO (1998)[14]	EGIR (1999)	NCEP-ATP III[14] (2001)	AACE (2003)	1DF (2005)[15]
Insulin resistance		IGT, IFG, Type 2 DM or Insulin resistance	Plasma insulin ≥75th percentile	None	IGT or IFG	None
		*	*	†	§	
Body weight	M	WHR>0.90	WC ≥94 cm	WC ≥102 cm	BMI ≥ 25 kg/m^2	Increased WC (population specific)
	F	WHR >0.85 and/or BMI >30 kg/m^2	WC ≥80 cm	WC ≥ 88 cm		
Lipid		TG ≥150 mg/dl (1.7 mmol/l)	TG ≥150 mg/dl (1.7 mmol/l)	TG ≥150 mg/dl (1.7 mmol/l)	TG ≥150 mg/dl (1.7 mmol/l)	TG ≥150 mg/dl (1.7 mmol/l)
	M	and/or HDL-C <35 mg/dl (0.9 mmol/l)	And/or HDL-C <39 mg/dl (1.0 mmol/l)	HDL-C <40 mg/dl (1.03 mmol/l)	And HDL-C <40 mg/dl (1.03 mmol/l)	HDL-C <40 mg/dl (1.03 mmol/l)
	F	HDL-C <39 mg/dl (1.0 mmol/l)	HDL-C <39 mg/dl (1.0 mmol/l)	HDL-C <50 mg/dl (1.3 mmol/l)	HDL-C <50 mg/dl (1.29 mmol/l)	HDL-C <50 mg/dl (1.3 mmol/l)
Blood pressure (mmHg)		≥ 140/90	≥ 140/90	≥ 130/85	≥ 130/85	≥ 130/85
Glucose		IGT, IFG or Type 2 DM	IGT or IFG (but not diabetes)	>110 mg/dl [6.1 mmol/l] (including DM)	IGT or IFG (but not DM)	≥ 100 mg/dl (5.6 mmol/l) [includes DM]
Others		Micro-albuminuria			Other features of insulin resistance	

M: Males, F: Females, IGT: Impaired glucose tolerance, IFG: Impaired fasting glycemia, WC: Waist circumference, WHR: Waist hip ratio, DM: Diabetes mellitus, HDL-C: High-density lipoprotein cholesterol. *Plus any 2 of the following, †but any 3 of the following, ‡plus any of the following based on clinical judgment

1.1.6.2 Adipogenesis and obesity: a healthy or unhealthy process

The very recent literature has not still characterized how white adipose tissue develops from the early stages, although it results clear that subcutaneous and visceral adipose tissue originate from different cellular lineages [163]. From birth to adolescence, human WAT continues to develop in terms of number and volume, while it remains nearly constant during adulthood with a lower turnover [164,165].

However, regardless of the derivation lineage of different depots, PPARγ has been recognized as central regulator of preadipocytes maturation to adipocytes [166].

Models of dysfunctional adipose tissue, so called lipodystrophy, underline the importance of physiological development of WAT. The features of lipodystrophy are various and deleterious, including impaired triglycerides synthesis, accumulation of fatty acids (FA) in non-adipose tissue [167]. The ectopic deposition of lipids in the heart, skeletal muscle, liver and pancreas, contributes to develop dangerous consequences, first of all insulin resistance, which causes diabetes. These conditions of dysfunctional development of adipocytes in the complexity of adipose tissue correlate with metabolic disorders and, particularly, obesity [168,169].

Obesity represents a dangerous condition associated with several physiological and metabolic alterations, but interestingly it is important to distinguish that there are different types of obesity, and fat accumulation position and adipose metabolism play a pivotal role in differentiate “pathologic obesity” from “metabolically healthy obesity” [170].

The capacity of adipose tissue to expand in response to increased caloric absorption to store excessive energy is a physiological condition. However, when constantly exceeding in food intake, the physiological mechanism of fat accumulation may lead to complications such as insulin resistance and cardiovascular disease, often associated to obesity [171]. To characterize the pathological state of obesity, it is first necessary to consider WAT distribution: studies demonstrated that subjects with subcutaneous expansion of the WAT are less metabolically compromised when compared to individuals with fat accumulation in the intra-abdomen [172] (Figure 7). The visceral WAT has a direct connection to the liver through the portal torrent, so exceeding free fatty acids can flow from the adipose tissue to the liver thus contributing to ectopic fat accumulation [173].

Another feature that needs to be considered when discussing different location of WAT, is how adipocytes expand in the tissue when responding to growth signals. WAT is subjected to *hypertrophy* when enlarging adipocytes size due to lipid accumulation, and to *hyperplasia* when increasing adipocytes number [174]. Individuals with metabolic syndrome present a WAT mainly hypertrophic, associated with other pathological aspects of dysfunctional adipose tissue derivation, such as hypoxia and a pronounced inflammatory state characterized by abundance of macrophages. In contrast, healthy subjects show a WAT with smaller adipocytes in a major amount, which are well served by blood vessels, thus ensuring a better oxygen restoration [8,175].

In rodent models with diet-induced obesity, it has been possible to follow the expansion of the WAT: high-fat diet (HFD) mice showed an enlargement of adipocytes in the intra-abdomen in terms of number and size (hyperplasia and hypertrophy), while subcutaneous WAT results more exposed to hypertrophy. The receptor PDGFRβ might be, at least in part, associated to visceral adipocytes expansion in HFD mice [176,177].

However, it remains not completely defined whether obesity represents a cause or a consequence of impaired adipogenesis. Excessive calories surely lead to a saturation of adipocytes capacity and a consequent disequilibrium in WAT composition, thus favoring immune cells infiltration [178]. At the same time, hypertrophic adipocytes promote a dysfunctional maturation of preadipocytes. However, these considerations depend on different fat depots, as the processes of preadipocytes differentiation and proliferation may vary [179].

Therefore, characterizing the homeostatic regulation of WAT and its relation of coordination and interaction with other cohabitant cell types, constitutes a big challenge for the research against

obesity. A better characterization of the anatomically different adipose regions may represent an important front for developing new strategies for metabolic syndrome.

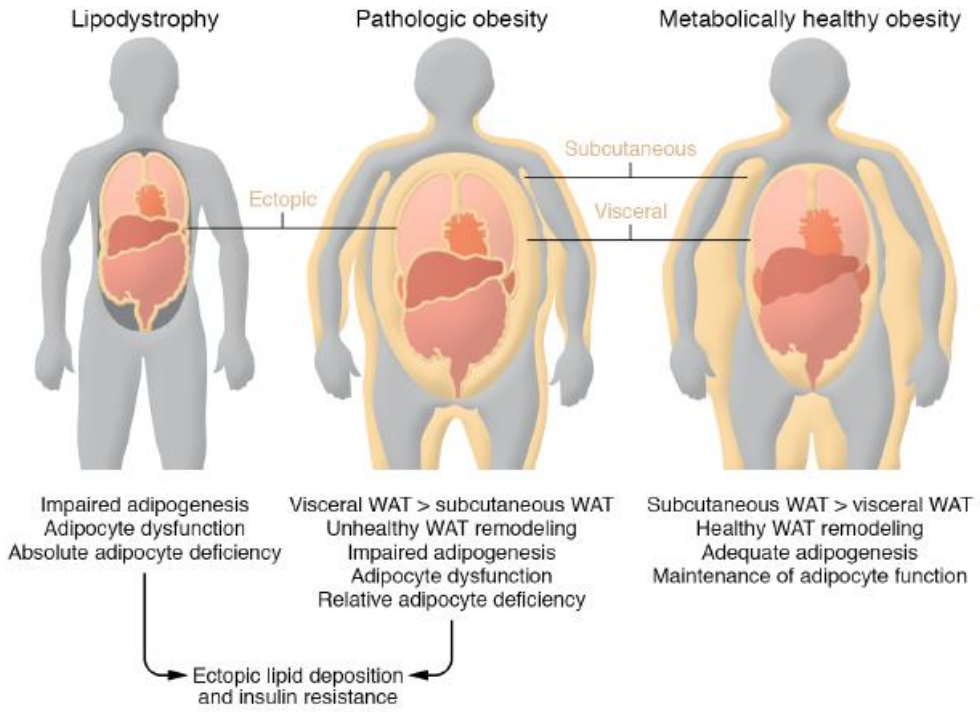


Figure 7*. Expansion of white adipose tissue: healthy versus pathogenic fat deposition* [170]*.*

1.1.6.3 Body composition: fat mass versus muscle mass

The human body can be devided into several constituent components. At tissue level, adipose tissue accounts for ~20%-30% respectively for men and women, however ~42-38% (respectively for men and women) of body mass is represented by muscle mass [180]. Adipose tissue has a complexity given by its multiple involvement in calorie storage, nutrient homeostasis, release of fatty acids during fasting and endocrine functions [3]. Muscle tissue, whose mass highly vary during life span with marked decrease during aging [181], is essential for body structural maintenance and movements [180], but it is a pivotal metabolic organ due to its high sensitivity to insulin [182] and it is responsible for 30% of energy expenditure [183]. Fat and muscle mass have a critical clinical relevance, in fact adipose tissue has been reported to associate to metabolic syndrome (MetS) [147], but important findings also related low muscle mass to increased risk of insulin resistance and cardiovascular disease [184,185].

Nowadays, several parameters are used in the attempt to define anthropometric indicators for predictive analyses, such as waist circumference (WC) and body mass index (BMI) [186,187], however body composition is highly variable among individuals with same BMI, thus this measure does not discriminate from fat and lean mass [188]. Considering body composition in terms of fat and muscle

mass and their distribution, gives a major relevance and more accurate indication in relation to MetS parameters [189]. There is also the evidence that obesity often implicates an increase of muscle mass as well [190,191]. Generally, survival increases when weight loss is matched with muscle increase rather than weight gain with muscle loss [192,193].

Fat-to-muscle mass ratio was found importantly related to MetS components and to predict MetS in a Chinese cohort [194], and with a clinical relevance in the evaluation of insulin resistance presence [195]. A study on Korean people, showed muscle-to-fat ratio as an indicator to prevent MetS [196]. In fact, insulin sensitivity and glycemia are strongly modulated by metabolic and structural muscle mass influence [197,198]. A recent study supported fat-to-muscle ratio as an indicator of MetS in Colombian young adults [199]. Increased fat-to-lean mass ratio was also found positively associated to HOMA-IR in a study on mothers and relative childrens [200] and was confirmed in women with polycystic ovary syndrome [201]. However, differences in ethnicity, age and lifestyle has to be considered when comparing these clinical studies.

Relevantly, during aging increased accumulation of fat while muscle mass is reduced often occurs, sustaining a phenomenon recognized as sarcopenic obesity, importantly associated with morbidity and mortality [202,203]. Sarcopenic obesity is characterized by multiple interplaying factors, including diet, physical activity, endocrine, vascular, inflammatory and reactive oxygen species factors [204,205]. Although loss of skeletal muscle naturally occurs with aging (and is defined as sarcopenia) [206], associated fat increase entails metabolic disruption, particularly insulin resistance and type 2 diabetes (T2D), cardiovascular disordes and cancer [205,207]. Moreover, also fat distribution changes with aging, tendently increasing in visceral abdominal fat and decreasing in subcutaneous fat, thus worsening metabolic profile [208]. However, fat-to-lean mass ratio was also associated to inflammatory and nutritional status in patients with hemodialysis [209] and to cardiometabolic risk in elderly with sarcopenic obesity [210].

These studies together suggest that fat-to-muscle ratio may be an important and powerful predictor of metabolic alterations and syndrome, particularly in elderly subjects where alterations of this balance easily occur, and it may represent an alternative to more complex and specific examination.

1.1.6.4 Systemic effects of adipose tissue metabolism in obesity

Adipose tissue homeostasis plays a main role for whole body health, as a murine model with lack of WAT showed, highlinghting the metabolic importance and influence of adipose tissue [211]. Adipocytes alterations during obesity affect systemic metabolism [212]. First, an increase of FFA and

glycerol release occurs [213], which relates to insulin desensibilization of other metabolic organs, particularly muscle [214]. Moreover, high levels of circulating glucose and lipids increase the availability of energy substrates for metabolic processes in adipose and nonadipose tissues, with consequent enhanced production of oxidative free radicals and ROS [215]. A copious number of *in vitro*, in vivo and epidemiological studies related alteration of redox state to obesity and the consequence damage caused to mitochocondria entails a cluster of related metabolic alterations, in particular inflammation and insulin resistance [216] (Figure 8). Pro-inflammatory factors production is in fact strongly increased in obesity [212], particularly TNF-α and IL-6 were found increased consistently with superoxide anion production [217]. Increase of macrophages infiltration in obese adipose tissue is also responsible for most of the TNF-α expression [16]. In agreement, increase of macrophages and related pro-inflammatory cytokine release, strongly promotes insulin release [218]. TNF-α high concentrations in adipose tissue, are associated with obesity and insulin resistance [219,220]. Eventually, these features importantly predispose to diabetes development and stroke [221,222].

Collectively, obesity pathogenesis and the cluster of predominant features involved, constitute a major issue for public health, thus supporting increased risk for several chronic illness, particularly including type 2 diabetes, cardiovascular complications, hypertension and heart attak, arthritis, hypertriglyceridemia and some forms of cancer [223].

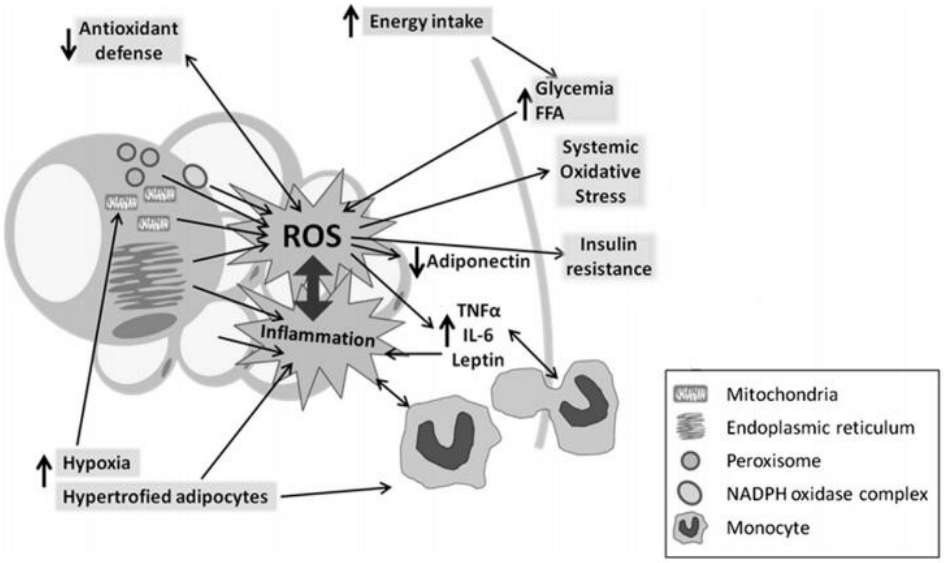


Figure 8. *Oxidative stress and inflammation in the contex of obesity and consequences. Obesity associates with increase of energy intake, thus increasing glycemia and FFA levels. This leads to increased ROS production and pro-inflammatory cytokines release, hypoxia and hypertrophy of adipocytes, and increased*

macrophages infiltration. Eventually, this results in systemic oxidative stress and inflammation and insulin resistance. Adapted from [216].

1.1.6.5 Oxidative stress in obesity

Reactive oxygen species result as a toxic product of metabolism. Their increased or, worst, chronic production activates a series of defense mechanisms involved in the elimination of these molecules [224]. Redox sensitive-mechanisms particularly include the ratio of reduced-to-oxidized glutathione, as oxidized glutathione (GSSG) is formed at the expense of reduced glutathione (GSH) in response to oxidative stress increase [225] and superoxide dismutase activation, which are SOD enzymes that blunt the oxidation cascade activated by superoxide [226]. Obesity induces oxidative stress in many ways, mainly through mitochondrial and peroxisomal oxidation of fatty acids and alteration of oxygen metabolism caused by lipid-rich diets [227]. Persisting obesity eventually decreased SOD activity [228], and SOD levels were also decreased in obese compared to lean humans [229]. Many studies reported *in vivo* evidence supporting oxidation stress link to insulin resistance [230–232] but also to non-diabetic obesity and more generally to metabolic syndrome [181,233–235], consistent with the hypothesis that oxidative stress may represent a cause other than a consequence of chronic hyperglycemia. Surely, hyperinsulinemia and increased FFA enhance insurgence of oxidative stress [40,236], whose effect influences all the insulin-sensitive organs. Importantly, antioxidants were reported to improve insulin sensitivity in animal models of diabetes [237]. Furthermore, skeletal muscle was often reported to be subjected to impaired insulin sensitivity in rodents and human models of altered mitochondrial function and redox state [238–240], but important findings also underlined adipocytes mitochondria fitness as an essential quality against insulin resistance [241]. Concordant with these findings, ROS were shown to be crucial in adipocytes development of insulin resistance mediate by TNF-α, suggesting the involvement of cytokines-mediated inflammation in the pathogenesis of diabetes and obesity [242]. Therefore, no doubt is left that mitochondrial oxidative stress is pivotal for whole-body metabolic homeostasis and more precisely for insulin sensitive organs. What remains not completely understood, is how this mechanism occurs. Insulin receptor substrate (IRS) proteins have been central in the induced decrease of insulin action under stress conditions [243]. IRS proteins are finely modulated by phosphorylation. Serine/threonine phosphorylated forms of IRS are more sensitive to degradation [244–247]. TNF-α has been associated to stimulated activation of c-Jun N-terminal kinase (JNK) and stress-activated protein kinase (SAPK), which phosphorylate IRS-1 serine and reduce insulin pathway activation [248,249], and this was confirmed in a model of JNK lacking mice that reported

decreased adiposity and increased insulin sensitivity, supporting stress and inflammation as negative regulators of insulin sensitivity [250].

Ikβ kinase B (IKKβ), another kinase mainly involved in stress-sensitivity, was found to mediate IRS-1 serine phosphorylation leading to insulin resistance [251]. In addition, oxidative stress was also found to enhance p-38 mitogen activated protein kinase (MAPK), an inhibitor of insulin-stimulated glucose transport [252]. Finally, mTOR was showed to be targeted by stress-activated kinases, promoting IRS-mediated insulin resistance [245].

1.1.6.6 Mitochondrial dynamics in obesity

Mitochondria, as a cellular power station, regulate energy homeostasis and are considered a marker of insulin sensitivity [230]. Mitochondrial capacity critically moderates metabolic processes, and its dynamics allow the maintenance of functional mitochondria. Specific stimuli induce mitochondrial biogenesis, fusion, fission and mitophagy, and alteration of these processes often relate obesity and T2D [253].

Unbalanced metabolic homeostasis leads cells toward increased stress, which translates in mitochondrial impaired oxidation and decreased content as well as increased ROS production, however continuous fusion and fission help to mitigate mitochondrial stress thus diluting and reducing stress products [253]. Diabetes-associated hyperglycemia stimulates glucose oxidation within the cells and increases NADH and pyruvate generation; pyruvate in turn is utilized in mitochondria for the TCA cycle to finally increase electron transport flux. Excessive glucose oxidation provokes unbalanced electrochemical potential, which translates in promoted ROS generation at complex I and interfaced ubiquinone and complex III [253]. Fusion and fission interchange (respectively from small to big and from big to small) is regulated by GTPase proteins, essential for mitochondrial dynamics and metabolic control. Mitofusin (Mfn) 1 and 2 regulate outer membrane fusion, while Optic Atrophy 1 (OPA1) is responsible of inner membrane fusion [254,255]. The cytosolic Dynamin-related Protein 1 (Drp1) is required for the opposite mechanism of mitochondrial division known as fission [256], where Drp1 molecules assembled to form a helical structure which constricts and divides mitochondrion [257] (Figure 9).

Several studies reported that mutations of some mitochondrial dynamics regulatory genes correlate to metabolic diseases, and Mitofusin, OPA1 and Drp1 were found particularly involved in tissues dysfunction. Mfn2 levels are decreased in skeletal muscle of T2D, which leads to decreased oxidative phosphorylation and impaired fusion [258,259]. Mfn2 expression is lower also in skeletal muscle of obese humans [260]. Mnf1 liver ablation showed an increase in mitochondrial respiration

capacity and improved complex I activity, and lipids resulted the preferential energy source; in these liver Mnf1 knockout (KO) mice, Mnf1 lack showed a protective role against insulin resistance development [261]. HFD also impaired mitochondrial dynamics and decreased Mnf1 levels in obese mice skeletal muscle, with consequent damaged mitochondrial respiratory capacity [262]. Not much is reported about mitofusins and adipose tissue, however a recent study showed that mice exposed to 12 weeks of HFD had a remarkable decrease of Mfn1 and Mfn2 in WAT [263]. Interestingly, the same work also showed that transgenic mice for adipocyte-specific Mnf2 deficiency increased food intake compared to control and developed obesity, and finally impaired glucose homeostasis and insulin sensitivity, suggesting that mitochondrial fusion is important for glucose oxidation and Mnf2 may play as an interactor to allow lipids utilization in the mitochondria [263].

OPA1 involvement in obesity and diabetes was also showed as OPA1 knockdown (KD) reduces lipolysis in adipose tissue and 3T3-L1 adipocytes [264,265]. Moreover, OPA1 ablation in pancreatic β-cells impairs glucose-induced ATP production and insulin release [266], while in skeletal muscle cells OPA1 deletion or silencing reduces oxidative phosphorylation and inhibit mitochondrial respiratory capacity [267,268].

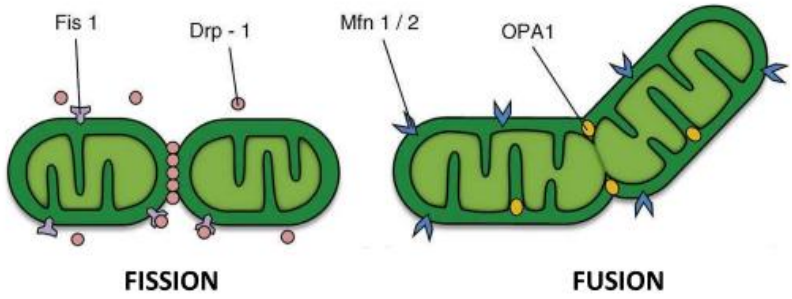


Figure 9. *Fission and fusion processes (Adapted from [269])*

The mitochondrial fission under Drp1 main regulation plays a key role in mitochondrial oxidative mechanisms and, thus, in obesity and insulin resistance [270]. Mitochondrial division mediated by Drp1 decreases ATP content and reduces glucose uptake in human skeletal muscle, but decreased Drp1 activation increases lipid oxidation and insulin sensitivity [271]. Drp1 KO mice supported increased skeletal muscle and systemic insulin sensitivity [272]. Also, diabetes-susceptible cybrid cells treated with Drp1-inhibitor showed decreased oxidative stress and increased insulin-induced

glucose uptake [273]. Drp1 deficiency in the liver also was found protective against obesity, thus reducing fat mass and promoting energy consumption [274].

Mitochondrial modulating GTPases represent thus important players to monitor in the pathogenesis of obesity and T2D.

1.1.6.7 Inflammation in obesity

Accumulating studies identify a pivotal role of chronic inflammation in the pathology of obesity-related metabolic alterations [121,122,275]. Experimental and clinical studies unanimously reported that inflammation is associated to metabolic diseases, particularly obesity and T2D [276,277]. TNF-α overexpression in obese mice adipose tissue gave an early evidence linking inflammation to obesity and diabetes [125]. TNF-α genetic suppression showed improved insulin sensitivity in both *in vitro* and *in vivo* models, and studies in humans as well supported the idea of TNF-α as a main regulator of insulin resistance, particularly in obesity pathogenesis [278,279]. Consistent with these findings, insulin sensitivity increased after treatment with anti- TNF-α antibodies in lean and obese subjects [280,281]. However, nutritional status and obesity are directly linked to inflammation response, in fact FAs affect cytokine production. *n*-6 FAs showed a pro-inflammatory effect, while *n*-3 FAs are considered protective against inflammatory response [258].

TNF-α has specific receptors (TNFR1 and TNFR2) by which carries out its actions, and activation of TNFR1-induced signals is sufficient to impair insulin sensitivity [282].

TNF-α acts impairing phosphorylation and consequent activation of the insulin receptor stimulated by insulin in adipose tissue and muscle, thus inducing insulin resistance. In fact, animal obese models treated to reduce TNF-α expression showed an increased insulin sensitivity in adipose tissue and also in muscle [283]. The mechanism by which TNF-α reduces insulin sensitivity, involves activation of other factors. In fact, including TNF-α was found to stimulate NF-kB gene expression with consequent impairment of insulin signaling in *in vivo* and *in vitro* models [284,285]. In turn, NF-kB activates other factors such as suppressor of cytokine signaling-3 (SOCS-3) which prevent IRS-1 tyrosine phosphorylation, thus inhibiting glucose uptake [286]. In skeletal muscle as well, TNF-α acts decreasing insulin sensitivity by suppressing AMPK activity [287].

Furthermore, obesity is characterized by enhanced oxidative stress and mitochondrial dysfunction [121], which stimulate TNF-α generation. TNF-α in turn activates JNK and NF-kB factors, involved in ROS production, thus amplifying insulin resistance effect [288]. Oxidative stress and TNF-α production are linked in a two-ways relation, indeed TNF-α is upregulated under oxidative stress

condition, but TNF-α activation leads to expression of proteins that increases oxidative stress response [289].

TNF-α is also a potent inducer of other inflammatory cytokines release, particularly IL-6 and IL-1β [290]. Similarly to TNF-α, IL-6 is as well implicated in obesity, and clinical studies assert that IL-6 increases when increasing fat amount and waist circumference in humans [291], and decreases proportionally to weight loss [292]. Interestingly, IL-6 seems to be particularly related to visceral adipose tissue rather than subcutaneous adipose tissue [291]. While some studies reported that IL-6 affects adiposity but not insulin signaling [293], another study highlighted IL-6 involvement in obese-related insulin resistance [220] and diabetes, suggesting IL-6 as a predictive factor for diabetes and other metabolic dysfunctions [221]. Probably, systemic increase of IL-6 may promote insulin resistance, whereas short-term IL-6 increase may be controlled by homeostatic processes [294]. Moreover, IL-6 functions appear to be dependent on tissue and metabolic state; in skeletal muscle and particularly during exercise, IL-6 promotes skeletal muscle glucose uptake thus increasing hypertrophy and AMPK-mediated FA utilization, thus showing an anti-inflammatory effect [295]. In contrast, IL-6 works in liver and adipose tissue as a pro-inflammatory cytokine, supporting insulin resistance SOCS3-mediated [296]. In WAT, IL-6 promotes mesenchymal stem cells proliferation and inhibits adipogenesis [297]. In pancreatic cells, IL-6 was found to stimulate insulin secretion, suggesting that IL-6 increased levels in obese individual may be a mechanism to increase insulin release in condition of obesity-induced insulin resistance [294]. Additional studies reported IL-6 as an inducer of insulin resistance in both *in vivo* and *in vitro* [298].

Among the main pro-inflammatory cytokines, IL-1 relation to insulin resistance has not been characterized as much as TNF-α. Patients with diabetes mellitus showed altered levels of IL-1 in pancreas [299]. IL-1β also correlates with metabolic syndrome [300], and shows increased levels in visceral adipose tissue of obese subjects [301]. *In vitro* studies also showed both IL-1α and IL-1β to impair adipocytes insulin signaling [302,303].

IL-1α$^{-/-}$ mice showed a decrease of glucose fasting levels and improved insulin sensitivity [304]. IL-1α also, together with IL-6, reduced AKT phosphorylation with consequent inhibition of insulin signaling, while IL-1β treatment showed to reduced insulin-mediated glucose uptake and lipogenesis [305]. HFD was also found to increase mRNA expression of IL-1β in epididymal fat of obese mice [305]. In human, combined concentrations of IL-1β and IL-6 resulted as predictors for T2D risk [306].

1.1.6.8 Insulin resistance in adipose tissue and obesity

The insulin hormone is well known as a modulator of metabolism, essential for glucose and lipid homeostasis. Constant hyperglycemia is identified in diabetes chronic pathology. Congenital destruction of pancreatic β-cells defines type 1 diabetes mellitus, however food industrialization of the last century with increased consumption of food products densely caloric and high in fat and sugar, combined with an accentuate sedentary lifestyle, led toward an increment of type 2 diabetes mellitus and, of course, obesity [53]. Nowadays these two pathologies are often associated, and the consequent co-morbidities represent a serious epidemic issue and a big medical challenge. Insulin role's is tissue specific, in fact in skeletal muscle promotes glucose transport and utilization, while in the liver it enhances lipogenesis and glycogen synthesis thus decreasing gluconeogenesis. In insulin-resistant subjects, at normal plasma insulin levels, insulin-sensitive tissues are unable to restrain endogenous glucose production thus favoring increased glucose uptake [307–310], but they need stronger insulin levels in plasma to compensate this diminished sensitivity [311]. Insulin resistance refers to an impairment of insulin cascade and insulin-mediated glucose disposal, in response to normal or even elevated insulin level and correlates to metabolic syndromes [312]. The reason behind decreased response to insulin stimuli has been discussed and investigated since the discovery of insulin and its effectors. Firstly, researchers attention focused on insulin receptor (IR), which presented defects in adipocytes but also other cell types of obese and diabetic humans and rodents [313,314]. Thus was found that obese mice and humans presented decreased level of IR in adipocytes due to increased expression of the ubiquitin ligase MARCH1 involved in IR ubiquitination and degradation and FOXO1 inhibition [315]. According to tissue-specific insulin effects, insulin resistance shows as well tissue-specific consequences. Skeletal muscle is highly sensitive to insulin, therefore its insulin signaling results often altered in obese and type 2 diabetes patients. In skeletal muscle, insulin mediated-glucose uptake is required for glycogen synthesis, which results halved in T2D patients [55,316]. Many studies reported that defects in glucose transport mostly account for impaired glycogen synthesis in diabetic patients [58,62,317], and that insulin signaling is particularly altered in the proximal steps that involve IS, IRS-1, PI3K, AKT and GSK activity [53].

In the liver, where insulin entails glucose uptake and suppression of glucose production, it seems that decreased gluconeogenesis may also be mediated by external insulin-mediated signals [60,318]. Insulin suppresses lipolysis in WAT, thus reducing fatty acids flux toward the liver and consequent hepatic β-oxidation. Glycerol, a product of triglycerides oxidation implicated in gluconeogenesis,

diminishes in response to insulin-induced lipolysis abolition, thus reducing hepatic glucose production [60]. Impaired insulin-induced inhibition of hepatic glucose production may thus depend not only on liver insulin resistance, but rather on defects of WAT insulin response. Several *in vivo* studies also provided the evidence that inhibition of FOXO1 transcription factor, a potent gluconeogenetic activator, may also be a key mechanism to modulate *Insr*$^{-/-}$-induced hepatic glucose intolerance [319,320]. Increased FOXO1 was found to correlate with a homeostatic model assessment of insulin resistance (HOMA-IR) [321]. Additionally, Samuel et al., showed that chronically fat-fed mice knocked-down for hepatic and WAT FOXO1 showed an improvement of insulin signaling [322]. Insulin action on liver is also involved in lipid metabolism and particularly in lipogenesis stimulation, mainly through SREBP-1c [323,324]. However, although insulin resistance associates with decreased hepatic *de novo* lipogenesis [325] (DNL), paradoxically subjects with insulin resistance present hepatic steatosis, and net lipogenesis results increased in insulin resistant-liver [326,327], suggesting that the liver is selectively resistant to insulin-mediated glucose branch (mediated by FOXO1) but not to the lipid one SREBP-1b-mediated. FOXO1 and SREBP-1b pathways may thus present different insulin sensitivity [328], and particularly overfeeding may lead to lipogenesis activation independently of insulin action, but the interconnection of these mechanisms is not completely clarified yet.

In WAT insulin acts as a promoter of lipogenesis and inhibits lipolysis [53]. In particular, mesenteric and omental adipose tissue are considered associated to hepatic insulin resistance as the portal vein directly connects these white depots to the liver, thus exposing it to all the metabolic factors that affect insulin signaling impairment released by the adipose tissue [329]. Insulin receptor presents a decreased amount and functionality in humans with T2D in the WAT as well [330,331]; however, weight loss can contribute to rescue this condition [330], although the molecular mechanism behind this process remains undetermined. Contrary to skeletal muscle, adipose tissue does not stand out for insulin-induced glucose disposal [332]. Withal, Abel et al., showed that skeletal muscle and liver insulin sensitivity resulted impaired when GLUT4 was specifically deleted in mice adipose tissue with no effects on fat mass [333], suggesting that adipocytes affect - even indirectly - insulin sensitivity and glucose uptake in other insulin sensitive organs. Interestingly, this indirect connection between adipocytes and other insulin-sensitive organs may be regulated through the lipogenic activator Carbohydrate Response Element Binding Protein (ChREBP) [78]. In addition, the glycerol-3-phosphate generated by adipocytes glucose uptake mediates fatty acids

esterification, canalizing lipid storage in adipose tissue and preventing ectopic fat accumulation in liver and muscle mass [334].

The pathophysiological relevance of adipocytes lipolysis suppression determined by insulin is significant since adipose tissue is crucial for NEFA plasma release, for liver gluconeogenesis and thus for T2D determination. Insulin resistant adipocytes are in fact no longer able to suppress lipolysis, and T2D patients showed elevated plasma NEFA concentrations [310,335], and higher plasma NEFA levels resulted predictive of T2D insurgence [336,337]. Karpe et al., also clarified that obesity does not seem to be correlated with increased circulating NEFA levels *per se*, but rather the latter might be a consequence of indirect physiological alteration, such as insulin resistance [338]. However, bariatric surgery on obese patients implicate a decrease of adipose tissue lipolysis along with improved HOMA-IR [339]. A clinical study on 300 subjects showed that adipose tissue insulin resistance increased in relation to increased glucose intolerance [340].

Essentially, it seems that insulin resistance in adipose tissue impedes to promote lipolysis during fasting and to support lipid accumulation during feeding, as showed by a human study [341]. Insulin resistance thus impairs the normal fat storage in WAT, probably even deflecting toward ectopic fat deposition.

Although WAT is evidently related to insulin resistance, the molecular mechanisms regulating this process are much less characterized if compared to liver and skeletal muscle. What is surely noteworthy is that the inflammatory system is clearly involved in insulin resistance development of the adipose tissue. HFD only took three days to impair the inflammatory profile of rats [342], suggesting that cytokines are a consistent component of insulin sensitivity impaired process.

As already mentioned, NEFA esterification shows a consistent role in adipocytes insulin resistance as well. Defects in NEFA esterification may be causative of impaired lipolysis in insulin resistance. Insulin promotes adipocytes glucose uptake; when glucose enter the cells, undergoes to glycolysis, thus forming a series of intermediates. Among these, glycerol-3-phosphate is important for NEFA esterification and lipid storage, and this process may be part of the insulin resistance-induced metabolism alteration [343].

These clues combined, underline how adipose tissue is actively involved in T2D pathology. WAT high sensitivity to insulin becomes critical when low defects in this pathway may also affect skeletal muscle and liver insulin sensitivity. However, although the evidence correlating dysfunctional adipose tissue to damaged insulin signaling, T2D and obesity is clear and widely

proved, it remains an open debate to define the mechanisms modulating the interconnection between the parties.

1.1.6.9 Adipocytes remodeling in obesity

Adipocytes size is considered a relevant marker of adipose tissue-related metabolic condition. In fact, adipocyte remodeling is a dynamic process subjected to environmental and nutritional stimuli, and reflects its morphology and function [344]. Obesity is a condition that strongly affects adipocyte morphology and hypertrophy, and translates in increased inflammation, hypoxia and, finally, insulin resistance [345]. Studies on humans reported a correlation between adipocytes size and insulin sensitivity and glucose tolerance [346,347]. In the same work, Weyer et al. defined adipocytes hypertrophy as a predictor of type 2 diabetes. Enlarged adipocytes associate to worsened functionality of the adipose tissue [348]. Early studies soon related increased adipocytes size to reduced insulin sensitivity and glucose uptake [349–351]. Mice treated with high-fat diet for a short-time, presented enlarged adipocytes which also resulted less responsive to insulin signal as IRS-1 and AKT stimulation were attenuated, even though the molecular mechanism behind this association remains unknown [352,353]. Actin cytoskeleton is in fact directly involved in GLUT4 transport under insulin stimulation, because vescicles containing GLUT4 move along actin filaments after AKT activation [354,355]. Moreover, hypertrophic adipocytes and consequent adipose tissue remodeling showed important implications in metabolic alterations at systemic level [329]. Increased adipocytes size was associated to increased cell necrosis, which in turn impairs adipose tissue functionality and enhances inflammation [356,357]. In fact, expression of the major pro-inflammatory cytokines including TNF-α, IL-6, MCP-1, was found increased in hypertrophic adipocytes [125,358]. These factors in turn entail insulin resistance and sustain immune response [359–361]. In addition, in enlarged adipocytes in obese models, basal lipolysis was found increased, thus supporting the release of FFAs [362,363]; importantly, the final result of adipocytes metabolism disruption is the ectopic lipid accumulation and lipotoxicity in non-adipose tissues [364]. Furthermore, adipose tissue inflammation and immune cells recruitment after HFD, induce inflammation also in other metabolic tissues, in particular liver and skeletal muscle [365]. Therefore, although excess of energy intake in obesity primarily regard WAT, it lately has an impact at systemic level, inducing inflammation and insulin resistance also in other organs and affecting whole body metabolic homeostasis [329].

Adipocytes increased size requires a remodeling of actin cytoskeleton, pivotal for the maintenance of cellular size and shape [366]. This cytoskeleton remodeling is determined by its principal

component actin, and its ability to shift from globular monomeric (G-actin) to filamentous polymeric (F-actin) state. F/G-actin dynamics were found to be related to metabolic processes, including glucose uptake, fat utilization and insulin signaling [367,368], although the modalities of dynamics regulation are not fully understood. Lipid droplets characterize adipocytes structure, and their size affects adipocyte morphology and F/G-actin dynamics [344]. Actin remodeling is supported by several regulatory factors, including Arp 2/3 complex, Cofilin-1, Profilin-1 and some RhoGTPases [369]. These proteins intervene during adiopcytes differentiation and when nutritional status induces an increase of lipid accumulation within the cell that enhances adipocyte expansion [370]. Furukawa et al., reported that Rho kinases involved in adipocytes remodeling are also able to phosphorylate IRS-1 serine site and insulin response [371]. HFD expectedly corresponds to expansion of WAT and increased size of adipocytes, as well as impaired insulin signaling [372]. In an interesting study, Hannson et al., demonstrated that HFD-feeding mice presenting increased adipocytes size, showed a re-organization of actin structure as well [352]. In fact, Arp2/3, Cofilin-1 and Profilin-1 resulted increased in epididymal fat of obese mice compared to wild-type. In agreement, profilin-$1^{+/-}$ model resulted protected against obesity-associated glucose intolerance and reduced inflammation in terms of macrophages infiltration and TNF-α levels [373]. These findings add new interesting cues in the articulate plot that regulates adipocytes metabolic homeostasis.

In conclusion, adipocytes size gives an important indication of adipose tissue health status, and its remodeling characterizes obesity [344,352]. Importantly, increase of fat cell size may represent an indicator and predictor of adipose tissue metabolic dysfunctions [374].

1.2 GHRELIN

1.2.1 Biology and functions

In 1999 Kojima et al. first published an article that introduced ghrelin for the first time, a gastric hormone isolated from rat stomach [375376]. Ghrelin is a 28 amino acids peptide, firstly found to present a n-octanoyl at serine 3, thus conferring the acylation to this hormone. The name ghrelin originates from the root of *ghre*, which means "grow" since the peptide was individuated for its ability to stimulate growth hormone (GH) release from the anterior pituitary [375]. In the previous years, some research teams already characterized a growth-hormone secretagogue receptor (GHS-R), a G-coupled protein involved in GH secretion, although its ligand was unknown [377,378]. Since then, many studies focused on discovering ghrelin activities and implication and, little by little, all its potential started to rise, thus increasing the interest of many researchers on this emerging hormone.

The initial works concerning ghrelin described this peptide as a gastrointestinal signal involved in communication with the central nervous system (CNS) assigned to regulate food intake, glucose metabolism, adiposity and energy expenditure, hence suggesting ghrelin fame as the "hunger" hormone [379–381]. However, ghrelin's copious activities revealed through the years, reconsidered and amplified its role far beyond its view of "hunger" hormone: ghrelin was found to be involved in metabolism regulation in many ways, but also in modulating sleep [382,383], gastric motility [384,385], brown adipose tissue thermogenesis [386–388], muscle atrophy contrast [389,390], anxiety modulation [391,392] and consistent implication in cardiovascular functionality [393–395].

1.2.1.1 Structure and genetic regulation of ghrelin

Relevance of ghrelin peptide is remarked from the evidence that its gene and structure and its receptor are conserved across mammalian, as well as birds, amphibian, and fish [396].

Ghrelin gene in human is located on the short arm of the third chromosome, where it presents six exons, two of which are noncoding (Figure 10) [397]. The first and the second exon contain the genetic code for the 28 amino acids (aa) that constitute ghrelin peptide [398,399]. From this gene, a fist transcript is translated to 117 aa so-named preproghrelin. The 23 aa N-terminal of preproghrelin is removed to obtain the remaining 94 aa proghrelin, which is then processed to obtain the 28 aa ghrelin, plus the 66 aa named C-ghrelin [400,401]. The C-ghrelin is as well processed to generate obestatin [402], another peptide of 23 aa: many studies showed an anorexic effect of

obestatin, although there is no evidence that it affects food intake, neither alone or by influencing ghrelin activity [403–405].

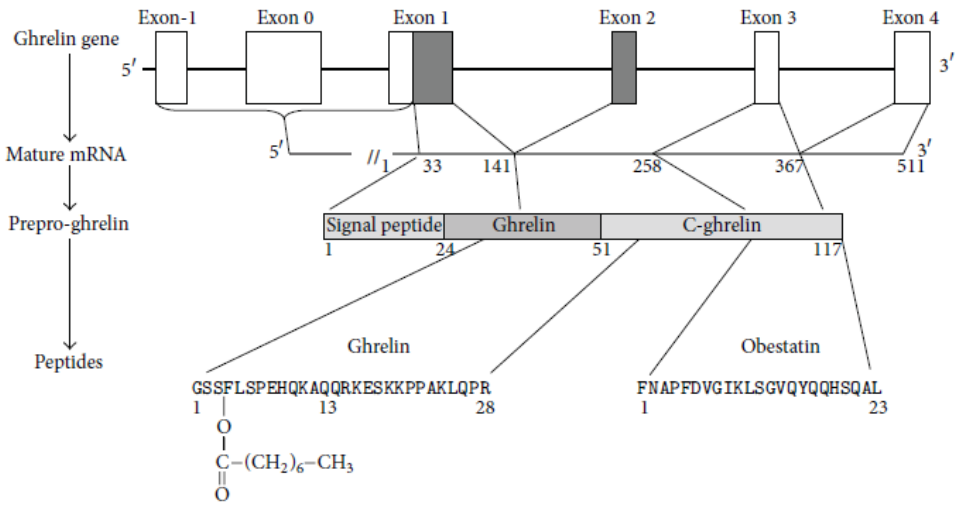


Figure 10. *Ghrelin structure gene and its processing to obtain ghrelin and obestatin peptides.* [397].

1.2.1.2 Post-translational modification of ghrelin

The post-translational modification that characterizes ghrelin is the acylation on the hydroxyl group of Ser3 [375]. The enzyme responsible for this acylation is ghrelin O-acyl transferase (GOAT), which principally utilizes octanoyl group (C8:0) as a substrate, but in rare cases it may also use decanoyl (C10:0) or (C10:1) group [406]. The acylation process is supposed to take place in endoplasmic reticulum [407].

GOAT enzyme localization was found in the X/A like cells in the fundus of the stomach, thus supporting the gastric origin of ghrelin production [408]. GOAT knockout mice display a total lack of ghrelin acylated form and no significant differences in body weight and composition and food intake when compared to wild-type in a standard diet feeding. At the same time, GOAT knockout mice fed with high-fat diet showed lower body weight and increased food intake compared to wild-type ones [409], suggesting a role GOAT in the modulation of food endurance. Moreover, GOAT regulation is subjected to leptin levels, and its expression increases in fasted rats treated with leptin injection [410].

Ghrelin peptides was found, in both forms, to be also subjected to phosphorylation on Ser18 by protein kinase C [411]. However, the role and regulation of ghrelin phosphorylation is yet to be determined, so additional studies are required to define this secondary ghrelin modification.

1.2.1.3 Ghrelin receptor

Ghrelin in its acylated form, was characterized to perform its action through a G-protein coupled receptor known as growth hormone segretagogue receptor (GHS-R). This protein is generated from GHR-S gene constituted by two exons and one intron, which encode for the 7 trans-membrane receptor [377]. The GHR-S gene is subjected to alternative splicing that generates two different GHR-S mRNAs, which lead to two different receptors, identified as GHRS-1a and GHRS-1b. GHRS-1a is the one containing all the 7 trans-membrane domain, and it is the most relevant because of its affinity to acylated ghrelin, while GHRS-1b is shorter, with 5 trans-membrane domain [378]. The expression of GHRS-1b is quite wide, however less is known about its protein expression and function [412]. It was proved that GHRS-1b is unable to bind both forms of ghrelin (acyl and desacyl) [377], but it can affect GHRS-1a activity through the formation of a heterodimer with the receptor that reduces GHRS-1a functionality [413,414]. On the other hand, GHRS-1a shows a more defined expression mainly in the CNS and in the pituitary, but also in pancreas, lung, kidney, cardiac and adipose tissue [415,416]. Acylation of ghrelin on Ser3 is necessary to activate GHRS-1a, which then drives phospholipase C activation in the calcium pathway and GH release [417]. The hypothesis that ghrelin may act through other receptors is still taken into consideration, although it has never been proved.

1.2.1.4 Ghrelin secretion and distribution

Most of the circulating ghrelin derives from the stomach, where X/A-like cells produces the 60-70% of total ghrelin [418]. Some other organs produce the remaining part, particularly hypothalamus, pituitary, and pancreatic cells, and its mRNA was found in many other tissues [376,419]. Total plasma level of ghrelin oscillates depending on feeding state: it increases when fasting and decreases right after meals [420]. The body composition as well indicates a variance in ghrelin presence, characterizing a low grade of ghrelin obese people, while increasing in lean subjects [396]. Ghrelin regulation also depends on dietary food composition, as lipids apparently reduce ghrelin levels compared to other macronutrients [421].

Although AG was firstly considered the only metabolically active and so the most important form, it only accounts for approximately the 5-10% of total circulating ghrelin [422]. Lately, value of UnAG has been reconsidered, and more and more studies have started validating UnAG relevance and its potential metabolic influence. So far, several data either recognize UnAG independent activity in support or contrast to AG functionality.

1.2.1.5 Physiological functions of ghrelin

Ghrelin relevance derives from its copious activities and implications that were observed during the past 20 years.

GH release. As discovered at first, ghrelin induces GH release in the pituitary and in the hypothalamus [375]. The importance of GHS-R1a in mediating ghrelin effect on GH release, suggested the acylated form as the one designated to this role; however, is not excluded that UnAG may as well affect GH stimulation by regulating GH-insulin growth factor axis [423].

Appetite and food intake. Ghrelin notoriety also derives from its consistent effect as a regulator of appetite. Its levels strongly increase during fasting, to decrease then after feeding [420]. Administration of ghrelin at central or peripheral level relate to increased hunger and augmented food intake and body weight, both in rodents and human [379,424,425]. Ghrelin orexigenic activity takes place via the vagal nerve, which connects stomach secreted-AG ghrelin to the central nervous system [426]. Here, ghrelin stimulates in the hypothalamic neurons other orexigenic peptide: neuropeptides Y (NPY), agouti-related peptide (AGRP), thus favoring sense of appetite and food intake [427]. Apparently, this ghrelin effect is mediated by 5' AMP-activated protein kinase (AMPK), which acts as a sensor of the energy status thus regulating ATP production/consumption. AMPK is so considered a regulator of food intake and energy balance [428].

Energy homeostasis. Ghrelin studies have soon highlighted this peptide's involvement in body weight, and it clearly plays a role in the regulation of glucose homeostasis. Plasma ghrelin increases when losing weight, while it decreases with overfeeding or fat diet [429,430]. Besides, several *in vivo* studies also showed ghrelin's ability to induce adiposity [379,387] thus promoting carbohydrates utilization and preserving lipids oxidation [431], suggesting ghrelin's correlation to body mass as well as body composition.

A direct involvement of ghrelin in glucose metabolism is supported by the presence of GHRS on the α-cells of pancreas that, according to increased level of glucose when ghrelin is administered in human, show AG as a direct glucagon stimulator [391,432,433]. Correlated studies evaluated also insulin modulation in response to ghrelin administration and found a negative correlation between the two hormones: insulin plasma level are lower when AG is given, and this theory was also validated with animal studies [434–436]. AG-mediated inhibitory effect on insulin secretion appears to be differently driven. On one side, Dezaki et al. showed that ghrelin acts on the regulation of Ca^{2+} and K^{+} channels which modulate insulin release [437]; on the other side, ghrelin seems to mediate insulin production by the AMPK-UCP2 pathway in the pancreatic β-cells [438].

However, in contrast with these findings, other researches support the thesis of ghrelin as a promoter of insulin secretion [439,440]. This divergence of information may depend on experimental conditions (in terms of ghrelin concentration and exposition) [397] but also on AG/UnAG ratio, as UnAG appear to contrast AG effect hence promoting insulin secretion [441,442] (Figure 11). Although many aspects of ghrelin effects remain not fully understood, it is important to analyze the specificity of ghrelin forms in the different tissues.

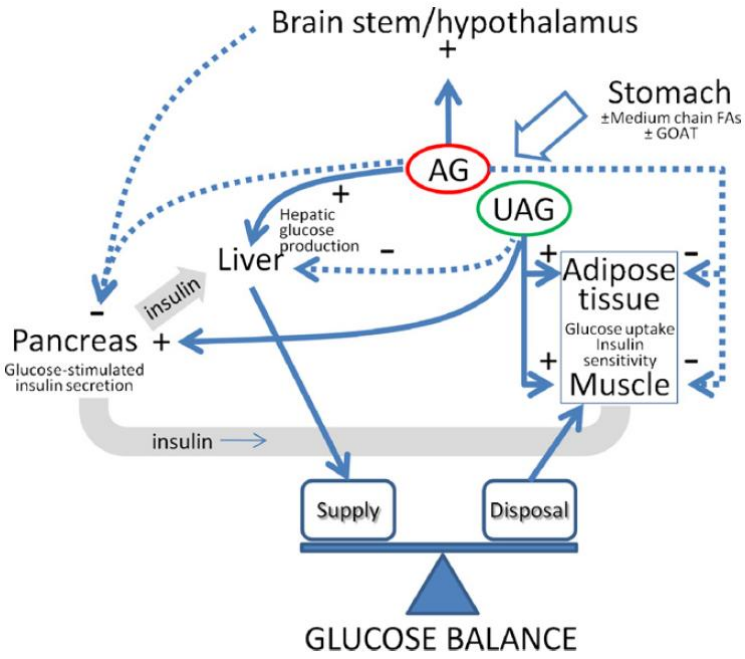


Figure 11. *AG and UnAG opposite effect in regulation of glucose balance in pancreas, liver and other tissues.*

1.2.2 UnAcylated Ghrelin versus Acylated Ghrelin: an independent metabolic modulator

1.2.2.1 Effects on liver

UnAG and AG hepatic regulation has been analyzed in multiple *in vivo* and *in vitro* studies.

On *in vitro* hepatocytes, UnAG acted as a suppressor of gluconeogenesis, but the same study showed an opposite role of AG, which was found to stimulate gluconeogenesis and glucose release [443]. The latter finding was supported by other works, which showed that AG treatment on rats supports gluconeogenesis activation through PGC1α induction in the liver, while decreasing insulin signaling [433]. Additionally, AG causes rats lipogenic genes activation and triglycerides accumulation in liver tissue, thus reducing AMPK guided lipid oxidation pathway [444].

In a model of healthy rats with 4-days of UnAG infusion and in a mice model overexpressing UnAG, this peptide did not show any modification of redox state, mitochondrial activity, insulin signaling and triglyceride content [445,446]. Nonetheless, UnAG may play an interesting protective role in the

liver against mitochondrial impairment and apoptosis induction in a model of ischemia/reperfusion liver injury [447]. However, also HFD-feeded rats with impaired inflammatory state and increased oxidative stress, presented an amelioration of those conditions in the liver as a result of AG administration [448,449]. Conceivably, this correlates with a decrease of AKT signaling activation regulated by insulin, which may promote lipogenesis, oxidative stress and inflammation in the liver of fat overfed rats [448] and cultured hepatocytes [450].

1.2.2.2 Effects on skeletal muscle

Ghrelin in its both acylated and unacylated forms implications in the musculoskeletal system has been quite well investigated. To date, there are many articles available reporting interesting metabolic beneficial effects of ghrelin forms on the skeletal muscle by acting in several ways, from preventing muscle strength to reduce inflammation and protecting against muscle atrophy to supporting mitochondrial functionality and insulin sensitivity, thus conferring an important role of prevention for many pathological conditions [451]. The two-ghrelin forms share in part some effects on the muscle: first, both of them promote myoblast C2C12 proliferation [389]. *In vitro* experiments also demonstrated that UnAG and AG induce myocytes glucose uptake, reasonably by increasing GLUT4 production, even though this was confirmed in terms of gene but not protein expression [452]. AG in particular was found to prevent insulin signaling and consequent glucose uptake impairment due to excessive fatty acids (FAs) diet on rat's myoblast [453]. These AG-treated cells presented in fact, in presence of palmitate, reduced TAG content. Ghrelin plays a role in modulating muscle FA metabolism: muscle cells treated with AG and UnAG showed increased FA oxidation [454]. UnAG in particular promoted FA utilization and protected insulin sensitivity and glucose uptake in FA-treated rats by stimulating AMPK/ACC pathway. However, this protective role was lost in high fat-overfed rats in the same conditions [455]. AG influence on lipid metabolism and oxidation was also proved in rats [456]. At the same time, UnAG appears as well – or even more involved in skeletal muscle preservation. UnAG positive effect on skeletal muscle insulin signaling was also confirmed in both *in vitro* and *in vivo* rat models [433,457].

UnAG and AG effects were also evaluated in mitochondrial activity. Non orexigenic doses of AG showed a positive effect on skeletal muscle of healthy rats by increasing uncoupling protein (UCP2) which contrasts ROS production [458]. In rat models of physiological alterations including chronic kidney disease and chronic heart failure, AG as well ameliorated mitochondrial oxidative capacity [459,460].

UnAG consistently reported an effect in reducing mitochondrial oxidation and ROS production [445] and resulted a myogenic regenerative inductor of satellite cells after ischemia and ROS-induced impairment [461]. In both the studies, UnAG appeared able to enhance superoxide dismutase's antioxidant activity, thus improving skeletal muscle cells oxidative stress. UnAG protective role against ischemia and comparable injuries was confirmed in other studies, that agreed on a correlation between UnAG treatment and an improvement of the damaged tissue condition [462,463]. Also *in vitro* studies on myocites bolstered UnAG-induced decrease of ROS release [464]. A further study of Gortan Cappellari et al., supported UnAG action in lowering ROS generation of skeletal muscle in a model of transgenic mice overexpressing UnAG, and this improvement in redox state persisted in analogus mice treated with HFD to induce obesity [445].

Few studies showed evidence that UnAG and AG may also be involved in the inflammatory system regulation. In fact constant administration of AG in HFD-induced obese rats induces a reduction of inflammation by decreasing TNF-α and NF-kB levels [456]. In line with this finding, the unacylated form of ghrelin hormone as well was detected to decrease the skeletal muscle's inflammatory state in rodents [445,465].

Additional evidence correlates ghrelin effects on skeletal muscle to autophagy and apoptosis; firstly an *in vivo* study showed that AG administration induced an increase of autophagy [466], but UnAG as well presented a positive correlation to the autophagic process [445,446,467]. Interestingly, UnAG seems to favor mitophagy activation in particular [468], according to UnAG role in improving mitochondrial function thus decreasing oxidative stress and ROS generation and stimulating mitochondrial regeneration. Moreover, UnAG prevents apoptosis in both *in vivo* and *in vitro* cardiomyocytes subjected to an ischemic damage [464]. Eventually, UnAG also showed to protect skeletal muscle against atrophy in *in vitro* and *in vivo* models of fasting and denervation-induced damage (Porporato et al., 2013), supporting the versatility and importance that this hormone can play.

1.2.2.3 Effects on adipose tissue

All the implications of ghrelin hormone in food intake and metabolism yield inevitable evidence to associate such peptide to one of the main metabolic organs, which is the adipose tissue.

However, while AG effects on adipose tissue have been pretty well investigated, much less is known about unacylated form of ghrelin. Few articles report a parallel action of AG and UnAG in promoting lipoprotein lipase (LPL) levels and lipid accumulation in human and rodents [470–472]. UnAG, as much as AG, was found to block isoproterenol-induced lipolysis [473]. UnAG infusions in

human volunteers reduced free fatty acids level for 16 hours, supporting the idea of promoted accumulation of triglycerides in WAT depots [474]. However, UnAG but not AG induced decreased release of glycerol and nonesterified fatty acids in 3T3-L1 adipocytes [475]. UnAG and AG were both found to blunt lipolysis in rodents subcutaneous and visceral adipose tissue *ex vivo*, however this effect was no longer reported in UnAG and AG administration *in vivo* [476]. Several studies concordingly reported that AG orexigenic function affects weight gain and adiposity, particularly by striking brain control [427], with consequent promoted adipogenesis and triglycerides intake and reduced fat utilization and lipolysis [477,478]. Right after ghrelin's discovery, Tschöp et al in 2000 reported on *Nature* the adipogenic effect of AG in rodents. AG infusion caused increased appetite with consequent enhanced food intake and adipogenesis [379]. *In vitro* studies soon brought to light a direct effect of AG on adipocytes, where it stimulates preadipocytes differentiation by increasing the main adipogenetic factor PPAR-γ production [479]. The same work showed that administrated AG to rat isolated adipocytes contrasts isoproterenol-induced lipolysis revealing that, somehow, AG can also modulate adipose tissue metabolism directly. Peripheral administration of AG for 7 days on mature male mice confirmed ghrelin-induced weight gain for increased adiposity, but without affecting food intake. The increased body weight corresponded to increased WAT caused by a major accumulation of triglycerides in adipocytes, while ectopic lipid accumulation in other organs including heart and kidney remained absent [480]. Ablation of ghrelin or of its receptor GHR-S in mice resulted in a protective effect against diet-induced obesity [481].

Eventually, a consistent lack of data persists regarding UnAG effect on WAT, leaving a complex but interesting chapter open to new studies and research.

1.2.2.4 Effects on mitochondrial function

Mitochondria as powerhouse organelles have been widely described. Mitochondria provide for oxidative phosphorylation and ATP production [482], and several processes ensure mitochondrial homeostasis in physiological conditions. A balance between fission and fusion is surely required for proper mitochondrial functionality and efficient respiration [483]. In addition, cellular bioenergetics are crucially modulated by mitochondrial reactive oxygen species production and essential for organ functionality [484].

The majority of articles investigating UnAG on mitochondria function, reports studies on skeletal muscle, one of the main metabolically active organs. Early studies showed AG ability to improve mitochondrial enzyme activities in skeletal muscle after 4-days of exogenous administration in healthy rats [444], and the same AG treatment also improved mitochondrial oxidative capacity in

pathological rodents model of uremia and heart failure [459,460], and preserved muscle from triglyceride accumulation in obese rodents [433]. AG-treatment also ameliorated mitochondrial activity in skeletal muscle in a model of sarcopenic mice [485].

Conversely, UnAG showed to reduce ATP synthesis in rodents model of UnAG sustained administration and constitutive overexpression [445]. However, AG and UnAG enhanced muscle anabolism and were protective against muscle atrophy in an aging mice model, but curiously UnAG potential raised major interest as no obesogenic side effects were reported [486]. Furthermore, UnAG was found to prevent mitochondrial dysfunction in liver after ischemia/riperfusion injury through affecting a mitochondrial fusion mediator [447].

1.2.2.5 Effects on oxidative stress

Free radicals are produced by mitochondria respiration, the main source of reactive oxygen species generation. Unbalanced ROS production contributes to worsen mitochondrial function and eventually reflects metabolic stress conditions [484]. Accordingly to UnAG role findings on mitochondrial function, UnAG showed to negatively affect oxidative stress: *in vitro* studies reported UnAG to reduce mitochondrial ROS generation in myocytes [464]. Also *in vivo* studies showed a role of UnAG but not AG in contrasting skeletal muscle oxidative stress induced hindlimb ischemia through dismutase SOD-2 action [461], and to protect vessels against ROS toxicity in mice with peripheral artery disease [487]. In support, additional studies confirmed that UnAG lowers mitochondrial ROS generation and improves redox statein skeletal muscle both *in vitro* and *in vivo* and particularly in an obese mice model [445,468].

On the other hand, UnAG but also AG were protective against oxidative damage in MC3T3-E1 cells [488]. Accordingly, AG showed antioxidant properties in 3T3 L1 preadipocytes [489], rat ovary [490,491] and rat kidney [491]. However, contrasting findings showed that AG treatment was not able to decrease oxidative capacity in obese rats [456].

1.2.2.6 Effects on inflammation

Since inflammation is a cellular response to harmful stimuli, several studies investigated the role of AG on inflammation, while only recently UnAG was also considered for its potential involvement in inflammation regulation. The already cited articles that reported UnAG beneficial effects on mitochondrial function and redox state, also supported UnAG anti-inflammatory role in myotubes and in rodents skeletal muscle and liver [446,445,468]. In agreement, UnAG was also found to reduce age-associated chronic inflammation in skeletal muscle of aged mice; curiously though, the same

study reported that also in a mice model of ghrelin knockout, systemic inflammation was decreased [492]. In addition, UnAG suppressed TNF levels, a main cytokine inflammatory marker in stromatic macrophages of brest adipose tissue [493].

AG role on inflammation instead, has been much more studied and characterized. Accordingly though, AG as well showed anti-inflammatory effects in several different models. Chum et al., showed that both UnAG and AG present a protective role against inflammation in diabetic humans [494]. AG exerts anti-inflammatory activity on human T lymphocytes through GHS-R [495]. In a rodent model of obesity, AG-treatment was also found to lower TNF-α and inflammation markers [449,456]. Moreover, anti-inflammatory action of AG was considered therapeutic in rodent models of colits and gastric inflammation [496,497] but also in humans [498], as well as in *in vitro* and *in vivo* model of skin disease [499]. Importantly, AG acts also on adipose tissue lowering inflammation in mice with AT atrophy [500]. AG administration improved inflammation in non-alcoholic fatty liver disease [448], but also in lungs, myocardial and kidney injury [501,502,503]. Eventually, UnAG and AG are surely involved in lowering inflammation in different districts with different modalities; most of the cited studies reported AG to act through its receptor, which has no proven affinity for UnAG.

1.2.2.7 Effects on insulin resistance

Ghrelin levels modulation gives an important cue to understanding its metabolism: circulating total ghrelin levels increase before a meal, to decrease then after food ingestion in rodents and human [379,504]. Postprandial suppression of plasma ghrelin is influenced by diet composition. Protein-enriched meals repress AG production the most, on the contrary lipids give the mildest effect in lowering AG levels while carbohydrates showed a double effects thus first strongly abolishing ghrelin release to then stimulate it at a later time [505].

Inevitably, such a metabolic hormone also was found to play a role in modulating insulin sensitivity. Early studies described total ghrelin to be inversely related to insulin resistance, reporting low levels of total ghrelin in subjects with type 2 diabetes compared to normoinsulinemic [506,507], even though not all the articles presented the same results [508]. However, the molecular mechanisms regulating UnAG and AG in relationship to insulin sensitivity are still under investigation. Barazzoni et al. showed that in obese metabolic syndrome patients AG positively correlated to HOMA-IR, an index of insulin resistance, as well as BMI and waist circumference. UnAG instead, was negatively related to these parameters in the same patients [433]. UnAG infusion was not found to affect insulin sensitivity in men with type 2 diabetes [509], and did not stimulate insulin secretion in healthy humans, both alone or co-administred with AG [510,511].

Conversely, co-administration of UnAG and AG improved insulin sensitivity and prevented AG alone-induced decrease of insulin sensitivity [512]. Consistenly, UnAG also showed a protective role against insulin signaling impairment in skeletal muscle of diabetic rodents [445,467]. However UnAG and AG were not found to affect directly glucose transport in skeletal muscle of rodents [513].

Interestingly, UnAG levels and insulin sensitivity were increased after physical activity [514] and after weight loss in obese subjects [515]. A human clinical study showed that total and unacylated but not acylated ghrelin negatively associated HOMA-IR in a 5-year follow-up, suggesting a role of UnAG as a potential predictor of insulin resistance development [457].

Glucose and consequent insulin metabolism are strictly related to liver metabolic dynamics, leading research focus on AG and UnAG effects on hepatic-related insulin signaling. Administration of AG seems to improve hepatic inflammation and oxidative stress in fat-induced liver impairment, but effects on insulin pathway appear controversial [448,449]. UnAG instead, exhibited beneficial effects in protecting glucose homeostasis in HFD-treated mice [516]. Ghrelin hepatic implications are also reflected on the liver-adipose tissue axis. While ghrelin may stimulate AKT-GSK3β insulin pathway in the liver, this may also turn in enhanced lipogenesis, which eventually may favor insulin resistance [450]. AG [420,478,517] and perhaps UnAG [518] may have lipogenetic effects on adipose tissue, but how it could affect insulin signaling is yet to be determined. Insulin resistance represents another metabolic feature often strongly related to obesity, and that surely deserves consideration in this articulate framework.

1.3 UNACYLATED GHRELIN EFFECTS ON ADIPOSE TISSUE METABOLISM AND IN OBESITY

On one side, ghrelin hormones are renowned for all their complex and global implications in metabolism network, including GH secretion, appetite, food intake, body weight, glucose homeostasis and energy consumption [421]. On the other side, obesity represents a critical feature that results from a set of factors, such as genetic, behavioral and environmental aspects, with consequent alteration of the metabolic and physiological system [65]. Hence derives the interest in evaluating ghrelin implications in such a disease. Disparate studies were carried out in an attempt to individuate ghrelin-signaling alteration driving obesity at a genetic level, aiming to find in ghrelin gene but also in its promoter and receptor, sequence variance that may directly link obesity pathology, but results were disparate and often in conflict with one another [519]. In particular, the unacylated form of ghrelin and the metabolic effects on adipose tissue with particular regard to obesity pathogenesis, which is characterized also by dysfunctional adipose tissue [348], are yet to be determined.

Lately, UnAG has shown to be an interesting player in metabolism [516] and few studies already suggested an implication in obesity and obesity-related disorders [520–522], however UnAG implications on adipose tissue and particularly in the weight gain process, remain largely uninvestigated.

UnAG plays a role in inhibiting food intake [523,524], and its circulating levels are reduced in obese rodents [525,526] and humans [527]. Consistently, anorexic patients reported higher levels of UnAG compared to control, but after short-term feeding treatment UnAG were recovered near the normal range [528]. Curiously, patients with Prader-Willi Syndrome (PWS) represent an exception to these correlations: this syndrome is a genetic disorder with common obesity, GH deficiency, hyperphagia and cognitive deficit [529]. Although obese, PWS patients present unchanged UnAG levels but increased AG-to-UnAG ratio [530], probably justified by the absence of GH secretion, which in turn results in stimulated ghrelin production [531,532].

Importantly, UnAG administration resulted in decreased fat pads mass [523]. Consistently, Barazzoni et al., showed that individuals with metabolic syndrome, in comparison to healthy subjects, presented lower levels of UnAG [433].

However, studies investigating UnAG effect on adipose tissue are only few and sometimes controversial. Delhanty et al. suggested a lipogenic effect of UnAG on mice adipose tissue [518], and this finding was supported by the evidence of UnAG as inhibitor of lipolysis in rats adipose tissue

[476]. In agreement, human visceral adipocytes showed enhanced lipid accumulation induced by UnAG [471]. In a transgenic mice model, UnAG overexpression induced a decrease of WAT and improved glucose tolerance and insulin sensitivity [533]. These findings were in agreement with *in vitro* studies which reported UnAG to improve glucose uptake in mature adipocytes from retroperitoneal fat of rats [534], while not supported by another study which showed that UnAG is not affecting glucose uptake in adipocytes from perirenal adipose tissue [535].

Finally, additional studies should clarify UnAG effects on adipose tissue and its influence in obesity and related metabolic alterations.

2. AIM OF THE STUDIES

Increasing evidence is supporting the hypothesis that UnAG plays a role in a complex cluster of metabolic tissues and function. Some recent studies have addressed UnAG association to skeletal muscle, where UnAG was found to positively modulate oxidative stress, inflammation and insulin sensitivity in association with improved systemic insulin resistance, but very little is known about specific UnAG effects on adipose tissue.

To this purpose, the current study aims to:

1) Investigate UnAG effects on white adipose tissue metabolism of healthy rats after 4-day of sustained exogenous administration in terms of mitochondrial activity, oxidative stress, inflammation and insulin signaling.

2) Compare these results with UnAG effects on white adipose tissue metabolism of transgenic mice presenting UnAG systemic upregulation, to evaluate UnAG in both short-term sustained injection versus constitutive overexpression in rodents adipose tissue

3) Evaluate UnAG effects on white adipose tissue of transgenic mice with UnAG systemic upregulation with high-fat diet-induced obesity, to test whether UnAG affects metabolic alteration on mitochondrial function and dynamics, redox state, insulin sensitivity, adipocytes size and remodeling machinery, factors known to be modified and associated to metabolic derangements in obesity.

In addition, this study also aims to test clinical relevance of UnAG in a human population from the North-East of Italy. In particular:

1) In an elderly cohort, we verify the potential negative association between UnAG and BMI through cross-sectional analyses, and the prediction of UnAG to muscle mass wasting after 5 years in subgroups of partecipants undergoing a 5-year follow-up

2) In a general population cohort, we test potential association between UnAG and body composition.

3. MATERIALS AND METHODS

3.1 Animal models

3.1.1 Administration of UnAG in rats

The presented study takes part of a follow-up of a published previous study [445].

With Animal Studies Committee of University of Trieste approval, twenty 12-weeks old male Wistar rats (Harlan-Italy, San Pietro al Natisone, Udine, Italy) were housed for two weeks at the University of Trieste Animal Facility. Rats were divided in individual cages with free access to water and standard chow (Harlan 2018, 14.2 kJ/g), in a 12 hours light/dark cycle conditions. Animals were randomly divided in two groups: a first Control group (Ct n=8) received subcutaneous injection of saline solution (NaCl 0.9% weight for volume), whereas 200-mg UnAG was administered to a second group (n=8) (Bachem, Bubendorf, Switzerland) twice a day for 4-days, in doses based on previous studies [444]. Rats were daily monitored for body weight and food intake; three hours after the last injection, animals were killed by anesthesia (tiobutabarbital 100mg/kg, tiletamine/zolazepam [1:1] 40 mg/kg i.p.) and retroperitoneal adipose tissue was surgically isolated – as well as other organs and blood by heart puncture – and frozen at -80°C for following analysis (Figure 12).

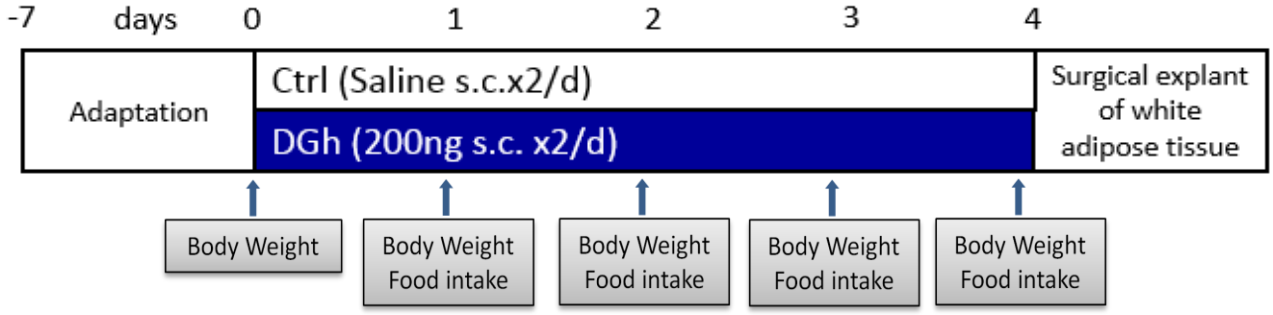


Figure 12. *Study protocol of healthy rats with saline solution or desacyl ghrelin (DGh) administration and time frame.*

3.1.2 UnAG overexpressing mice

The presented study takes part of a follow-up of a published previous study [445].

Transgenic mice overexpressing ghrelin (Tg Myh6/Ghrl) were obtained in collaboration with a research group of Università del Piemonte Orientale, Novara. Tg model was obtained by cloning ghrelin gene under control of the cardiac promoter sequences of the β-myosin heavy chain 3' UTR and the first 3 exons of the α isoform Myh6, as described in Porporato et al. [469]. UnAG overproduction in heart also resulted in fortyfold increased UnAG circulating level, while AG

concentration remained unchanged. Both wild-type and transgenic mice were randomly selected to undergo high-fat diet feeding (with 60% calories from fatty acids, Research Diets, New Brunswick, NJ) or standard diet (with 10% calories from fatty acids, Research Diets, New Brunswick, NJ) for 16 weeks (n=5 or 6 animals/group) (Figure 13). Body weight and food intake were monitored daily. Insulin was injected intraperitonealy (Humulin R 3 nmol/kg; Eli Lilly, Indianapolis, IN) after 4 hours of fasting in 15 weeks aged mice, in order to perform Insulin Tolerance Tests (ITTs), and blood was collected from the tail to perform glucose measurements (ACCU-CHEK Active; Roche, Basel, Switzerland) immediately before insulin injection and at 20, 40, 60 and 80 min.

Lastly, mice were killed in anesthesia (tiobutabarbital 100 mg/kg, tiletamine/zolazepem [1:1] 40 mg/kg i.p.) and tissues including both gastrocnemius muscle and left epididymal WAT were collected after careful surgical dissection, weighted, snap frozen in liquid nitrogen and kept at -80°C for further analyses. Plasma was separated by centrifugation, aliquoted and frozen at -80°C.

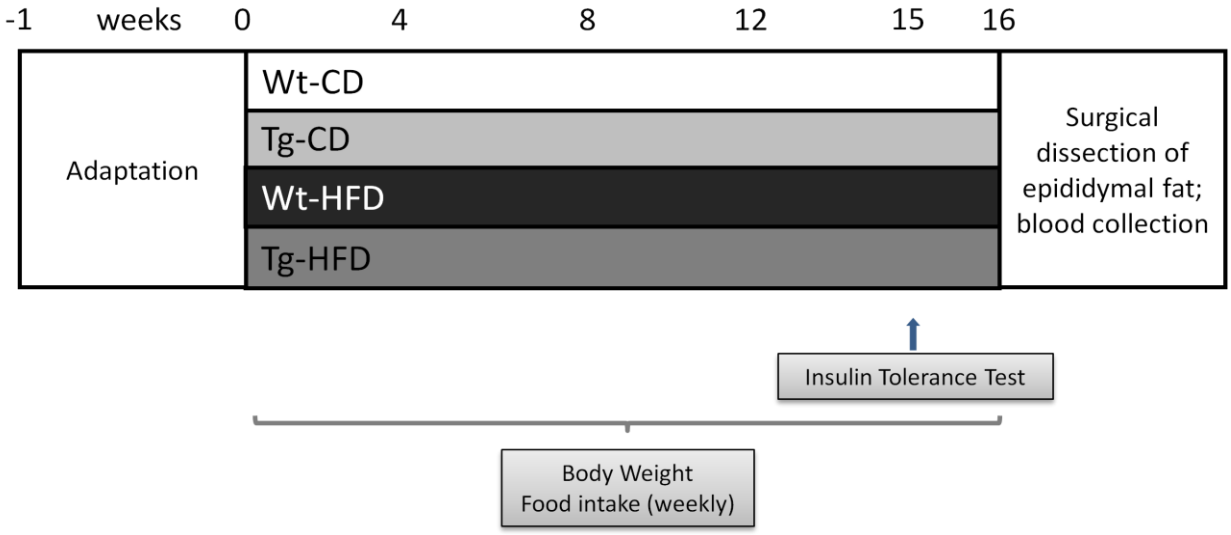


Figure 13. *Study protocol of UnAG overexpressing mice. Partition of the 4 mice groups (Wt-CD: wild-type with control diet; Tg-CD: Transgenic Myh6/Ghrl with control diet; Wt-HFD: wild-type with high-fat diet; Tg-CD: Transgenic Myh6/Ghrl with high-fat diet) and time frame.*

3.1.2.1 Plasma measurements

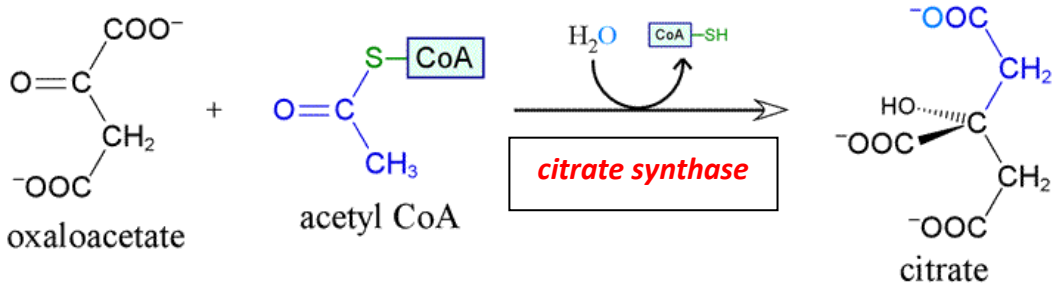
Plasma insulin concentration was measured by ELISA (Ultrasensitive ELISA; DRG, Springfield, NJ). Plasma glucose and nonesterified fatty acid (NEFA) concentrations were derermined by standard enzymatic colorimetric assay [536,537].

3.1.3 Enzymatic assay on mitochondrial activity

Enzymatic extracts were prepared from animal frozen tissues. Adipose tissues were sectioned in pieces in liquid nitrogen, and lately hand homogenized for 10 minutes in PBS (Phosphate Buffer Saline pH 7.4, Sigma); homogenation buffer was calculated as 4µl buffer/tissue mg. Tissue's homogenate was transferred in eppendorf and centrifuged at 600g for 10 minutes at +4°C. Centrifugation induced lipid accumulation on the top of the solution and cellular heavy structures precipitation on the bottom. Surnatant was extracted leaving pellet and lipids ring. A second centrifugation followed at same conditions to ensure lipid elimination. Surnatant was collected and frozen for the following analyses.

3.1.3.1 Citrate Synthase assay

Citrate synthase (CS) is a transferase enzyme which catalyzes the first reaction of the citric acid cycle, also known as Krebs cycle. It allows citrate production from oxaloacetate in the following reaction:



CS activity is calculated spectrophotometrically by a colorimetric technique that exploits CoA-SH, a released product from Oxaloacetate catalysis, which in turn interacts with 5,5'-Dithiobis 2-nitrobenzoic acid (DTNB) through its sulphydryl group thus yielding TNB, a yellow product. TNB is so detectable in spectrophotometric measurements in microplate, with an absorbance at 412 nm, and TNB development is directly proportional to CS kinetic [538]. The spectrophotometer reads the kinetic absorbance of the samples and generates a straight line whose slope indicates CS activity.

The assay was done by adding each adipose tissue extract sample in a microplate well and mixed with an Assay Buffer composed of: TrisBase Buffer 200 mM pH 8.2, Acetyl CoA 0.25 mM, DTNB 0.025 mM, Triton solution X-100 10%. Subsequently, Oxaloacetate solution (Oxaloacetate 7.6 mM

plus TrisBase Buffer 200 mM pH 8.2) was added to the Sample+ Assay Buffer mix right before the spectrophotometrical read. Spectrophotometer was set at 30°C on kinetic read at 412 nm at intervals of 10 seconds for 15 minutes. The absorbance in the different time steps for each sample generates a line with right ascension. The slope of this line is representative of CS activity concentration based on the formula:

$$Ec = \frac{\Delta A\lambda}{Cc} \cdot \frac{Vr}{Vc \cdot \varepsilon b \cdot l \cdot vb}$$

Where:

Ec= sample enzyme concentration

ΔAλ= delta of wavelength absorbance

Cc= sample total protein concentration

Vr= reaction volume

Vc= sample volume of reaction

εb= DTNB extinction coefficient (13.6 mM^{-1}/cm)

l= optical path length

vb= stoichiometric number (=1)

Delta value of the linear phase was taken for analysis after blank subtraction and after normalization based on sample protein content (from BCA protein assay- Thermo Scientific Pierce).

3.1.3.2 Cytochrome C Oxidase assay

Cytochrome C Oxidase (COX), also known as Complex IV, is the terminal enzyme of the respiratory chain. In the mitochondrion, COX allows energy production by coupling electron transport through the Cytochrome chain to the Oxidative Phosphorilation (Figure 14).

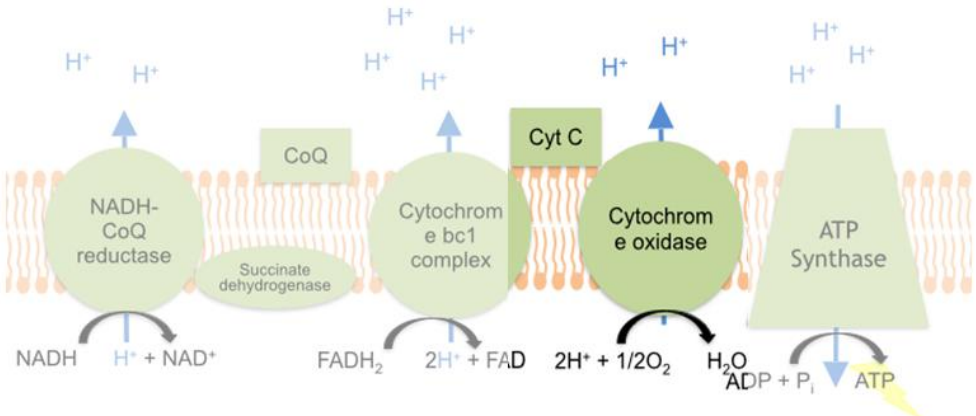


Figure 14. *Electron transport chain reactions in the mitochondrial inner memebrane.*

COX in the sample reduces its substrate Cytochrome C from ferrous to ferric state in a time-consuming reaction detectable by the spectrophotometer analysis at wavelength of 550 nm [539].
Adipose tissue extracts were loaded on the microplate and mixed with the Assay Buffer (constituted by BSA 0.65 mM, $MgCl_2$ 10.3 mM, KPI Buffer 50 mM and Cytochrome C 0.05 mM). The microplate is then read at the spectrophotometer preset at 30°C and 550 nm in kinetic mode (reads each 10 seconds for 10 minutes). Absorbance reads are translated in dots on a Cartesian plane that constitute a descending line. The slope of this line is representative of COX activity concentration based on the same formula previously described for CS.
Delta value of the linear phase was taken for analyses after blank subtraction and after normalization based on sample protein content (from BCA protein assay- Thermo Scientific Pierce).

3.1.4 Protein assay

3.1.4.1 Western blot analysis

Adipose tissue samples were sectioned in pieces in liquid nitrogen and stored at -80°C for following handling.
For healthy rat models, adipose tissue samples were hand homogenized for 10 minutes in Cell Extraction Buffer (Invitrogen) with addition of Phenylmethilsulfonyl fluoride (PMSF 1M) and 4.8% of Protease Inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO, USA); homogenation buffer was calculated as 4 µl buffer/tissue mg. Tissue homogenate was transferred in eppendorf and centrifuged at 13000 rpm for 10 minutes at +4°C, thus causing lipids compaction on top and organelles precipitation as a pellet. Surnatant was collected and frozen for further analyses.
For wild-type and transgenic (Myh6/Ghrl) mice, adipose tissue samples were hand homogenized for 10 minutes in PBS (4 µl buffer/tissue mg). Tissue omogenate samples were transferred in eppendorf and solutions were completed by adding 1% SDS, PMSF 1M and 4.8% of Protease Inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO, USA) to the extracts. The samples were then vortexed for 1 minute and centrifuged at 13000 rpm for 10 minutes at +4°C, thus causing lipids compaction on top and organelles precipitation as a pellet. Surnatant was collected and frozen for further analyses. Different detergents are reported to cause different effects on actin polymerization [540,541]. In particular, SDS is able to induce actin filament breakdown [542], and this resulted in our WAT mice samples, which presented a final SDS concentration ten-fold higher than conventional cell extraction buffer. This finally resulted in increased depolimerization of

filamentous (F)-actin which remains suspendend in the extracts, thus generating F-actin enriched fraction. Because of that, actin resulted no longer a valid normalizator for our WAT mice salmples.

For all our rodents WAT samples, a small amount of extract was used to detect protein concentration by BCA method [543]. This allowed to load the same amount of protein content for each sample maintaining a common final volume by dilution with PBS, in order to reach gel wells capacity. In the samples final volume, addition of 6x Laemmli Loading Buffer [544] (TrisBase 375 mM pH 6.8, SDS 9%, Glycerol 40%, Bromophenol blue 0.03%, β-mercaptoethanol 9%) was also considered. Final samples were denaturated at 95°C for 5 minutes and then loaded on a 12% polyacrylamide for a SDS-PAGE protein separation. Samples run first through a Stacking Gel (Tris 0,15 mM pH 6.8, SDS 0,05%, acrylamide 3.6%, Ammonium Persulfate 0,1%, TEMED 0,1%) and, once compacted, entered the Running Gel (Tris 0,4 M pH 8,8, SDS 0,05%, acrylamide 12%, Ammonium Persulfate 0,05% TEMED 0,01%). Gel was soaked in Running Buffer (TrisBase 0.25M, Glycine 1.92M, SDS 0.1%), and electrophoresis run at 80-100V and 10mA. After protein separation, gel was placed in a semi-dry transfer system in contact with a 0.2 µm nitrocellulose membrane to create a sandwich and wet with Transfer Buffer (TrisBase 0.25M, Glycine 1.92M, Methanol 10%). Transfer system was set at constant 17V. Final membrane was first stained with Ponceau S (Sigma) to preliminary check the correct protein transfer and the uniformity of protein content. After Ponceau S removal with PBS washes, membrane was incubated with Blocking Buffer (PBS, powder Milk 5%, Tween 0.05%) to saturate the membrane thus preventing non-specific binding of the antibodies to the free sites.

Primary Antibodies were then diluted to the appropriate concentration in Blocking Buffer and incubated with the membrane overnight (Table 2). Washing cycles in Washing Buffer (PBS, Tween 0.05%) followed membrane treatment, with subsequent re-incubation with Secondary Antibody horseradish peroxidase (HRP)-linked (Table 2), which allows final detection by exposing membrane to X-ray films for the requested time and consecutive develop first in developing solution, and second in fixing solution. X-ray films were acquired with densitometric scan (GS-700 Bio-Rad) and revealed protein bands were quantified by Quantity One image analysis software (Bio-Rad). Equal load was verified by Ponceau S staining first, and confirmed by Coomassie Blue staining.

In fact acylamide gel after membrane transfer was recovered as well and dyed with Coomassie Blue to visualize protein and further ensure equal protein content in the wells, thus granting correct normalization of detected protein of interest. The method requires a first incubation of the gel in Fixing Solution (H_2O mQ 65%, Acetic Acid 10%, 2-Propanol 25%) for 30 minutes, followed by

overnight incubation in Coomassie Blue solution (Coomassie blue SERVA 0.05%, Acetic Acid 10%) and a consecutive destaining (first destainer: H_2O mQ 90%, Acetic Acid 10%; second destainer: H_2O mQ 88%, Acetic Acid 7%, Methanol 5%) in two solvents that progressively reduce the background and leaves only the blue marked protein bands. Presented data were normalized with protein content.

Table 2. *Antibodies list.*

	Antibody	*Code*	*Manufacturer*
Insulin signaling	Phospho-AKT (Ser473)	9271	Cell Signaling Technology, Danvers, MA, USA
	AKT	9272	Cell Signaling Technology, Danvers, MA, USA
	Phospho-GSK-3β (Ser9)	9336	Cell Signaling Technology, Danvers, MA, USA
	GSK-3β	9315	Cell Signaling Technology, Danvers, MA, USA
Redox state	Cu/Zn SOD	SOD-101	Stressgen, Ann Arbor, MI
	MnSOD	SOD-110	Stressgen, Ann Arbor, MI
Mitochondrial dynamics	Mitofusin-1	ABC41	Millipore
	OPA1	80471	Cell Signaling Technology, Danvers, MA, USA
	Drp1	5391	Cell Signaling Technology, Danvers, MA, USA
Inflammation	TNF-α	sc-52746	Santa Cruz Biotechnology, INC.
Adipocytes remodeling	Arp2	3128	Cell Signaling Technology, Danvers, MA, USA
	Cofilin1	5175	Cell Signaling Technology, Danvers, MA, USA
	Profilin2	P0101	Sigma-Aldrich, Saint Louis, MO, USA
	β-actin	A3853	Sigma-Aldrich, Saint Louis, MO, USA
Secondary antibody	Anti-Rabbit IgG HPR-linked	7074	Cell Signaling Technology, Danvers, MA, USA
	Anti-Mouse IgG HPR-linked	NA931	GE Healthcare Life Sciences, Little Chalfont, UK

3.1.4.2 xMAP analysis

Luminex technology was used to determine insulin cascade activation at different levels. xMAP technology enables multiplex protein assays based on an antibodies-mediated recognition by color coded magnetic beads known as microspheres, which are read by the analyzer. Different beads for different analytes are characterized by different fluorophores which allow microsphere discrimination based on different concentration of fluorescent dyes within the beads. The microsphere passes through a red laser which, at 635 nm, excites the internal dyes, thus distinguishing the microsphere set. Another green laser at 532 nm excites the fluorescent dye on the reported molecule, giving the result of the assay. This method allows the read of multiple proteins all at the same time for a single sample (Figure 15).

Adipose tissue frozen samples were homogenized for 10 minutes in Cell Extraction Buffer (Invitrogen) completed with phenylmethylsulfonyl fluoride (PMSF) 1M and 4.8% of protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 13000 rpm at 4°C for 10 minutes, thus causing lipids compaction on top and organelles precipitation as a pellet. Surnatant was collected and frozen before analysis with xMAP technology (MAGPIX; Luminex Corporation, Austin, TX). Insulin signaling profile was measured with commercial kits validated by the manufacturer for multiplex profiling (LRC0002M, LHO0001M, LHO0002; Life Technologies, Carlsbad, CA) to detect total and phosphorylated insulin receptor (IR)$^{Y1162/Y1163}$, AKT^{S473}, GSK-3β^{S9}, pTSC2^{S939}, mTOR^{S2448}, p70S6K^{T421}, PTEN^{S380} and IRS-1^{S312}. Resulting data were interpolated to a standard curve using MILLIPLEX Analyst software (Millipore, Billerica, MA) and phosphorylation of each protein is expressed as phosphoprotein units per total in picograms.

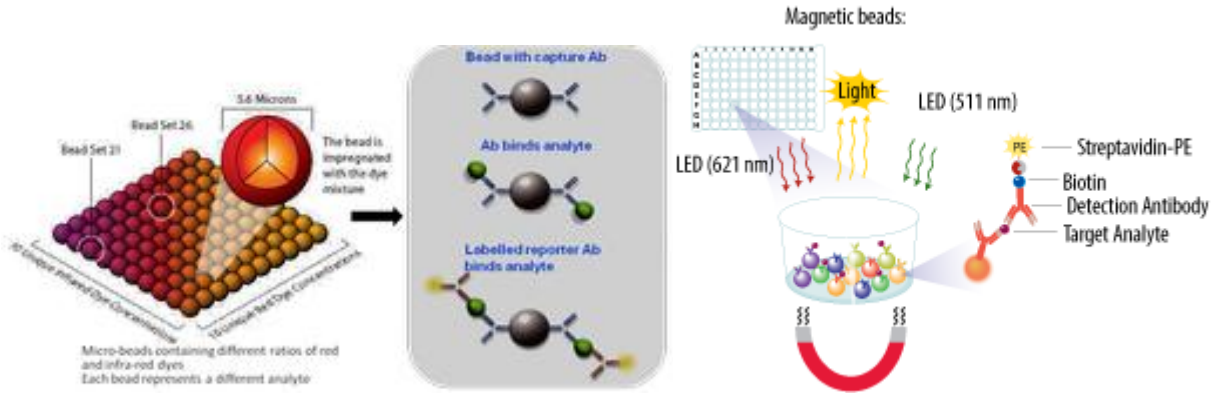


Figure 15. *xMAP technology. Samples containing the proteome of interest are added to dye-coded magnetic microsphere-antibody conjugated with target specific proteins. Then, secondary antibodies covalently bound with fluorescent labels are added. A magnet in MAGPIX analyzer capture and holds the magnetic beads in a monolayer, while two spectrally distinct LEDs illuminate the beads, thus one identifying the bead-related analyte, and the other determining the intensity of streptavidin-PE indirectly linked to the target analyte signal. Images adapted from www.bioassaysys.com*

3.1.5 Oxidized and Total Glutathione assay

Glutathione is a major player in cellular antioxidant defence, with its levels strictly related to oxidative stress. However glutathione presents two forms, the reduced sulfhydryl one (GSH) and the oxidized disulfide (GSSG). GSH is designated to contrast oxidative stress in cell through detoxification pathways involving glutathione reductase (GR), glutathione peroxidases (GPx) and glutathione-S-transferase, which lead to neutralize dangerous effects of oxidative radicals such as ROS, superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and xenobiotics [545]. The increase of the such molecules tends to unbalance the GSH/GSSG ratio, thus decreasing it. Therefore, the opportunity to measure GSH and GSSG in the cell extracts gives a clue of its oxidative stress status. The amount of glutathione in both GSH and GSSG forms can be measured spectrophotometrically as described by Rahman et al. [545]. Basically, GSH is able to react with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) in presence of GR and NADPH, thus producing TNB (Figure 16). GR reduces GSSG to GSH; GSH then reacts with DTNB, and forms a molecule of GS-TNB and releases another molecule of TNB. GR in turn, uses NADPH to reduce GS-TNB as well as GSSG in GSH, thus releasing TNB. Therefore, TNB production is measured spectrophotometrically at 412 nm. This technique allows to quantify total amount of oxidized and reduced glutathione in the sample, since GR with NADPH converts also GSSG in 2 molecules of GSH (Figure 16), so total [GSH] = [GSH] + 2*[GSSG]. The value of total GSH in the sample is determined from the regression curve generated from a pool of GSH standards.

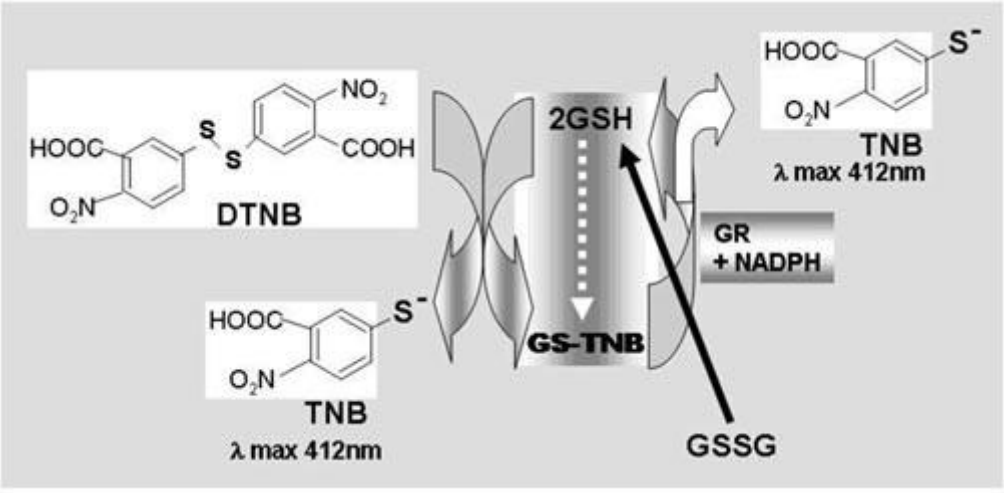


Figure 16. *The glutathione recycling reactions* [545].

Successively, GSSG is measured based on the same principle described, just samples are first treated with 2-vinylpyridine, a reagent which covalently reacts with glutathione only in its reduced

form (GSH), thus only the GSSG will react with GR and NADPH to release TNB. Excess of 2-vinylpyridine can be neutralized by adding triethanolamine.

Adipose tissue samples were homogenized in for 10 minutes in 5% metaphosphoric acid (weight/volume; 4µl of solution every mg of tissue), and then centrifuged at 12000g at 4°C for 15 min. Surnatant was separated from compacted fat ring and stored at -80°C for following analysis.

Samples were incubated at dark in 96-wells microplate for 30 seconds with Reaction Buffer (Glutathione Reductase 3.33 U/ml Sigma; DTNB 0.84 mM Sigma; KPE Buffer pH=7.5: EDTA 5 mM, KH_2PO_4 81.5 mM) in proportion Sample:Reaction Buffer= 1:6 to allow samples glutathione reduction. Subsequently, β-NADPH was added in solution with KPE (β-NADPH 0.8 mM in KPE buffer 0.1 M) to start the reaction, to quickly start the kinetic spectrophotometrical read with intervals of 60 seconds for 15 minutes. As previously described, these measures gave the amount of total [GSH] = [GSH] + 2*[GSSG] obtained by interpolation with the curve generated from standard samples of known GSH concentration and then normalized based on WAT pieces weight.

To determine GSSG levels, samples were incubated for 10 minutes with 4-vinilpiridine diluted 1:10 in KPE Buffer. In the following steps, samples were managed as previously described for total glutathione measurement. Finally, GSH was obtained by the difference of total GSH minus the GSSG amount.

3.1.6 Adipose tissue morphological analysis

Epididymal adipose tissue from wild-type and UnAG overexpressing mice with standard diet and HFD was analyzed histologically. Fat pieces were fixed in 10% Formalin for 12 hours and then embedded in paraffin. 5-µm of adipose tissues were sectioned by microtome, deparaffinized in xylene and rehydrated. Sections were stained first in Hematoxilyn (Sigma-Aldrich), and secondly counterstained in 0.25% Eosin (0.25 Eosin (Sigma-Aldrich), 80% v/v EtOH). After dehydratation in ethanol and clearing in xylene, sections were covered with a lipophilic solution and coverslip slides.

Adipose tissue sections were examined by light microscopy (Carl Zeiss-Jeneval) and digitalized with a high-resolution camera (Q-Imaging Fast 1394). The stained area was evaluated using Image-Pro Plus 6.3 (Media Cybernetics). Adipocytes area was measured with ImageJ Adiposoft plug-in. A range of 40-60 adipocytes were counted *per* image, and an average of 25 images *per* animal. Adipocytes size was reported as the area obtained in μm^2, where 1 µm corresponds to 2,16 pixels.

3.2 Human studies

3.2.1 MoMa study population and cohorts

Human analyses were part of an epidemiological study started in 2008 in MOntereale Valcellina and MAniago, two localities in the North-East of Italy, that gave the name MOMA to this clinical study. Supported by these municipals region, Friuli-Venezia Giulia, and realized by the University of Trieste in collaboration with GP outpatient clinic and the local health authority Azienda Sanitaria Friuli Occidentale (ASFO), the project aimed to investigate metabolic syndrome and related factors in these communities. Therefore, 2500 people aged between 18 and 69 were randomly selected from population lists to take part to this study, and among these, 865 male and 971 female for a total of 1836 participants agreed and were included in the study. The study protocol was approved by the competent Pordenone Hospital Ethics Committee and by Comitato Etico Unico Regionale FVG, and each subject gave written informed consent to participate.

The study started with the collection of participants clinical history, anthropometric measurements and a blood sample after 10-12 hours of night fasting. All variables were measured in duplicate, and the average of two measures was used for patient classification. Plasma samples were collected on the same day and used for biochemical profile assessment. Plasma aliquots were separated by centrifugation, frozen and kept at -80° until further analysis.

3.2.1.1 Ghrelin and muscle mass in aging

Among the individuals taking part to the MoMa basal cohort, a subgroup of randomly selected 450 elderly subjects (Age>65y; M/F: 179/271) was considered for analyses aimed to evaluate relations between ghrelin forms and muscle mass in lean to obese subjects during aging.

These subjects were divided in lean and overweight + obese based on their BMI (Lean: BMI < 25 kg/ m^2, Overweight+Obese: BMI > 25 kg/ m^2) [546].

3.2.1.2 5-year follow-up recall

From the same elderly subgroup cohort, 133 representative individuals were recalled for a 5-year follow-up evaluation, aimed to determine the relation between 5-year muscle mass and ghrelin profile, and the potential independence from confounding factors.

3.2.1.3 Ghrelin and fat mass/muscle mass

Besides, another cohort of all-age MoMa participants (Age: 57.5±16.7y, M/F:93/83) was examined to determine the relation between ghrelin forms and body composition in terms of the ratio fat mass/muscle mass, as this parameter is associated to insulin resistance [195] and metabolic syndromes [194,196,199].

3.2.1.3.1 Anthropometric measurements and derived indexes

Anthropometrical parameters were measured as follows: body weight (to the nearest 0.1 kg), height (to the nearest 0.5 cm), waist circumference (measured on bare skin during midrespiration at the natural indentation between the 10th rib and iliac crest, to the nearest 0.5 cm), blood pressure (measured on the right and left arm using a standard mercury sphygmomanometer), as reported in Table 3.

Then, following index were calculated as defined below:

- BMI = body weight [kg]/ height [m^2]
- MMI = muscle mass [kg]/ height [m^2]
- HOMA = (Fasting Plasma Glucose [mg/dL] x Fasting Plasma Insulin [µU/mL])/405
- Mean Arterial Pressure (MAP) = (systolic blood pressure + 2 x diastolic blood pressure)/3

Subjects were considered lean, overweight or obese based on BMI:

- Lean: BMI < 25 kg/ m^2
- Overweight: 25 kg/ m^2 < BMI < 30 kg/ m^2
- Obese: BMI > 30 kg/ m^2

3.2.1.3.2 Definition of Metabolic Syndrome (MetS)

Metabolic alterations were considered based on ATP III classification thresholds (reported in the following table) or the presence of ongoing related therapy. Metabolic syndrome parameters in participants were defined following Table 3:

Table 3. *Metabolic parameters considered for subjects participating to MoMa study.*

Metabolic alteration	**Defined threshold**
Hypertension	systolic blood pressure ≥135 mmHg and diastolic blood pressure ≥85 mmHg
Hypertriglyceridemia	plasma triglycerides ≥150 mg/dl
Low HLD-cholesterol	HDL-cholesterol <50 mg/dl for female and <40 mg/dl for male
Hyperglycemia	plasma glucose ≥110 mg/dl

High waist circumference	waist circumference >88 for female and >102 for male
Hypercholesterolemia	plasma total cholesterol >200 mg/dl
Diabetes mellitus	fasting plasma glucose >126 mg/dl

3.2.1.3.3 Plasma measurements

Plasma triglycerides, high-density lipoprotein (HDL), cholesterol, glucose and insulin were measured using standard methods at the Pordenone Hospital Laboratory.

Plasma total and acylated ghrelin were measured using radioimmunoassay (Linco, St Charles, MO, USA). Plasma total and acylated ghrelin were measured using radioimmunoassay (Linco, St Charles, MO, USA) following manufacturer's recommendations. UnAG was calculated as total ghrelin subtracted of acylated ghrelin levels.

Plasma cytokine levels, measured through the previously described xMAP technology (Millipore, Billerica, MA, USA) with the Magpix reader (Luminex, Austin, TX, USA).

High sensitivity C Reactive Protein (hsCRP) was measured by ELISA (DRG, Germany).

3.2.1.3.4 Body composition assessment

Body composition was measured by multifrequence bioimpedance analysis (BIA101, Akern, Italy) which allowed to estimate muscle and fat mass; subsequently, fat and muscle mass data were calculated using manufacturer's proprietary software (BodyGram 3.0, Akern, Italy).

3.3 Statistical Analysis

3.3.1 Animal studies

Data are showed as mean ± SEM. Groups were tested by Student's t test, while for multiple comparison among groups was used the one-way ANOVA test followed by appropriate post-hoc tests. Multiple comparisons were then corrected by the Bonferroni method. Results with $p < 0.05$ were considered statistically significant. All the analyses were performed using SPSS v.17 software (SPSS Inc., Chicago, IL).

3.3.2 Human Studies

Data distribution for continuous variables was verified using Shapiro-Wilk test. The whole study population was compared to the subgroup that participated to the follow-up for statistical similarity and continuous variables were tested by Student's t test or Mann-Whitney u test as appropriate; then, χ-square test determined similarity for percentage expressed data.

Several parameters, including HOMA and BMI showed an asymmetrical distribution, thus correlation between variables was assessed by the Spearman test. Association strengths were compared using Meng, Rosenthal and Rubin method. Statistical associations were considered significant for $p < 0.05$. Relation between ghrelin forms, BMI and muscle mass also included confounding parameters in stepwise multiple regression analyses, thus considering variables potential interference. Multiple linear regression analyses were validated by determining the normality of residuals. Prediction of 5-year low muscle mass index was tested by logistic multiple regression.

The values of the variables tested presented different orders of magnitude, and regression coefficients of the ghrelin forms resulted very low in absolute values; therefore, ghrelin's regression coefficients are presented multiplied by a 1000x factor.

Continuous variables are presented as Mean ± standard deviation (SD).

All the analyses were performed using SPSS v.17 software (SPSS Inc., Chicago, IL).

4. RESULTS

4.1 UnAG effects on adipose tissue after 4-day UnAG administration in healthy rats

4.1.1 Animal characteristics

Characteristics of adult healthy rats treated with Control (Ct) or UnAG are reported in Table 4. As shown, 4-day UnAG administration did not modify body weight, weight gain, and caloric intake as well as plasma glucose, insulin and NEFA (Table 4) [445].

Table 4. *Animal characteristics. Effects of UnAG (200µg subcutaneous injection twice per day) versus saline (Control) sustained 4-day administration on body weight, weight gain during treatment, caloric intake, plasma glucose, plasma insulin, plasma NEFA. Data are presented as Mean ± SEM (n= 8/group). No statistically significant differences were reported between groups.*

	Control (Ct)	**UnAG**
Body weight	319.6 ± 3.6 g	324.1 ± 6.1 g
Weight gain during treatment	13.0 ± 1.4 g	11.6 ± 1.1 g
Caloric intake	76.9 ± 2.3 kcal/day	73.5 ± 1.8 kcal/day
Plasma glucose	118.6 ± 6.0 mg/dL	120.5 ± 7.5 mg/dL
Plasma insulin	12.8 ± 2.1 µU/mL	14.3 ± 2.9 µU/mL
Plasma NEFA	0.27 ± 0.06 mmol/L	0.21 ± 0.03 mmol/L

4.1.2 4-day exogenous administration of UnAG effects on WAT mitochondrial function

In UnAG-treated rats, mitochondrial function was tested by enzymatic assays of Citrate Synthase (CS), the first transferase enzyme which takes part to Krebs cycle, and Cytochrome C Oxidase (COX), the terminal enzyme of the respiratory chain. The results show that no difference was found in CS and COX activity in UnAG-treated rats compared to control (Figure 17A-B), suggesting that 4-day UnAG exogenous administration has no effect on mitochondrial functionality of WAT.

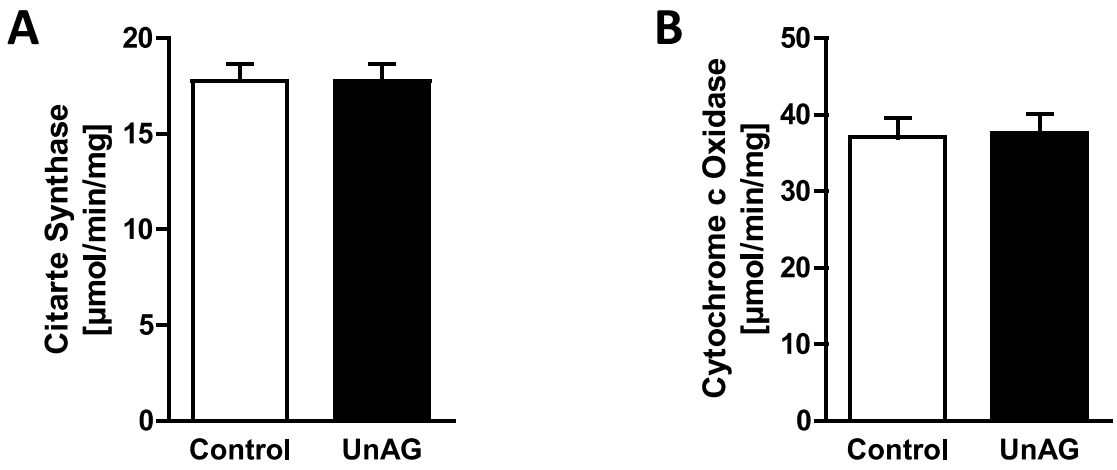


Figure 17. *UnAG and WAT mitochondrial function. Effects of UnAG (200µg subcutaneous injection twice per day) versus saline (Control) sustained 4-day administration on CS (A) and COX (B) mitochondrial enzyme activity. Data are presented as Mean ± SEM (n= 8/group). *p < 0.05.*

4.1.3 4-day exogenous administration of UnAG effects on WAT redox state

UnAG effect in adipose tissue was evaluated on oxidative stress by testing markers of the redox state. In adipose tissue, UnAG 4-day administration did not change total glutathione levels; however, the ratio oxidized-over-total glutathione decreased in WAT of UnAG-treated rats, a ratio that varies within the cell based on redox state as oxidative radicals induce an increase of oxidized-over-total glutathione levels (Figure 18A-B).

Moreover, UnAG treatment did not modify dismutase (CuZnSOD and MnSOD) levels in WAT, enzymes that play a major role in the defense against toxic oxygen species (Figure 18C-D).

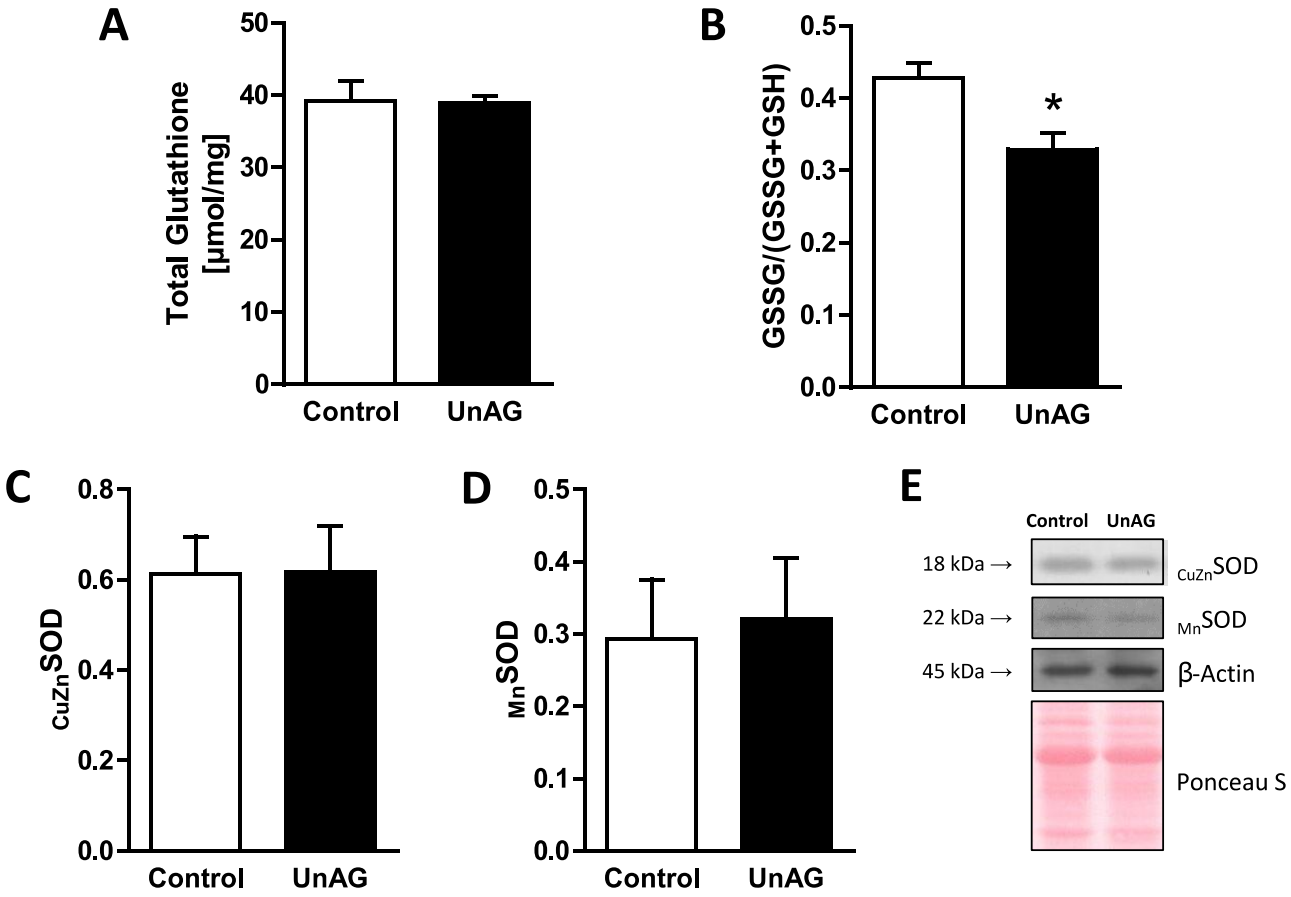


Figure 18. *UnAG and WAT redox state. Effects of UnAG (200µg subcutaneous injection twice per day) versus saline (Control) sustained 4-day administration on total (A) and oxidized (GSSG) over total (GSH+GSSG) tissue glutathione (B), and on dismutase levels of CuZnSOD (C) and MnSOD (D), with representative blots (E). Data are presented as Mean ± SEM (n= 8/group). *p < 0.05.*

4.1.4 4-day exogenous administration of UnAG effects on WAT inflammation

Western blot analyses were performed on rats WAT samples to check effects of UnAG 4-day administration on inflammation, and results showed no significant changes on TNF-α levels in UnAG-treated rats adipose tissue compared to control (Figure 19).

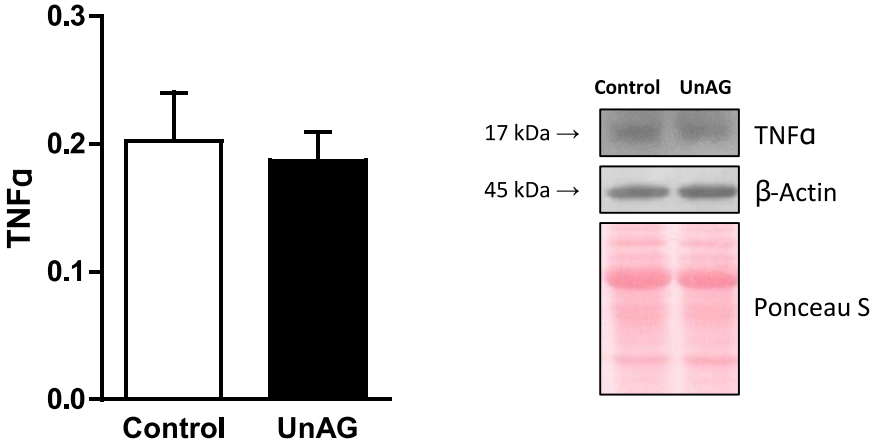


Figure 19. *UnAG and WAT inflammation. Effects of UnAG (200µg subcutaneous injection twice per day) versus saline (Control) sustained 4-day administration on TNF-α with representative blots. Data are presented as Mean ± SEM (n= 8/group). *p < 0.05.*

4.1.5 4-day exogenous administration of UnAG effects on WAT insulin signaling

Insulin signaling in WAT was tested by western blot analyses which showed that UnAG administration reduces phosphorylation of AKT^{S473} and GSK-$3\beta^{S9}$, two key mediators of insulin response (Figure 20A-B).

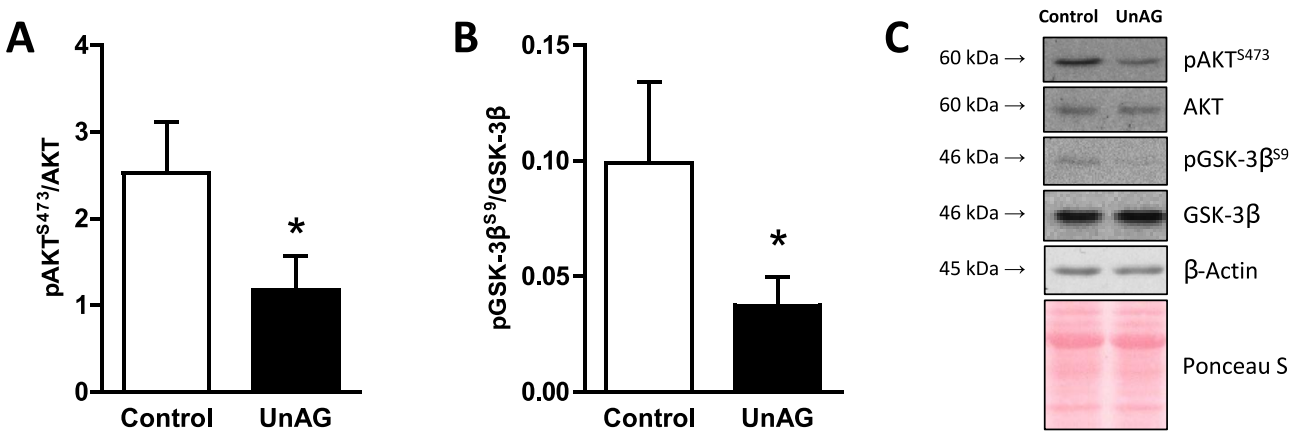


Figure 20*. UnAG and WAT insulin signaling. Effects of UnAG (200µg subcutaneous injection twice per day) versus saline (Control) sustained 4-day administration on $pAKT^{S473}$/AKT (A) and on $pGSK\text{-}3\beta^{S9}$/ GSK-3β (B), with representative blots (C). Data are presented as Mean ± SEM (n= 8/group). *p < 0.05.*

xMAP high throughput assays confirmed these results and allowed a deeper analysis of insulin pathway. UnAG showed decrease of insulin signaling by reducing phosphorylation of AKT^{S473}, GSK-$3\beta^{S9}$, $pTSC2^{S939}$, $mTOR^{S2448}$, $p70S6K^{T421}$ (Figure 21A-F). Phosphorylation was significantly reduced in

AKT^{S473}, GSK-3β^{S9}, p70S6K^{T421} and strongly decreased in pTSC2^{S939} and mTOR^{S2448}, but not at pIR$^{Y1162/Y1163}$ suggesting that UnAG effect on WAT may be possibly mediated by other signals that interact with insulin signaling downstream of its receptor.

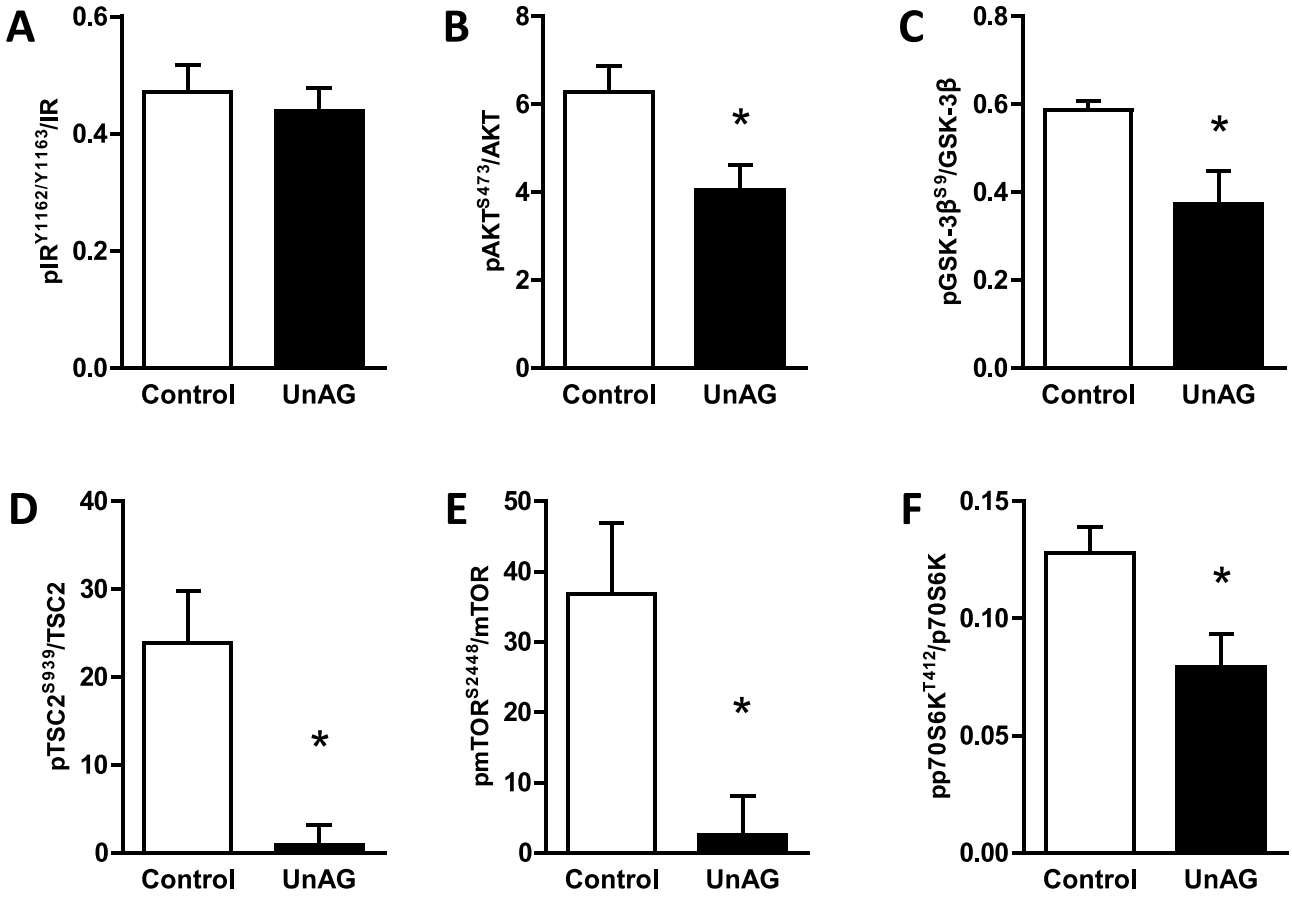


Figure 21. *UnAG and WAT insulin signaling. Effects of UnAG (200μg subcutaneous injection twice per day) versus saline (Control) sustained 4-day administration on pIR$^{Y1162/Y1163}$/IR (A), pAKT^{S473}/AKT (B), pGSK-3β^{S9}/ GSK-3β (C), pTSC2^{S939}/TSC2 (D), pmTOR^{S2448}/mTOR (E), pp70S6K^{T421}/p70S6K (F). Data are presented as Mean ± SEM (n= 8/group). *p < 0.05.*

To further investigate UnAG on insulin signaling of rats WAT, xMAP analysis was also assessed for insulin signaling modulator PTEN and negative feedback factor IRS-1. Phosphorylation of PTEN^{S380}, the major negative modulator of PI3K/AKT pathway, was unchanged (Figure 22A), while phosphorylation of IRS-1^{S312} (Figure 22B) which constitutes a negative modulatory feedback mediator for insulin signaling activation by disruption of insulin receptor (IR)-IRS1 bound, was found to be significantly increased in UnAG-treated rats, further supporting the hypothesis that UnAG 4-day administration reduces insulin signaling in rats adipose tissue.

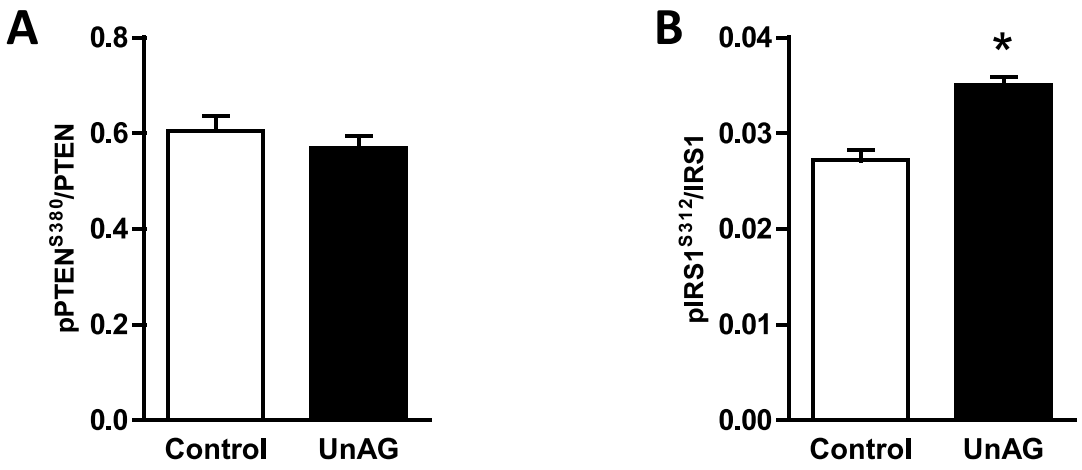


Figure 22. *UnAG and WAT insulin signaling. Effects of UnAG (200µg subcutaneous injection twice per day) versus saline (Control) sustained 4-day administration on pPTEN^{S380}/PTEN (A) and IRS-1^{S312}/IRS-1 (B). Data are presented as Mean ± SEM (n= 8/group). *p < 0.05.*

4.2 UnAG effects on adipose tissue in mice with constitutive systemic UnAG overexpression in standard diet or HFD-induced obesity

4.2.1 Animal phenotypes

UnAG myocardial constitutive overexpression (Tg Myh6/Ghrl) increased UnAG systemic levels and did not modify body weight and caloric intake in both control diet (CD) and HFD, as results were comparable between wild-type and transgenic (Tg) mice under the same diet [445]. Blood glucose and plasma insulin were comparable among lean groups but they resulted decreased in HFD-obese Tg Myh6/Ghrl compared to obese Wt; HFD was confirmed to induce hyperglycemia and hyperinsulinemia. No significant differences were found in plasma NEFA levels among all groups (Table 5).

Table 5. *Animal phenotype. Body weight (measured at Time 0 (T0) and at the end of the protocol, after 16 weeks (T16)), caloric intake, plasma glucose, insulin and non esterified fatty acid (NEFA) in wild-type (Wt) and transgenic mice (Tg Myh6/Ghrl) with systemic overexpression of UnAG fed with Control Diet (CD) or High-Fat Diet (HFD). Data are presented as Mean ± SEM. p < 0.05 among groups with different letters.*

	CD-Wt	CD-Tg	HFD-Wt	HFD-Tg
Body weight (T0)	23.5 ± 0.8 [a]	22.8 ± 0.7 [a]	21.2 ± 0.7 [a]	23.1 ± 0.7 [a]
Body weight (T16)	31.0 ± 2.1 [a]	28.7 ± 2.1 [a]	37.9 ± 3.0 [b]	36.6 ± 1.1 [b]
Caloric intake (kcal/day)	13.4 ± 0.2 [a]	14.3 ± 0.7 [a]	18.1 ± 0.4 [b]	17.6 ± 0.5 [b]
Plasma glucose (mg/dL)	106.0 ± 7.3 [a]	98.2 ± 8.0 [a]	161.9 ± 30.7 [b]	102.7 ± 11.6 [a]
Plasma insulin (µU/mL)	13.3 ± 1.8 [a]	14.5 ± 3.1 [a]	25.1 ± 2.2 [b]	16.5 ± 1.6 [a]
Plasma NEFA (mmol/L)	0.32 ± 0.06 [a]	0.38 ± 0.08 [a]	0.27 ± 0.05 [a]	0.32 ± 0.10 [a]

In the first phase of this study protocol, as already described in a work from our group [445] systemic insulin sensitivity was tested by insulin tolerance test (ITT)-induced blood glucose changes (Figure 23A) and measured from the resulted area under the curve (Figure 23B). Moreover, those findings showed that UnAG overexpression in Tg Myh6/Ghrl transgenic mice recovered systemic insulin sensitivity in obese mice, in association with a profound impact on skeletal muscle tissue metabolism [445].

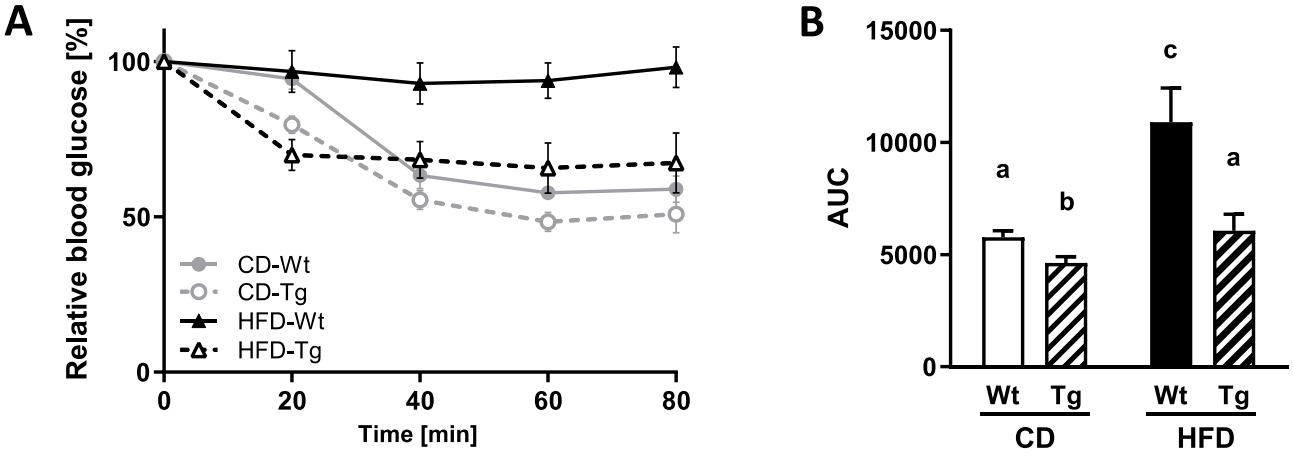


Figure 23. *UnAG overexpression on systemic insulin sensitivity. Effects of UnAG overexpression in Tg Myh6/Ghrl (Tg) versus wild-type (Wt) mice fed 16 weeks with control diet (CD) or HFD* [445] *on relative blood glucose (A) and the corresponding area under the curve (AUC) (B). Data are presented as Mean ± SEM. p < 0.05 among groups with different letters.*

4.2.2 UnAG overexpression effect on mitochondrial function in mice WAT in CD and HFD condition

UnAG overexpression does not significantly change CS enzymatic activity in UnAG transgenic mice compared to wild-type with control diet (CD), and recovers CS reduced levels caused by HFD (Figure 24).

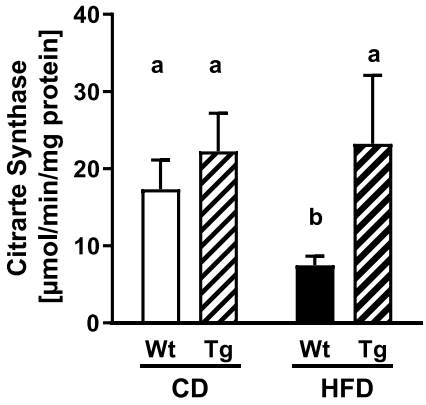


Figure 24. *Effect of systemic UnAG overexpression on adipose tissue mitochondrial function. Effects of UnAG overexpression in Tg Myh6/Ghrl (Tg) versus wild-type (Wt) mice fed 16 weeks with control diet (CD) or HFD on citrate synthase (CS) activity. Data are presented as Mean ± SEM. p < 0.05 among groups with different letters.*

4.2.3 UnAG overexpression effects on mitochondrial dynamics in mice WAT in CD and HFD condition

Following up on the results on mitochondrial function, UnAG effect on mice WAT was evaluated in terms of impact on mitochondrial dynamics, which is essential for mitochondrial homeostasis,

particularly under stress condition. UnAG upregulation did not cause significant changes in mitochondrial fusion markers level (Mitofusin-1 and OPA1) in control diet, while wild-type HFD-induced obese mice reported a significant decrease of both Mitofusin1 and OPA1, in agreement with findings from other authors [263]. Interestingly, UnAG systemic overexpression increased the levels of both mitochondrial fusion markers in obese mice (Figure 25A-B).

In agreement with these results, UnAG constitutive overexpression did not alter Drp1 levels, a marker of mitochondrial fission, while restoring Drp1 levels lowered by HFD (Figure 25C).

Mitochondrial dynamics were also evaluated as Mitofusin-1 : Drp1 ratio, and results confirmed that UnAG does not modify fission and fusion balance in standard diet condition, but it recovers HFD-induced lower fusion to fission rate in mice WAT (Figure 25D).

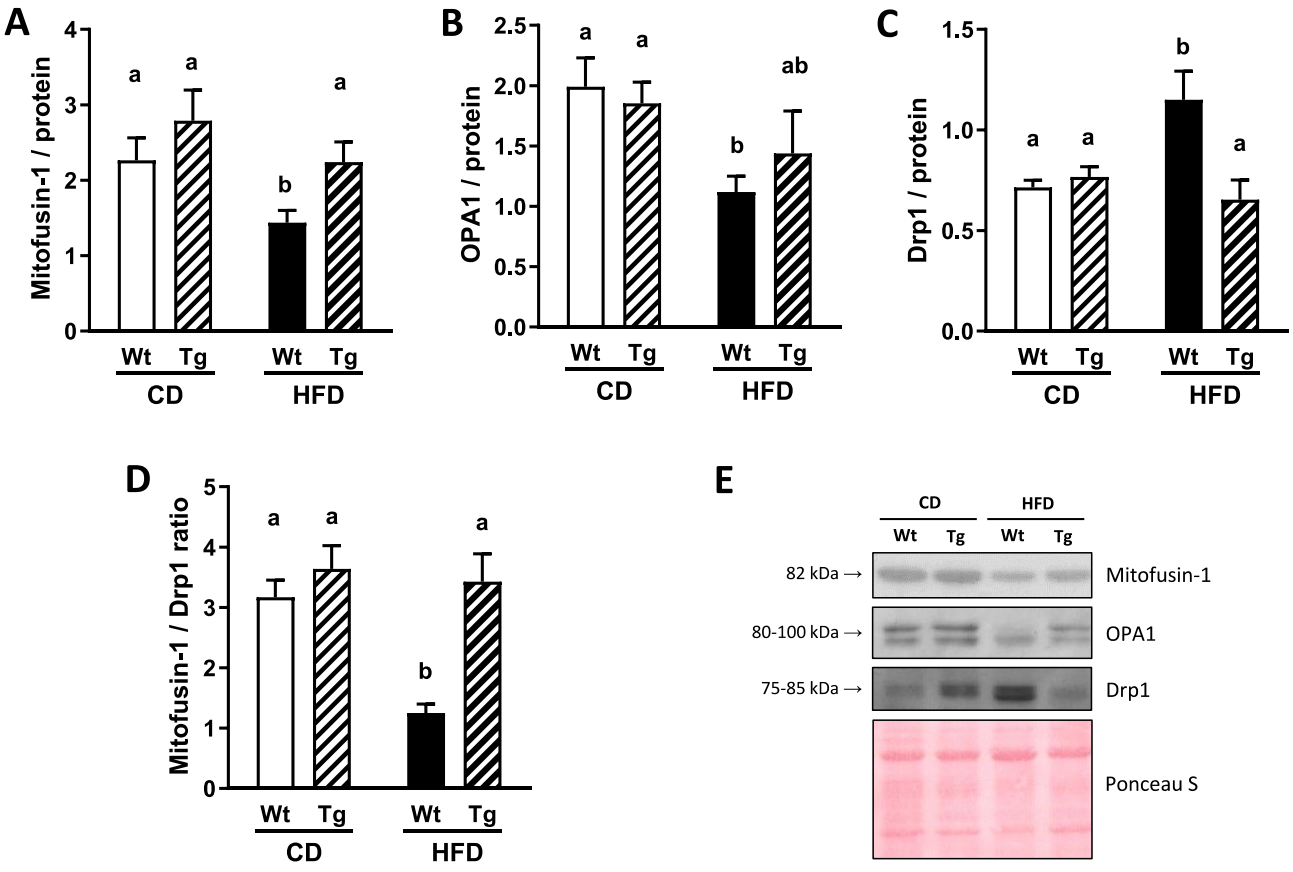


Figure 25. *Effect of systemic UnAG overexpression on adipocytes mitochondrial dynamics. Effects of UnAG overexpression in Tg Myh6/Ghrl (Tg) versus wild-type (Wt) mice fed 16 weeks with control diet (CD) or HFD on mitochondrial fusion markers Mitofusin-1 (A) and OPA1 (B), on Drp1 fission marker (C), and on Mitofusin-1 : Drp1 ratio (D), with representative blots (E). Data are presented as Mean ± SEM. p < 0.05 among groups with different letters.*

4.2.4 UnAG overexpression effects on redox state in mice WAT in CD and HFD condition

UnAG systemic upregulation effect on mice WAT tissue redox state was tested by dismutase isoforms protein expression levels. UnAG overexpressing mice with CD reported the same levels of MnSOD and CuZnSOD compared to wild-type ones. HFD was found to decrease cytoplasmatic CuZnSOD levels (Figure 26A) and to increase mitochondrial MnSOD levels (Figure 26B). However, UnAG overexpression did not significantly change MnSOD levels in obese mice, although Tg mice with HFD showed a trend to lower the enzyme expression to values also comparable to CD. Besides, UnAG overexpression clearly and completely recovered CuZnSOD expression levels (Figure 26A-B).

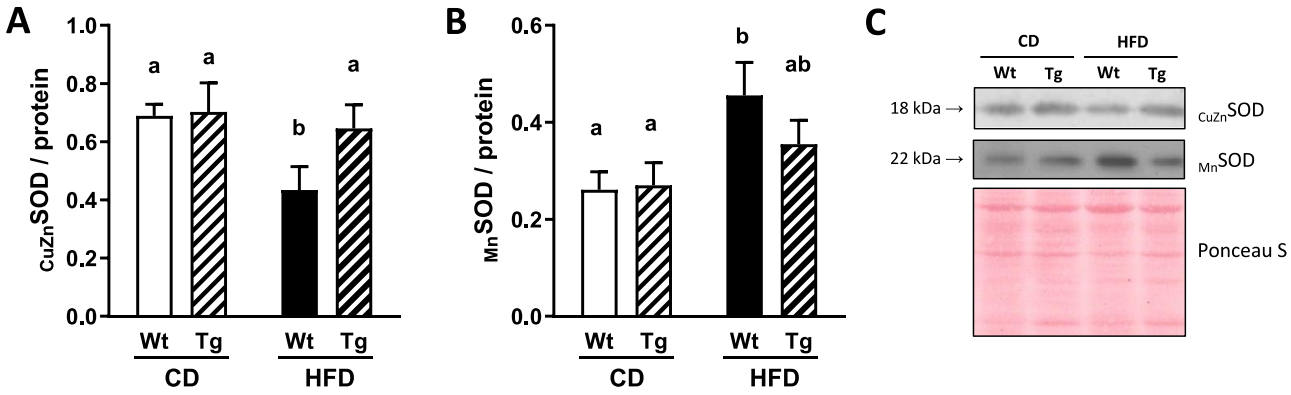


Figure 26. *Effect of systemic UnAG overexpression on adipose tissue oxidative stress. Effects of UnAG overexpression in Tg Myh6/Ghrl (Tg) versus wild-type (Wt) mice fed 16 weeks with control diet (CD) or HFD on protein expression levels of CuZnSOD (A) and MnSOD (B), with representative blots (C). Data are presented as Mean ± SEM. p < 0.05 among groups with different letters.*

4.2.5 UnAG overexpression effects on inflammation in mice WAT in CD and HFD condition

UnAG systemic overexpression did not change TNF-α levels, one of the main pro-inflammatory cytokine, in CD-fed mice adipose tissue (Figure 27). Accordingly to previous findings in the literature [125], HFD raised inflammation in adipose tissue with increased TNF-α levels; interestingly though, UnAG overexpression decreased TNF-α levels of obese mice WAT, showing TNF-α protein concentration comparable to the ones of the control diet models (Figure 27).

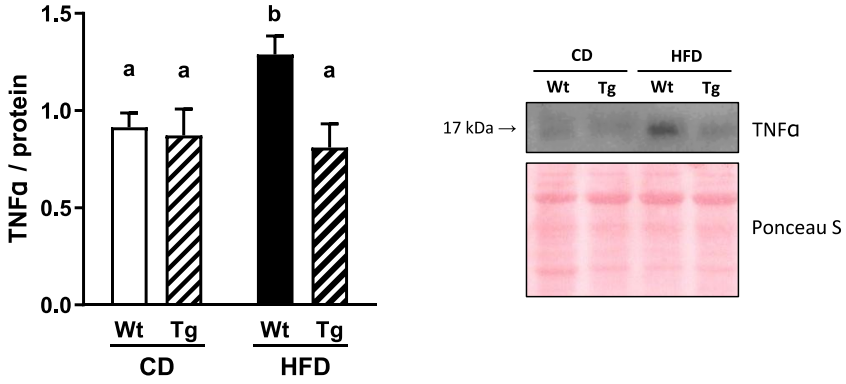


Figure 27. *Effect of systemic UnAG overexpression on adipose tissue inflammation. Effects of UnAG overexpression in Tg Myh6/Ghrl (Tg) versus wild-type (Wt) mice fed 16 weeks with control diet (CD) or HFD on TNF-α cytokine levels, with representative blots. Data are presented as Mean ± SEM. p < 0.05 among groups with different letters.*

4.2.6 UnAG overexpression effects on insulin signaling in mice WAT in CD and HFD condition

HFD-fed mice reproduced a model of insulin resistance that showed as expected a strong decrease of AKT^{S473} and GSK-$3\beta^{S9}$ phosphorylation in WAT. Importantly, UnAG overexpression led to a significant increase of $pAKT^{S474}$/AKT (Figure 28A) and $pGSK\text{-}3\beta^{S9}$/ GSK-3β (Figure 28B) in obese animals, thus protecting from HFD-induced impairment of insulin signaling.

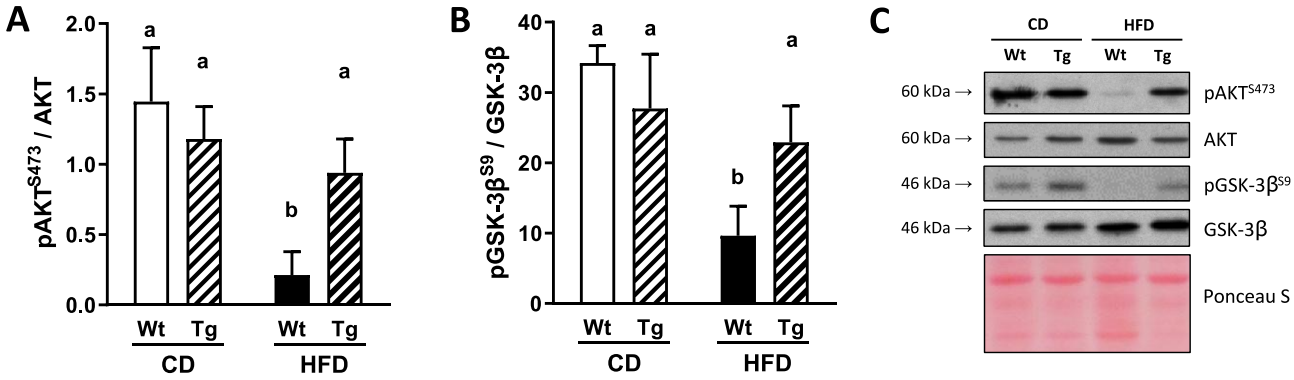


Figure 28. *Effect of systemic UnAG overexpression on adipose tissue insulin signaling. Effects of UnAG overexpression in Tg Myh6/Ghrl (Tg) versus wild-type (Wt) mice fed 16 weeks with control diet (CD) or HFD on $pAKT^{S473}$/AKT (A) and on $pGSK\text{-}3\beta^{S9}$/GSK-3β (B) levels, with representative blots (C). Data are presented as Mean ± SEM. p < 0.05 among groups with different letters.*

4.2.7 UnAG overexpression effects on adipose tissue mass and adipocytes morphology in mice WAT in CD and HFD condition

UnAG upregulation did not change epididymal fat pads mass, which weighted similarly among genotypes in both treatment with CD and HFD in absolute values. However, in line with expectations, HFD led to a significant increase of fat mass pads in Tg and Wt mice compared to respective CD models (Figure 29A). Interestingly though, when fat pads mass was compared in terms of relative weight to gastrocnemius muscle mass, constitutive UnAG overexpression resulted in lower values after HFD (Figure 29B). This result is consistent with the evidence that fat-to-muscle mass, an index of a clinical relevance, is in fact increased in insulin resistant humans [195] in association with diagnosis of metabolic syndrome [194,196,199].

Histological analysis allowed to evaluate the effects of UnAG systemic overexpression on adipocyte size. UnAG overexpression did not modify adipose cell area in lean mice. In obese mice adipocytes are shown to be considerably enlarged, and this result agrees with the known effect of high-fat diet to increase lipidic content in adipocytes [344]. Importantly, UnAG constitutive overexpression prevented HFD-induced adipocyte expansion, with a marked decrease in adipocyte size in HFD Tg Myh6/Ghrl mice compared to HFD wild-type (Figure 29C-D). Further analysis also showed that HFD-Tg presented a cell size frequency pattern comparable to that of CD mice, at variance with HFD-Wt which were characterized by lower in small but higher in large adipocyte frequency compared to all other groups (Figure 29E).

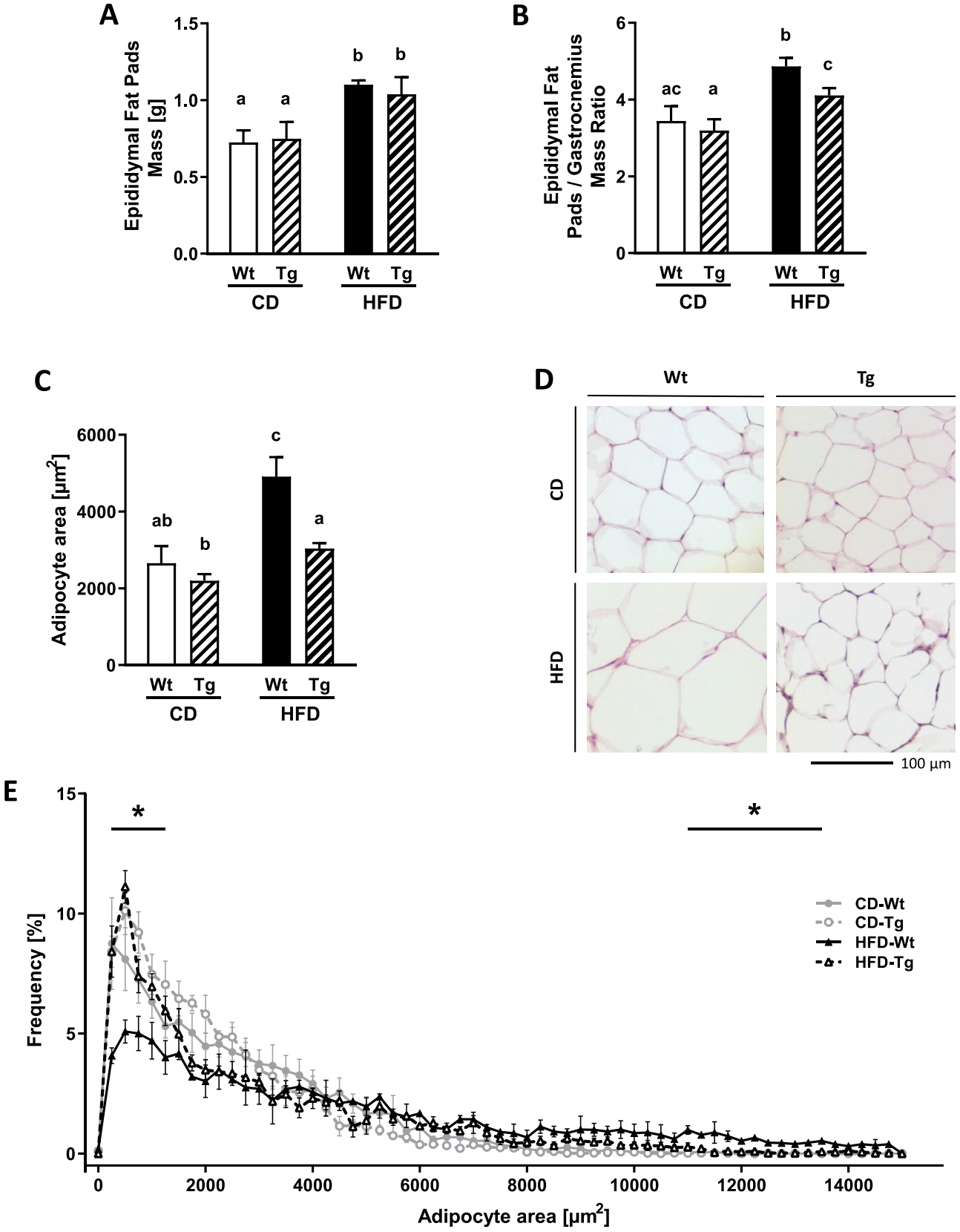


Figure 29. *Effect of systemic UnAG overexpression on epididymal adipose tissue mass and adipocytes morphology. Effects of UnAG overexpression in Tg Myh6/Ghrl (Tg) versus wild-type (Wt) mice fed 16 weeks with control diet (CD) or HFD on absolute (A) and relative to muscle (B) epididymal fat pads mass, adipocyte area (C), with representative histological images (D) and adipocyte size frequency distribution of epididymal*

*fat in the 4 groups (E). Data are presented as Mean ± SEM. p < 0.05 among groups with different letters. *p<0.05 HFD-Wt vs. all other groups.*

4.2.8 UnAG overexpression effects on actin remodeling in mice WAT in CD and HFD condition

Based on previously described histological findings, we further investigated molecular factors involved in remodeling of adipocytes. Actin and particularly its F-fraction, was shown to lead adipocyte remodeling thus associating adipocyte size, and to be itself a relevant mediator for obesity-related inflammation and metabolic complications [352]. The protein extraction of our samples of adipose tissue, which allowed to obtain F-enriched fractions of actin, suggested that UnAG was able to impede obesity-induced actin remodeling in mice adipocytes (Figure 30D). To further investigate this finding, we assessed protein measurements of several factors that regulate and activate actin-mediated remodeling machinery. To this purpose, protein levels of Arp-2, Cofilin-1 and Profilin-2 were tested, as such factors mediated actin-induced cytoskeleton re-organization. In control diet conditions, UnAG overexpression was found to not modify Arp-2, Cofilin-1 and Profilin-2 and F-actin levels compared to wild-type (Figure 30A-D). As expected, HFD induced a significant increase in Arp-2, Cofilin-1 and Profilin-2 levels and, in agreement with previous studies [352], showed that HFD causes actin re-organization thus entailing adipocytes expansion. Importantly, UnAG systemic overexpression was found to mitigate HFD effects on actin remodeling machinery, and markedly decreased Arp-2, Cofilin-1 and Profilin-2 in agreement with F-actin levels, restoring them towards or at control diet levels (Figure 30A-D).

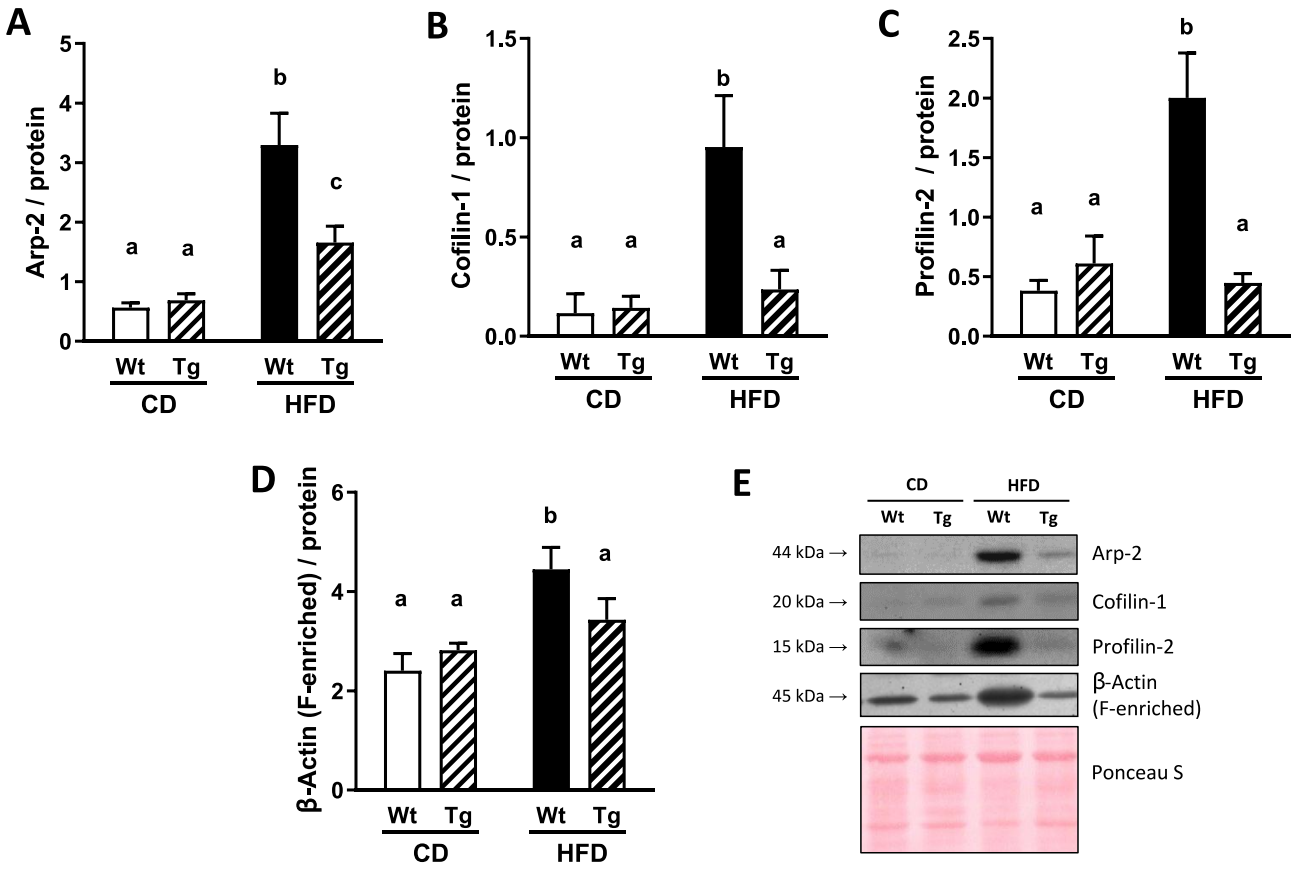


Figure 30. *Effect of systemic UnAG overexpression on adipocyte cytoskeleton remodeling. Effects of UnAG overexpression in Tg Myh6/Ghrl (Tg) versus wild-type (Wt) mice fed 16 weeks with control diet (CD) or HFD on Arp-2 (A), Cofilin-1 (B), Profilin-2 (C), F-Actin (D), with representative blots (E). Data are Mean ± SEM. p < 0.05 among groups with different letters.*

4.3 UnAG plasma levels decrease with obesity and predict 5-year low muscle mass in elderly humans

4.3.1 Anthropometric and metabolic parameters and plasma ghrelin forms

Study population characteristics are reported in Table 6. The individuals were divided in two subgroups, lean and overweight + obese based on their BMI. As expected, the two subgroups were different for most of the tested metabolic and metabolic-related variablese. Subsequently, the representative subgroup from this cohort which was selected for follow-up analysis, did not present any difference from the whole basal study cohort (Table 6).

Table 6. *Study population. Sex, age, body mass index (BMI), waist circumference (WC), high sensitivity C Reactive Protein (hsCRP), tumor necrosis factor α (TNF-α), interleukin 10 (IL-10), triglycerides, high density lipoprotein-cholesterol (HDL-cholesterol), glucose, insulin, homeostasis model assessment of insulin resistance (HOMA), mean arterial pressure (MAP) and BMI>25 in whole study cohort, in lean and overweight+obese subgroup and in subject that subsequently underwent 5-year follow-up evaluation. Data are presented as Mean ± standard deviation (SD). *P < 0.05. **p < 0.01.*

	Lean subgroup	Overweight Obese subgroup	Whole study group	Subgroup with 5-y follow-up
n	190	260	450	133
Sex (M/F)	75/115	104/156	179/271	52/81
Age [y]	68.5±3.8	68.8±3.9	68.7±3.3	68.8±3.4
BMI [kg/m^2]	23.6±1.9	31.3±4.2**	28.2±5.1	28.1±5.1
WC [cm]	88.1±7.8	104.3±11**	97.8±12.6	97.5±12.4
hsCRP [mg/L]	39.1±88.4	82.4±113.7**	64.9±106.2	69.8±209.9
TNFα [pg/ml]	0.64±0.68	0.83±0.67*	0.76±0.67	0.84±0.78
IL-10 [pg/ml]	1.84±2.15	2.07±3.03	1.97±2.97	2.01±3.45
Triglycerides [mg/dL]	124.6±70.6	149.4±75.3**	139.4±74.4	136.2±63.7
HDL-Cholesterol [mg/dL]	61.1±16.2	53.5±13.3**	56.6±15	57.3±14.5
Glucose [mg/dL]	98.5±24.4	106.3±25.4*	103.1±25.3	104.9±21.1
Insulin [µI/mL]	7.5±3.6	14.3±9.9**	11.6±8.7	12.5±7
HOMA	1.8±1.1	3.8±2.9**	3.0±2.6	3.2±1.8
MAP (mmHg)	99.2±11.3	103.9±12.0**	102.0±12.0	101.3±11.0
BMI>25 [%]	0	100**	58	59

4.3.2 Cross-sectional analyses

4.3.2.1 Ghrelin levels in lean and obese-overweight individuals

Ghrelin plasma profile was characterized for the whole study group at the baseline. Consistent with other studies on different populations [442,527], total, acylated and unacylated ghrelin were also reduced in elderly overweight+obese subjects compared to lean (Figure 31).

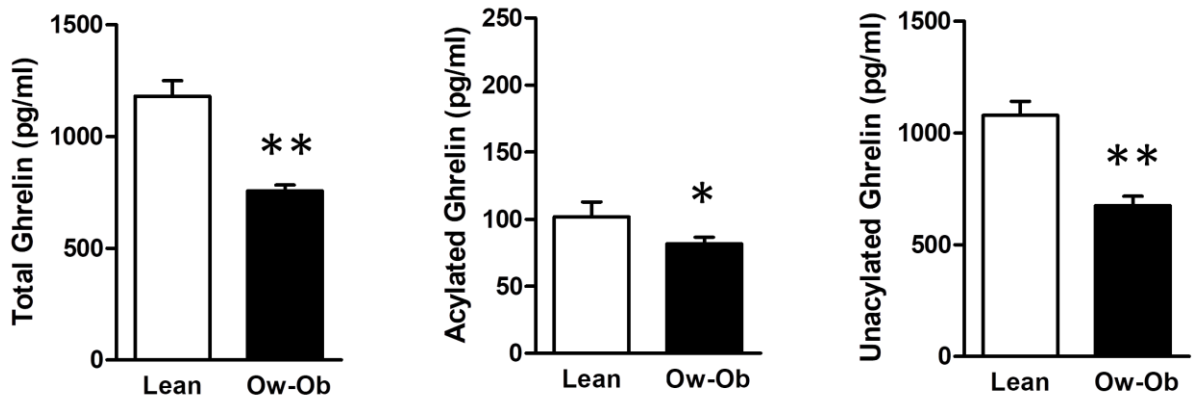


Figure 31. *Ghrelin plasma profile in lean and overweight+obese subjects. Total, acylated and unacylated ghrelin plasma levels in lean (n=190) and overweight+obese (Ow-Ob, n=260) subgroups. Data are presented as Mean ± standard error (SD). *p < 0.05. **p < 0.01.*

4.3.2.2 Associations between plasma ghrelin forms and body mass index

Association studies revealed that total, acylated and unacylated ghrelin are negatively associated to body mass index (BMI) (Figure 32A-C). Interestingly, the strength of this association resulted higher for TG and UnAG and lower for AG, according to Meng, Rosenthal and Rubin method.

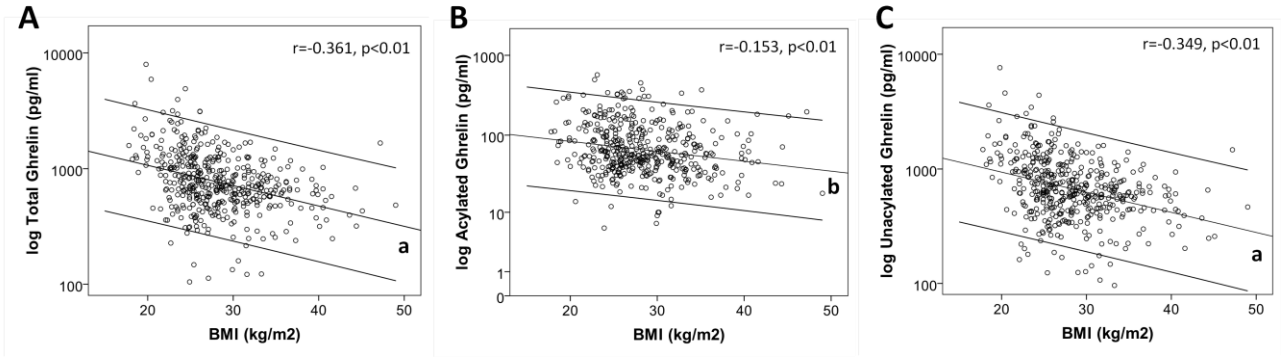


Figure 32. *Association study. Association between body mass index (BMI) and total (A), acylated (B) and unacylated (C) ghrelin in the whole study group. Different letters near regression line indicate p < 0.01 in association strength comparison.*

Furthermore, as BMI was also associated with anthropometric and metabolic parameters in linear regression analyses (Table 7), multiple regression analyses were used to test the independence of

the association between ghrelin forms and BMI from the identified potential confounding variables.

Table 7. *Linear regression association analyses for potential confunders. Association between body mass index (BMI) and sex, age, waist circumference (WC), plasma high sensitivity C Reactive Protein (hsCRP), tumor necrosis factor α (TNF-α), interleukin 10 (IL-10), triglycerides, high density lipoprotein-cholesterol (HDL-cholesterol), glucose, insulin, homeostasis model assessment of insulin resistance (HOMA), mean arterial pressure (MAP) in whole study cohort (n=450).*

	ρ	p
Sex (M)	0.044	0.352
Age	-0.026	0.589
WC	0.841	<0.01
hsCRP	0.408	<0.01
TNFα	0.128	<0,05
IL-10	0.008	0.878
Triglycerides	0.272	<0.01
HDL-Cholesterol	-0.298	<0.01
Glucose	0.279	<0.01
Insulin	0.591	<0.01
HOMA	0.592	<0.01
MAP	0.232	<0.01

Intriguingly, negative correlations between BMI and plasma total and unacylated ghrelin also resulted independent of metabolic syndrome parameters (Table 8), while the weaker but statistically significant negative association between BMI and AG was no longer significant after adjustment for confounding variables in multiple regression analyses.

Table 8. *Multiple regression analyses. Multiple linear regression analyses between total (TG), acylated (AG) and unacylated ghrelin (UnAG) plasma levels and body mass index (BMI) in the whole study group (n=450) in different statistical adjustment models. B: Coefficient; SE: standard error; t: t-value.*

		BMI			
		B	SE	t	p
TG	Model 1	-39.472	10.996	-3.590	<0.01
	Model 2	-28.758	11.265	-2.553	<0.05
AG	Model 1	-3.863	1.850	-2.088	<0.05
	Model 2	-3.318	1.919	-1.730	NS
UnAG	Model 1	-35.609	10.142	-3.511	<0.01
	Model 2	-25.440	10.386	-2.449	<0.05

Data adjustments:
Model 1: sex, BMI
Model 2: Model 1 + Triglycerides, HDL-Cholesterol, Glucose, Mean Arterial Pressure, high sensitivity C Reactive Protein (hsCRP), Tumor Necrosis Factor α (TNFα).

4.3.3 Longitudinal analyses with 5-year follow-up recall

A subgroup (n=133) from the basal cohort study participated to a 5-year follow up where basal total, acylated and unacylated ghrelin were related to 5-year low muscle mass in elderly subjects. Logistic regression analyses were used to evaluate plasma ghrelin profile in low muscle mass prediction. Results showed that lower basal total and UnAG but not AG predicted low muscle mass (MM)-index as $p < 0.05$ also after adjustment for metabolic confounders including sex, BMI, hsCRP, TNF-α, Triglycerides, HDL-cholesterol, glucose and MAP (Table 9).

Table 9. *5-year low muscle mass prediction analyses. Multiple logistic regression analyses between total (TG), acylated (AG) and unacylated ghrelin (UnAG) plasma levels and matching of low muscle mass diagnostic criteria at 5-year follow-up in the population subgroup undergoing 5-year follow-up evaluation (n=133) in different statistical adjustment models. B: coefficient; SE: standard error; z: Wald test; NS: non-significant*

		5-year low muscle mass prediction			
		B	SE	z	p
TG	Model 1	0.001	0.001	4.463	<0.05
	Model 2	0.002	0.001	3.880	<0.05
AG	Model 1	0.001	0.002	0.420	NS
	Model 2	0.001	0.002	0.080	NS
UnAG	Model 1	0.002	0.001	4.981	<0.05
	Model 2	0.003	0.001	4.661	<0.05

Data adjustments:
Model 1: sex, BMI
Model 2: Model 1 + Triglycerides, HDL-Cholesterol, Glucose, Mean Arterial Pressure, high sensitivity C Reactive Protein (hsCRP), Tumor Necrosis Factor α (TNFα).

4.4 UnAG reduces fat mass/muscle mass ratio in a human population study

4.4.1 Anthropometric and metabolic parameters and plasma ghrelin forms

In our experimental model adipose fat pad mass was not modified by UnAG in absolute terms, while presenting lower values in relative to mass ratio along with profound histological and metabolic alterations. We therefore tested the hypothesis that UnAG levels could also be associated with relative adipose mass in humans.

For this study cohort, anthropometric and metabolic parameters were measured along with UnAG and body composition in a cohort of 176 randomly selected subjects from the study population. Cohort characteristics are reported in Table 10.

Table 10. *Study population. Sex, age, body mass index (BMI), triglycerides, total cholesterol (Total-Chol), high density lipoprotein-cholesterol (HDL-cholesterol), glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), systolic (SBP) and diastolic blood pressure (DBP), diabetes, hypertension, dyslipidemia, muscle mass, fat mass, total, acylated and unacylated-ghrelin of the whole study cohort. Data are presented as Mean ± standard deviation (SD).*

	mean±SD
Sex (M/F)	93/83
Age [y]	57,5±16,7
BMI [kg/m2]	26,4±5,1
Triglycerides [mg/dl]	113,8±69,2
Total-Chol [mg/dl]	202,6±43,4
HDL-Chol [mg/dl]	55,6±16,6
Glucose [mg/dl]	98,3±19,3
Insulin [µU/ml]	13,6±11,8
HOMA-IR	3,6±4,4
SBP [mmHg]	131,8±19,2
DBP [mmHg]	81,7±9,1
Diabetes	9,5%
Hypertension	56,4%
Dyslipidemia	17,9%
Muscle mass [kg]	34,0±6,9
Fat mass [kg]	20,8±10,0
Total-Ghrelin [pg/ml]	1432±976
Acylated-Ghrelin [pg/ml]	87±119
Unacylated-Ghrelin [pg/ml]	1344±921

4.4.2 Associations between fat mass/muscle mass ratio and plasma ghrelin forms

Association studies showed that total and unacylated but not acylated ghrelin negatively correlates to fat/muscle mass ratio. (Figure 33A-C).

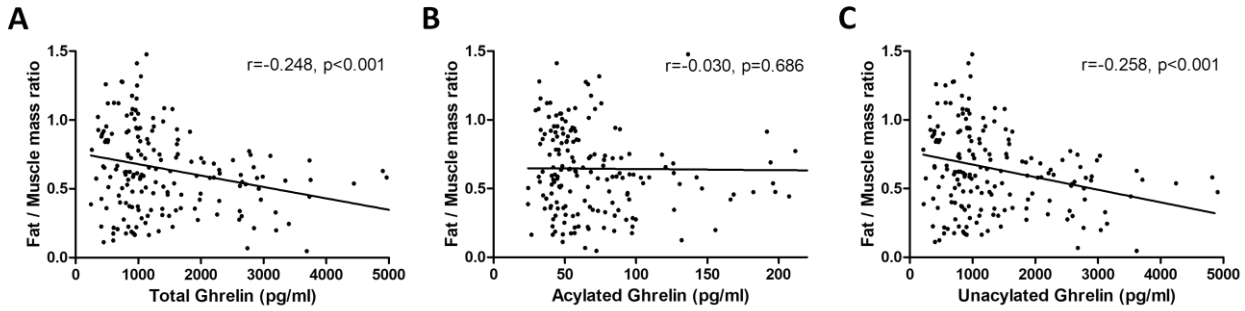


Figure 33. *Association study. Association between Fat/Muscle mass ratio and total (A), acylated (B) and unacylated (C) ghrelin in the whole study group.*

Moreover, as fat/muscle mass ratio was also associated with anthropometric and metabolic parameters in linear regression analyses (Table 11), multiple regression analyses were used to test the independence of the association between body composition as assessed by fat/muscle mass ratio and ghrelin forms from the identified potential confounding variables. In particular, fat/muscle mass ratio resulted negatively related to sex, but expectedly showed significant positive association to age and metabolic parameters, particularly body mass index (BMI) but also triglycerides, glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), systolic (SBP) and diastolic blood pressure (DBP) (Table 11).

Table 11. *Linear regression association analyses for potential confunders. Association between fat/muscle mass ratio and sex, age, body mass index (BMI), triglycerides, total cholesterol (Total-Chol), high density lipoprotein-cholesterol (HDL-cholesterol), glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), systolic (SBP) and diastolic blood pressure (DBP) in whole study cohort (n=176).*

	Fat/Muscle mass ratio	
	ρ	p
Sex (M)	-0,426	<0.001
Age	0,542	<0.001
BMI	0,736	<0.001
Triglycerides	0,250	<0.001
Total-Chol	0,052	0,488
HDL-Chol	-0,084	0,262
Glucose	0,297	<0.001
Insulin	0,560	<0.001
HOMA-IR	0,543	<0.001
SBP	0,428	<0.001
DBP	0,276	<0.001

At multiple regression analysis, plasma total and unacylated ghrelin but not acylated ghrelin remained associated to fat/muscle mass ratio even after adjustments for the above identified confounding anthropometric and metabolic variables (Table 12), suggesting that the association of higher UnAG with reduced fat/muscle mass ratio is at least in part independent from the identified confounding variables.

Table 12. *Multiple regression analyses. Multiple linear regression analyses between total (TG), acylated (AG) and unacylated ghrelin (UnAG) plasma levels and fat/muscle mass ratio in the whole study group (n=176) in different statistical adjustment models. B: Coefficient; SE: standard error; t: t-value.*

		Fat/Muscle mass ratio			
		B (1000x)	SE (1000x)	t	p
TG	Model 1	-0,0702	0,0187	-3,780	<0.001
	Model 2	-0,0224	0,0119	-1,878	0,062
	Model 3	-0,0236	0,0117	-2,012	0,046
AG	Model 1	-0,0123	0,1473	-0,083	0,934
	Model 2	-0,0401	0,0887	-0,452	0,652
	Model 3	-0,0492	0,0873	-0,564	0,574
UnAG	Model 1	-0,0783	0,0197	-3,980	<0.001
	Model 2	-0,0244	0,0127	-2,010	0,046
	Model 3	-0,0256	0,0124	-2,060	0,041

Model 1: age, sex
Model 2: Model 1 + BMI
Model 3: Model 2 + Triglycerides, Mean Arterial Pressure, HOMA-IR

5. DISCUSSION

5.1 UnAG effects on rodents adipose tissue

The current study investigates UnAG metabolic effects in white adipose tissue of different rodent models. These studies follow previous published findings from our research group of UnAG as an independent metabolic regulator in skeletal muscle [445]. UnAG impact was therefore tested in 1) sustained 4-day exogenous administration 2) transgenic systemic upregulation both in standard diet conditions and developing obesity with high-fat diet feeding. Our results globally show that UnAG has a profound influence on adipose tissue metabolic processes with particular major impact in obesity, where UnAG importantly improves and even recovers several key metabolic derangements.

To assess metabolic implication of UnAG in WAT, the presented animal models were initially evaluated in terms of adipose tissue mitochondrial function, oxidative stress and inflammation, factors known to be modulated by UnAG in skeletal muscle [445,447,464], and which are also known important metabolic players in adipose tissue [227].

Key in cellular metabolism and core of the main metabolic processes [25], mitochondria were tested to identify whether UnAG caused any change in their functionality, dynamics and redox state. Dysfunctional mitochondria are in fact known to produce excessive reactive oxygen species, with consequent unbalance of redox state [547]. UnAG was already reported to ameliorate mitochondrial activity, oxidative stress and inflammation, particularly in liver and skeletal muscle in diseases and metabolic alteration state [445,447,465,548,549], however much less is known about UnAG effect in adipose tissue. Our findings suggest that sustained UnAG administration does not modify WAT mitochondrial activity in healthy rats. Transgenic mice with UnAG overexpression confirmed this finding as the mitochondrial enzyme tested reported little but not significant increased activity compared to wild-type mice. However, in the obese model, which is known to be characterized by lower adipocyte mitochondrial function [547], UnAG constitutive overexpression completely prevented mitochondrial enzyme activity impairment caused by HFD. Consistently, obese wild-type mice also reported a marked alteration of mitochondrial dynamics, as fusion and fission represent two crucial processes for mitochondrial maintenance under stress condition [270]. HFD induces a decrease of fusion markers Mitofusin-1 and OPA-1 protein levels, in agreement with previous evidence of HFD as a negative modulator of mitochondrial homeostasis [263]. This finding is also in agreement with the notion that mitochondrial fusion enhances glucose oxidation, while

impaired fusion of mitochondria leads to glucose utilization as a substrate for lipogenesis, with consequent increase of lipid storage and fat depots. Moreover, mitochondrial fusion was found essential to mediate the interaction with lipid droplets, which ultimately allows fatty acid transfer from lipid droplets to mitochondria, site of fatty acids β-oxidation [263]. Importantly, consistent with previously described UnAG associated prevention of mitochondrial dysfunction in HFD, transgenic obese mice upregulating UnAG presented increased levels of Mitofusin-1 and OPA1, indicating an increase in fusion activation [253]. Further confirmation came from tests on Drp1, a main fission marker [256] whose protein levels were importantly recovered in obese transgenic mice after alterations induced by HFD. Collectively, these data support the idea that UnAG does not have a major impact on mitochondrial homeostasis in physiological conditions, but potentially ameliorate mitochondria alterations, a critical feature of obesity.

Consequently, oxidative stress was evaluated in terms of oxidizet-to-total glutathione levels, which increase under stress conditions and reflect cellular redox state [225], and at the same time by checking SOD enzymes as following antioxidant system to attenuate oxidative cascade [226]. UnAG in WAT of rats after 4 days of treatment showed to decrease oxidized-to-total glutathione amount without changing superoxide dismutase protein levels. Accordingly, mice with systemic UnAG overexpression presented similar dismutase levels to wild-type in WAT, supporting the hypothesis that UnAG does not alter antioxidant system in adipocytes.

Obesity has been associated with conflicting results with regard to SOD expression, with reports indicating increased or decreased expression in different models [228,550–552]. In our findings, while MnSOD levels, in line with some evidence in literature [550], were significantly increased in obese wild-type mice, CuZnSOD presented diminished levels in WAT after HFD treatment. This result is in agreement with previous findings from Galinier et al., reporting an opposite trend of the two superoxide dismutases in WAT in a model of obesity [551]. Most importantly though, our results essentially reveal a role for UnAG role in partly recovering HFD-induced dismutase alterations, bringing their levels similar or close to lean animals, implying a profound reversion of oxidative derangements in obesity.

UnAG in both models of 4-day exogenous administration and constitutive overexpression did not affect inflammatory state with control diet. However, and in parallel with the above results, UnAG was also shown to prevent WAT inflammation, one of the hallmarks that characterize obesity pathophysiology [121].

Numerous studies already provided the evidence that oxidative stress, dysfunctional mitochondria and inflammation are implicated in an elaborate network that finally contributes to insulin sensitivity modulation [122,445,537,553]. In particular, these features were in concordance often associated to obesity and type2 diabetes [276,277]. Elevated dietary intake increases fatty acid levels, with consequent increase of mitochondrial ROS production and inflammatory cytokines release [39]. In fact prolonged nutritional excess enhances insulin release, but attenuates its metabolic response in skeletal muscle, liver and, of course, adipose tissue, and eventually causes hyperglycemia [554]. Hyperinsulinemia and hyperglycemia promote unrestrained adipose tissue lipolysis [555], which is the primary event contributing to insulin resistance development [556].

Our results showed that sustained subacute UnAG administration globally decreases insulin signaling in adipose tissue at multiple levels, including AKT, GSK-3β and activation of downstream effectors such as TSC2, mTOR and P70S6K resulted severely reduced. In agreement, 4 days of UnAG administration does not seem to affect insulin receptor (IR) activation, strongly supporting the idea that UnAG may act through other mechanisms downstream of the receptor. Among the major molecular mechanism driving reduced insulin sensitivity, increased concentration of reactive molecules within the cell stimulates the serine/threonine kinase cascades which lead to insulin receptor which inactivates its substrate (IRS-1) through phosphorylation, thus triggering IRS-1 degradation and impairing insulin sensitivity as a negative modulation feedback [224]. PTEN as well, is a potent negative regulator of insulin action which attenuates downstream insulin signaling by impairing AKT recruitment [557]. In agreement with signaling downregulation, UnAG was found to modify IRS-1 phosphorylation, meaning an inhibition of insulin action as a result of IRS-1 disruption from IR in a downstream effect of mTOR activation [558]. At the same time, UnAG effect on insulin signaling does not seem to be modulated by PTEN-associated pathways as its levels were unchanged after UnAG-treatment.

At variance with previous results from our lab and others which showed that UnAG is a positive modulator of insulin sensitivity in skeletal muscle [445,447], under physiological conditions this hormone instead reduced insulin signaling activation in white adipose tissue in the same models. However, it is important to remark that insulin sensitivity has a highly tissue-specific meaning and response. While in skeletal muscle metabolism insulin is essential for glucose uptake and disposal, in WAT it promotes lipogenesis and inhibits lipolysis, in agreement with adipose organ role as a storage of calories in times of energy availability [53]. In adipocytes, mTOR results as a promoter of lipogenesis by activating sterol regulatory element-binding protein (SREBP) [559], a transcriptional

factor that activates genes involved in synthesis and uptake of fatty acids, sterols and triglycerides [560]. Furthermore, insulin resistance in WAT occurs to promote lipolysis during fasting and to support feeding-induced lipid accumulation [561]; since ghrelin and, more specifically, UnAG have been well characterized to be over-released post-prandrialy [421], UnAG exogenous administration may be perceived from the organism as a signal to prevent fat storage in favor of muscle anabolic fluxes as alternative to WAT destination.

A possible similar trend for a UnAG effect in decreasing insulin signaling in WAT was also detected in transgenic mice with UnAG overexpression, however this was not found significant in our model, possibly due to the fact that effects in constitutive overexpression models are likely to be affected by long term homeostasis. A severe impairment of insulin sensitivity induced by HFD was reported at systemic level by ITT in these mice in parallel with increased insulin resistance at skeletal muscle level [445]. Importantly, the current findings show that insulin signalling at AKT/GSK level is also depressed in adipose tissue following HFD. Importantly, HFD mice overexpressing UnAG showed insulin sensitivity levels comparable to to standard diet. Together, these data support UnAG as an important modulator of WAT insulin sensitivity with potential interconnections and crosstalks with other metabolic organs.

Interestingly, increasing evidence has shown a profund relationship between systemic insulin sensitivity and adipocyte size and shape which, in turn, also affects adipose tissue function and metabolism [348]. HFD induced obesity is characterized by an excess of fatty acids that are compartimentalized and stored in adipocytes as trigliceryde droplets [174]. Several studies confirmed adipocytes cell size to be predictive of adipose tissue dysfunction and metabolic alterations [374]. In agreement with the literature, also in our study HFD accumulates lipids within the cell inducing a rearrangement of adipocyte structure and a global cellular and tissue enlargement, with associated impairment of insulin sensitivity [346,347].

First, epididymal fat weight highlighted a difference in adiposity between lean (wild-type and Tg Myh6/Ghr) and obese (wild-type and Tg Myh6/Ghr) mice, as HFD markedly increased epididymal fat mass as expected. While genotype did not have an impact on absolute fat pads mass, intriguingly, a comparison of fat-to-muscle mass showed that UnAG significantly decreased this ratio in HFD mice, suggesting that UnAG may globally modulate the balance between fat and lean mass , with the potential to revert negative alterations of body composition in obesity and related complications [562]. Increase of fat mass with reduced lean mass is a frequent condition particularly in elderly, and recognized as sarcopenic obesity [205], which is generally associated to an

exacerbation of systemic inflammatory profile [563]. Consistently, it has become clear that lower lean mass in obese subjects reduces survival chances and increases risk for metabolic syndromes and cardiovascular diseases [192,193]. Moreover, fat-to-muscle ratio has been recently proposed as a validated clinical marker to investigate body composition in this setting, with the potential to better address the investigation of the obesity paradox, a phenomenon described by decreased mortality of overweight or moderately obese patients with several chronic disease, including metabolic complications, in comparison to lean patients [564,565].

Importantly, histological analyses gave a direct evidence of adipocyte morphology and showed that extreme cells enlargement caused by HFD was markedly rescued in UnAG overexpressing mice. Regarding related mechanisms, actin plays a pivotal role in regulating cytoskeleton organization and consequent adipocyte remodeling in response to lipid droplets expansion or shrinkage, thus affecting insulin sensitivity [344]. These pieces of evidence, led us to investigate UnAG impact on adipocytes size and remodeling. To dig deeper into this aspect, adipocytes remodeling was investigated at molecular level. Based on emerging interesting findings suggesting increase of filamentous (F)-Actin fraction in enlarged adipocytes of HFD-fed mice [352], we tested in lean and obese groups its content , in order to detect potential confirm actin remodeling. In fact, β-actin in the F-enriched fraction was found significantly increased in wild-type obese mice compared to lean. Importantly, and consistently with both histological and metabolic findings, UnAG constitutive overexpression was found to remarkably decrease its levels, which resulted comparable to control diet-fed animals. Moreover, analyses of regulatory proteins that modulate actin-mediated remodeling, importantly confirmed that in obese mice, UnAG contrasts HFD-induced adipocytes cytoskeleton re-organization by reducing actin remodeling machinery protein levels.

These findings are consistent with the evidence that in obesity, hypertrophic adipocytes are associated to increased TNF-α levels, a major pro-inflammatory marker [566,567] and decreased insulin sensitivity [346,351], due to lipid accumulation and droples enlargement which induce a disruption of actin filaments, thus causing impaired GLUT4 translocation to the cell membrane [354,355]. Eventually, these factors are reported to predispose to cardiometabolic pathologies, particularly hypertension, dyslipidemia, type 2 diabetes, metabolic syndromes and some type of cancer [374]. UnAG capacity to limit adipocytes hypertrophy and to improve inflammatory profile and insulin sensitivity, is consistent with induced decrease of fat-to-lean mass, remarkedly reported as an indicator of metabolic alterations [194,199], which in turn are linked to inflammation

and oxidative stress [148]. Thus, our findings support the hypothesis that UnAG could potentially lower the risk of metabolic complications in obese subjects.

Taken together, these results underline novel metabolic effects of UnAG on WAT in counteracting HFD-induced fat accumulation, with an hypothetical potential as inhibitor of basal lipogenesis, thus contrasting obesity-derived metabolic alterations including mitochondrial functionality and dynamics, oxidative stress, inflammation and adipocytes remodeling and enlargement (Figure 34), possibly readdressing fuel utilization to tissues designated for energy disposal. This hypothesis is consistent with previous findings in the same rodent models of UnAG treatment or systemic upregulation, in which the hormone was reported to induce increased mitochondrial functionality, decreased oxidative stress and inflammation as well as increased insulin signaling in skeletal muscle [445].

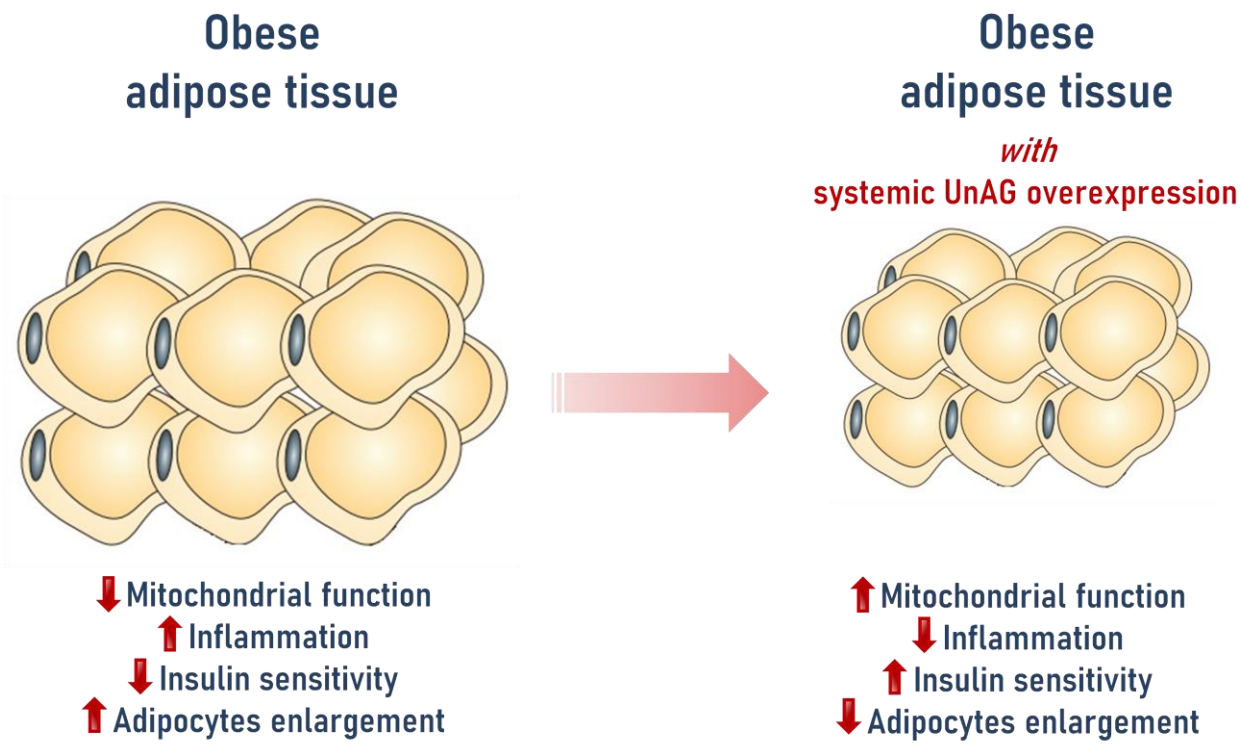


Figure 34. *Proposed interactions between UnAG and obesity-associated features and alterations in white adipose tissue. HFD-induced obesity in mice reduced mitochondrial functions and dynamics, increased oxidative stress and inflammation, lowered insulin signaling and enlarged adipocytes size. Constitutive UnAG overexpression contrasted these metabolic alterations in obese mice.*

As a limitation, our study has involved one single white adipose tissue district in each model. As WAT in subcutaneous and visceral districts may also present differences at anatomical, cellular, molecular, physiological and clinical levels [5,568], further investigation is required to assess whether and how different fat depots and generally subcutaneous adipose tissue behave under UnAG stimulation in physiological rather than metabolic altered conditions. Moreover, although for both human and rodents adipose tissue is a multi-depot organ, there are some anatomical differences that should be taken into consideration [1]. Adipose tissue of rodent models relatively represents human fat pads: epididymal fat is abundant in rodents and therefore the most frequently dissected, however it is less represented in human [569], with at least in part potentially different metabolic functions.

5.2 UnAG is associated with body composition in humans

Based on the results from animal studies showing a profound impact of UnAG on adipose tissue metabolism, we also aimed to investigate associations between UnAG and obesity and body composition in humans, eventually showing that 1) in a cohort of elderly subjects from the North-East of Italy, UnAG is lower in overweight-obese compared to lean and predicts 5-year muscle mass independently from BMI and 2) in a general population cohort from the same area, UnAG negatively correlates with fat/muscle mass ratio independently from BMI and other metabolic confounders.

The current study proposed, reported novel interesting cues that related TG and particularly UnAG to obesity and muscle waste in elderly subjects. Body composition assessment represents a pivotal parameter to evaluate metabolic status, and balanced fat and muscle mass are critical in the maintenance of metabolic homeostasis [570]. Changes in body composition naturally occur with aging, progressively leading to the decrease of lean mass in favor of increased fat mass, contributing to frailty and, potentially, to sarcopenic obesity; consistently, risk of metabolic syndrome development is accentuates in elderly subjects [571].

Consistent with literature knowledge [527], total (TG), acylated (AG) and unacylated ghrelin (UnAG) were decreased in overweight-obese subjects in comparison to lean in a cohort of elderly humans. In fact clear evidence supported ghrelin concentrations to be inversely related to obesity parameters such as BMI, waist circumference (WC), and body fat amount [572]. Total ghrelin levels were found increased in anorexic patients while decreased in obese ones [442].

Supporting these data, cross-sectional analyses allowed to assess plasma ghrelin forms correlations with body mass index (BMI) to find that TG, AG and UnAG were negatively associated to BMI. However, the test of Meng, Rosenthal and Rubin allowed to verify the strength of these association and importantly revealed that TG and UnAG are strongly more associated to BMI compared to AG. Following these results, multiple regression analyses confirmed that the significance of this association persists for TG and UnAG but not for AG if considering adjustments for anthropometric and metabolic parameters associated to BMI. It must be pointed out that TG result is mostly due to changes in UnAG, as the latter accounts for the majority of total circulating ghrelin [422]. These results support the hypothesis that UnAG and AG present different and partly independent roles also in obesity. Moreover, a decline in UnAG with BMI increase agrees with previous findings in human studies [456,573,574]. Also, in another study, UnAG was already shown to be independently and negatively associated to BMI in a small cohort of metabolic syndrome patients [575]. Supporting these findings, another work from our research group consistently found that UnAG is negatively associated to BMI in a cohort of metabolic syndrome patients, independently from confounding variables[457]. In accordance, the same study showed also an independent negative correlation between UnAG and insulin resistance (HOMA), eventually suggesting that UnAG decline may enhance obesity-associated insulin resistance [457]. Moreover, analyses of prospective association importantly corroborated the hypothesis of UnAG association to muscle mass, as results suggested UnAG to be predictive of 5-year muscle mass independently from BMI and metabolic-related confounders. Conversely, AG did not show association to any model of sex, BMI and other metabolic parameters in logistic regression results for predictive association.

These results find consistent basis in the available knowledge of UnAG effect on skeletal muscle anabolism which is, particularly in aging, drove by defects in mitochondria functionality, defective autophagy and mitophagy and inflammation [576]. UnAG was in fact shown to improve mitochondrial function thus reducing inflammatory cytokines thus enhancing autophagic flux in skeletal muscle [445]. Moreover, as a novel aspect, this study considers elderly individuals with different BMIs: aging process is normally associated with loss of muscle mass and frailty [206], but this condition is aggravated when combined with fat gain [205]. Reduced muscle mass and increased fat accumulation naturally occurs in elderly and is defined as sarcopenic obesity, which is associated with increased risk of morbidity and mortality [202,203]. Therefore it is becoming of primary relevance to understand and better characterize the pathogenesis of sarcopenic obesity,

and to determine predictive tools to finally prevent associated health risks. This study importantly introduces UnAG as a possible prediction marker to identify subjects with increased sarcopenic risk in an elderly general population cohort.

These results were followed by further analyses with the aim of evaluating UnAG in correlation to body composition in terms of fat-to-muscle mass ratio. In our data, fat-to-muscle mass ratio is found to be decreased as UnAG increase in a general population cohort, and this finding is independent of confounding variables. In contrast, no correlations were reported between AG and fat-to-muscle mass ratio, remarking and supporting UnAG as, at least in part, an independent modulator of fat-to-muscle mass ratio. Importantly, several studies have reported that fat-to-lean mass ratio is positively associated to metabolic alterations, thus representing a predictor of metabolic syndrome [194,196,199], insulin resistance [201] and cardiovascular risk [210], and increased muscle mass with decreased fat mass has been associated to a major survival [192,193]. Therefore, fat-to-muscle mass ratio gives a cue about the impact that body composition has on metabolic parameters, independently of the single contribution of fat or lean mass, which eventually are still mutually related [189]. Furthermore, our findings support the potential of UnAG in association to metabolic syndromes and eventually as a predictor of metabolic disruption risks.
These findings in humans are importantly in agreement with the results of animal studies showing UnAG is negatively associated to fat-to-lean mass also in obese mice and strongly suggest that UnAG may play a key role in the modulation of body composition.

However, as a limitation, it has to be considered that body impedence assessment (BIA) used to evaluate body composition, takes account of the hydration status. Therefore, unlike direct methods such as dual-energy X-ray absorption (DEXA) and computed tomography (CT), BIA method allows to estimate rather then precisely measure body composition.

In conclusion, the results proposed in the current study showed for the first time that UnAG is a major modulator in the homeostasis of white adipose tissue metabolism, with potential relevant impact in obese rodents model. In addition, these findings are also supported by evidence in human body composition. Taken together, our findings suggest that UnAG has a relevant impact on metabolic processes of the adipose tissue, such as insulin signaling, mitochondrial function and dynamics, oxidative stress and inflammation, also affecting adipocytes morphology. This broad spectrum of effects on adipose tissue, synergistically with those recently observed in skeletal

muscle and at systemic level, makes UnAG a potential relevant player in the complex network of interlinked metabolic alterations which characterize obesity, insulin resistance and related metabolic alterations. Further research may clarify its potential clinical relevance.

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