UNIVERSITY OF NAPLES FEDERICO II

SCHOOL OF MEDICINE

DEPARTMENT OF PHARMACY

PhD program in Pharmaceutical Sciences

XXXVII Cycle PON

INTERACTIONS AND SYNERGY BETWEEN N-ACYLETHANOLAMINES AND/OR POLYPHENOLIC COMPOUNDS IN THE CONTROL OF OBESITY AND RELATED DISORDERS

PhD student

Stefania Melini

Coordinator
Prof.
Rosaria Meli

Tutor
Prof. Rosaria Meli

Co-tutor
Prof. Giuseppina Mattace Raso

XXXVII CYCLE PON (a.y. 22-24)

SUMMARY

ABSTRACT

Metabolic dysfunction-associated fatty liver disease (MAFLD), obesity, and diabetes are closely interconnected, as changes in glucose and lipid metabolism play a vital role in the development of all these conditions. Lipid overnutrition and following positive energy balance contribute to the redistribution of fat within metabolic organs, causing the alteration of liver-adipose tissue axis, which plays a key role in maintaining lipid and glucose homeostasis.

Due to the multi-variant metabolic features of the above-mentioned disorders, more than one drug or natural product may be required to achieve proper therapeutic effects.

N-acylethanolamines (NAEs) are endogenous lipids involved in the control of inflammation and energy metabolism. On the other hand, classes of natural bioactive compounds, including polyphenols and flavonoids, provide strong protective effects against steatosis, oxidative stress, inflammation, and dysbiosis, which are linked to the progression of MAFLD and obesity.

The potential pharmacological effects of combining NAEs with each other or with these bioactive compounds in managing inflammation-based metabolic disorders have not yet been explored.

This PhD project aimed to investigate:

i) the efficacy of a formulation containing co-micronized palmitoylethanolamide and rutin (PEA-Rut) associated with hydroxytyrosol (HT), namely NORM3, against hepatic damage and metabolic alterations in high-fat diet (HFD)-induced diabesity in mice.

ii) the metabolic effect of OLALIAMID® (OLA), an olive oil-derived NAE mixture containing oleoylethanolamide (OEA), palmitoylethanolamide (PEA), stearoylethanolamide (SEA), and linoleylethanolamide (LEA), in limiting liver and adipose tissue dysfunction of high-fat diet (HFD)-fed mice.

iii) The possible capability of NAEs to modulate cellular responses induced by metabolic stress, including autophagy and senescence.

We have demonstrated that these two NAE-based formulations are effective in alleviating the metabolic disturbances associated with obesity. Specifically, NORM3 was found to counteract insulin resistance and hepatic dysmetabolism related to 'diabesity,' while OLA improved the dysfunction of the adipose tissue and liver interplay induced by obesity.

OLA, as well as NORM3, reduced body weight and fat mass of obese mice and decreased insulin resistance (IR), improving hepatic glucose and lipid metabolism, as shown by *in vivo* tolerance tests, homeostasis model assessment (HOMA) index, leptin/adiponectin ratio, and other *ex vivo* determinations (i.e Western blot, and real-time PCR). Moreover, both formulations improved serum lipid and hepatic profile and the immune/inflammatory pattern of metainflammation. In the liver, NORM3 limited macro- and micro-vacuolar steatosis, as revealed by morphological analysis, and reduced the associated hepatic inflammation. At molecular level, NORM3 treatments counteracted glucose and lipid dysmetabolism induced by HFD, restoring insulin signaling, and regulating mRNAs of key markers of involved in lipid homeostasis, including the expression of carnitine palmitoyl-transferase (CPT)1, a rate-limiting enzyme of fatty acid β-oxidation.

Relevantly, NORM3 exhibited a profound antioxidant activity by reducing reactive oxygen species and increasing detoxifying factors and enzymes, in both liver of obese mice and hepatocytes *in vitro*. Otherwise, OLA treatment improved hepatic lipid and glucose metabolism altered by lipid overnutrition and stimulated adipose tissue reprogramming deeply altered by HFD inducing the thermogenesis of brown adipocytes (iBAT) and the beigeing of subcutaneous white adipose tissue (scWAT). Notably, the NAE mixture also reduced inflammation in iBAT and promoted M1-to-M2 macrophage shift in scWAT of obese mice. The systemic anti-inflammatory effects of OLA along with the increased expression of glucose transporter 4 in scWAT contributed to the improvement of gluco-lipid toxicity and insulin sensitivity.

Finally, in *in vitro* models of metabolic damage we demonstrated the role of each NAEs constituting our formulations in improving cellular homeostasis. Not only increasing the protective activation of autophagy in response to metabolic stress, but also reducing cellular senescence and oxidative stress triggered by chronic lipid overnutrition.

Summarizing, all these findings suggest that NAE-based formulations could offer a promising therapeutic strategy for controlling obesity and its metabolic co-morbidities. Indeed, this multitarget approach may help slow the progression of complex metabolic disorders associated with lipid overnutrition, such as diabesity and MAFLD, by correcting the imbalances in glucose and lipid metabolism and restoring the functionality of the adipose tissue–liver metabolic network.

INTRODUCTION and AIMS

More than 1 billion people in the world are affected by obesity and its prevalence is increasing worldwide due to the spreading adverse lifestyle behaviors (Collaborators, 2024).

Obesity is a medical condition that results from an interplay of genetic factors and environmental influences, such as high intake of calorie-rich foods and decreased physical activity (Singh et al., 2024).

In the obesogenic environment, genetic differences significantly influence individual responses, leading to variations in body weight and susceptibility to obesity across the population (Sheikh et al., 2017).

The ability to store fat during periods of nutritional abundance was a beneficial trait selected during human evolution over thousands of years. According to the "thrifty gene" hypothesis, proposed by Neel et al., in 1999, this evolutionary trait allowed efficient fat storage to endure periods of famine. Neel's hypothesis also suggests that these same genes, which were beneficial in the past, may now contribute to obesity in modern society, where food is abundant and calorie dense (Spiegelman and Flier, 2001).

Body weight remains stable when energy consumed matches energy expended over a given period, in accordance with the laws of thermodynamics. In this perspective, obesity can occur when this balance is disrupted, as it results when energy intake consistently exceeds energy expenditure. This imbalance leads to the storage of excess energy in the form of fat in adipose tissue (AT) (Hill et al., 2012).

Over the past decade, it has been introduced the term "diabesity" to describe diabetes related to obesity, highlighting the pathological

interconnections between these two epidemics and their detrimental effects (Michaelidou et al., 2023).

Additionally, recently it has been proposed the new acronymous of MAFLD to indicate the hepatic damage correlated to metabolic disorders including obesity and therefore it has been defined as the hepatic manifestation of a broader systemic metabolic disorder (Alam and Fahim, 2021).

Food restriction is a common strategy used in the treatment of obesity, creating a negative energy balance, and leading to weight loss. However, this strategy can only be effective in the short term, because it may be challenging to maintain over the long term due to the body's adaptive responses, such as increased hunger and a slower metabolic rate. These factors can make it difficult to sustain weight loss and may contribute to weight regain after the restriction period ends (Laferrere and Panda, 2022).

Several promising therapeutic agents are being explored for obesity treatment, showing various mechanisms of action. Some act centrally by suppressing appetite and increasing feelings of fullness and satiety, while others work peripherally by affecting metabolic processes outside the brain. Among others, the most promising drugs are GLP-1 receptor agonists, which have demonstrated potential in improving key metabolic parameters such as insulin sensitivity, blood glucose levels, and body weight regulation (Popoviciu et al., 2023).

Anyway, all these agents show specific risks and side effects, particularly related to cardiovascular and psychiatric health. It is important to identify a treatment for obesity that includes multiple

targets and addresses the various aspects that characterize a complex condition like obesity.

AT plays a central role in various health issues, particularly in obesity. It is not just a passive fat storage site, but an active endocrine organ involved in metabolic regulation. Its dysfunction can significantly contribute to disease development, making it a critical therapeutic target, acting on metabolic regulation, endocrine function, inflammation and immune modulation(Balistreri et al., 2010).

Indeed, AT regulates energy balance by storing excess energy as triglycerides (TG)s and releasing free fatty acids (FFA)s during energy deficits. Dysregulated lipid storage and mobilization in AT can lead to ectopic fat deposition in organs like the liver and muscles, contributing to IR and metabolic syndrome(Snel et al., 2012).

AT secretes various adipokines (e.g., leptin, adiponectin, resistin, and pro-inflammatory cytokines) that impact on appetite, glucose metabolism, and inflammation. Obesity alters the balance of these adipokines, promoting a pro-inflammatory and IR state. AT undergoes chronic low-grade inflammation and immune cells, such as macrophages, infiltrate the tissue, releasing pro-inflammatory cytokines (i.e. TNF-α and IL-6) that contribute to systemic inflammation, IR, and cardiovascular risks(Luo and Liu, 2016).

Adipocytes are dynamic cells that can expand (hypertrophy), multiply (hyperplasia), or even transdifferentiate (e.g., white to beige or brown adipocytes) in response to metabolic needs or environmental cues (Sakers et al., 2022).

Indeed, obesity involves hypertrophy (increasing fat cell size) and hyperplasia (increasing fat cell number) of adipose cells. Hypertrophic

adipocytes are more prone to hypoxia, stress, and dysfunction, contributing to inflammation and metabolic disturbances.

Adipocyte plasticity allows it to adjust metabolic processes maintaining energy balance, expanding itself in response to long-term stimulation of energy intake, changing during cold exposure to generate heat, and even de-differentiating when needed (Sakers et al., 2022).

AT forms with liver a key metabolic axis able to maintain body homeostasis, particularly in lipid metabolism and energy storage. Under normal physiological conditions, these two tissues work synergistically to support metabolic stability by secreting adipokines and growth factors. However, the function of the liver-adipose axis can be disrupted by multiple factors and mechanisms associated with obesity (Duwaerts and Maher, 2019).

Several efforts are underway to reduce body weight gain through the development of nutritional or pharmacological treatments targeting both organs involved in metabolic homeostasis and promoting AT plasticity.

Non canonical endocannabinoid N-acylethanolamines (NAEs) represent a family of endogenous bioactive lipids, synthesized "on demand" from membrane phospholipids, which can exert many beneficial activities through different converging mechanisms of action. The NAEs can interact with different receptors which are involved in many physiological processes, playing an important role in the regulation of energy homeostasis (Borrelli and Izzo, 2009; Hansen, 2014). Among these, palmitoylethanolamide (PEA), oleoylethanolamide (OEA) and linoleolylethanolamide (LEA) can all

activate peroxisome proliferator-activated receptor alpha (PPAR-α), that can mediate their anorexic, analgesic and anti-inflammatory effects (Mock et al., 2023). Even if they were previously defined endocannabinoids, PEA and OEA, as well as the stearoylethanolamide (SEA), do not bind to either CB1 or CB2 receptors and, in many instances, their endocannabinergic-like pharmacological effects *in vivo* cannot be ascribed to activation of any of the known CB receptor subtypes.

Even if SEA and LEA have been less studied, they exhibit similar biological activities to OEA and PEA. The last ones just mentioned exert a plethora of biological effects, involved in inflammation, pain, and metabolism control, which are mainly due to its interaction with the PPARα (Mattace Raso et al., 2014a). This receptor regulates the expression of genes involved in lipid metabolic pathways in hepatocytes, controlling trafficking, delivery, and storage of FFAs or enzyme function involved in mitochondrial β-oxidation (Kersten, 2014; Lin et al., 2022).

Among the NAE family members, OEA plays a key role in regulating satiety and lipid metabolism but also has anti-inflammatory and neuroprotective effects (Schwartz et al., 2008; Yang et al., 2016), which may be mediated through receptors such as GPR119 and the TRPV-1 channel(Mock et al., 2023). Similarly, PEA improves energy balance and metabolic flexibility in obesity and diabetes models and can activate liver AMP-activated protein kinase (AMPK), a crucial regulator of metabolism in both brown and white ATs(Annunziata et al., 2020).

Another class of naturally compounds are the polyphenols, which are naturally present in plant-based foods (fruits, vegetables, and medicinal plants) and protect against chronic diseases such as cardiovascular issues and obesity (Ciumarnean et al., 2020). Research has shown that these bioactive compounds provide strong protective effects against steatosis, oxidative stress, inflammation, and gut dysbiosis, which are linked to the progression of MAFLD (Pirozzi et al., 2016) (Rana et al., 2022).

Notably, hydroxytyrosol (HT), a key metabolite of oleuropein found in extra virgin olive oil, and the flavonoid rutin (Rut), found in plants like buckwheat, passionflower, apple, and tea, play crucial roles in detoxifying free radicals in the liver (de Pablos et al., 2019; Mosca et al., 2021; Pirozzi et al., 2016).

Considering the multiple mechanisms involved in the progression of metabolic damage correlated to obesity and their multifaceted interrelations, it is inadequate to target a single player, and it would be naive to search for a unidirectional remedy. Finetuning of this complex pathological process could be achieved only by multitarget therapies with synergistic and converging effects.

Thus, identifying the combination of safe and useful natural active products could provide an appealing approach to counteract this multifactorial disorder and prevent hepatic disease progression. The aim of this PhD program, co-funded by the EPITECH group S.p.A., has been to evaluate the possible beneficial effect of the association of NAEs and/or polyphenolic compounds in obesity and related disorders. Specifically, we investigated the effect of two dietary supplements: NORMAST 3 (NORM3) a patent formulation containing

co-micronized PEA and Rut associated with HT (as diabetes relievers) and OLALIAMID (OLA) a NAE mixture containing different percentage of OEA, PEA, LEA, and SEA, obtained from olive oil, in counteracting obesity-related disorders including diabesity and MAFLD.

1. OBESITY AND RELATED METABOLIC DISORDERS

Obesity has been defined as an abnormal and excessive fat accumulation that may impair health. Clinically, obesity is diagnosed using body mass index (BMI), which serves as a surrogate of body fat percentage. Specifically, it is considered the result of an imbalance between energy intake and expenditure (Figure 1) and is usually classified by a BMI > 30 kg/m^2, according to the definition of the World Health Organization. The degree of obesity can be further subcategorized into class 1 (BMI of 30 to <35), class 2 (BMI of 35 to <40), and class 3 (BMI of >40) (Purnell, 2000).The accumulation of an excessive amount of body fat induces a plethora of metabolic diseases, including IR, atherogenic dyslipidemia (characterized by high plasma TGs and low HDL-cholesterol), nonalcoholic fatty liver disease (NAFLD), pancreatic β cell dysfunction, and type 2 diabetes (T2D) and mood disorders, including anxiety (Bean et al., 2008; Luppino et al., 2010). Therefore, it is associated with a significant increase in morbidity and mortality. The concept of energy balance is essential to understand the complexity of obesity. If an individual has no issues with nutrient absorption, stored energy increases only when calorie consumption exceeds total energy expenditure. Energy expenditure comprises physical activity, basal metabolic rate, and adaptive thermogenesis. Physical activity refers to voluntary movements, basal metabolism refers to the various biochemical processes required for producing energy and sustaining life, while adaptive thermogenesis, on the other hand, involves the energy consumed as heat in response to environmental changes, such as

exposure to cold or dietary alterations and restrictions (Spiegelman and Flier, 2001).

Physiologically, energy balance is tightly ensured by a complex interplay between the feeding regulatory centers in the central nervous system (CNS) and the fat storage and mobilization that maintains body energy homeostasis (Affinati and Myers, 2000). Consequently, learning about both the peripheral and central pathways involved in this process is crucial to comprehend obesity itself.

In a lean individual, lipid overnutrition promotes an increased fatty acid oxidation (FAO), due to an adequate metabolic flexibility, and leads to an increased transcription of genes involved in this process and fatty acid transport among metabolically active tissues(Battaglia et al., 2012). During obesity, the onset of metabolic inflexibility results in ectopic lipid accumulation and development of IR (Morino et al., 2006). Specifically, excessive caloric intake results in fat accumulation in AT, leading to adipocyte hypertrophy and hyperplasia. Then, when fat accumulation exceeds the storage capacity of adipocytes, the FFAs enter the circulation and forms ectopic deposits in other organs, giving rise to the visceral fat, able to impair vascularization and induce oxidative stress, that in turn plays a role in the pathogenesis of various obesity-associated diseases (Jin et al., 2023).

Another crucial regulating factor of weight gain is the central control of appetite and energy balance, mediated by neural systems in the hypothalamus, central brainstem, cerebral cortex, and olfactory regions. In this context, the neuropeptide Y (NPY) pathway is a prominent example of how the CNS influences metabolic regulation. It is widely and abundantly expressed in the CNS, particularly in the

arcuate nucleus of the hypothalamus, which is crucially involved in nutritional regulation; indeed, NPY levels increase during starvation, enhancing feeding behavior while reducing energy expenditure. This response promotes weight gain and can contribute to the onset of obesity (Huang et al., 2021).

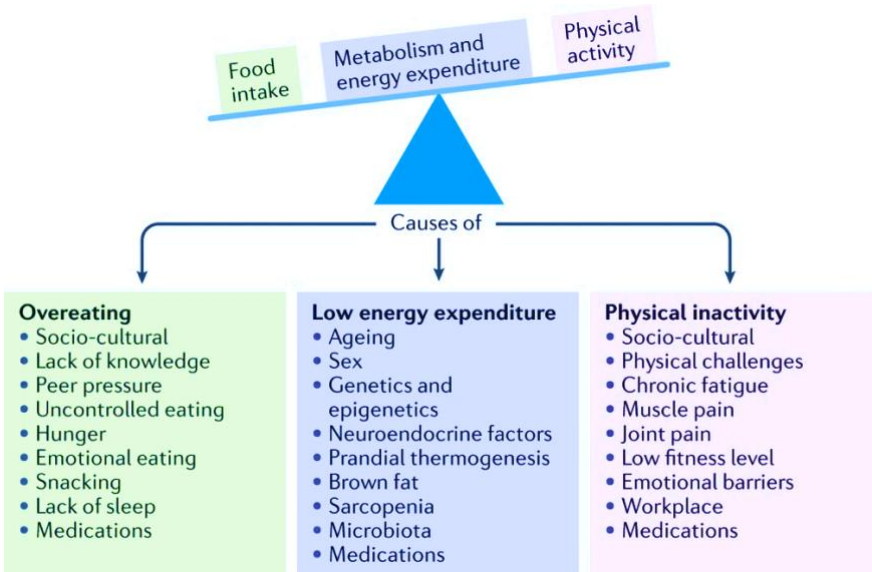


Figure 1. Factors that can contribute to a chronic positive energy balance, ultimately leading to obesity (Blüher M. et al., Nat Rev Endocrinol. 2019; doi:10.1038/s41574-019-0176-8)

1.1 Diabetes related to obesity (Diabesity)

As a result of the increasing prevalence of obesity, more than fifty comorbidities have emerged, posing significant threats to global health. Several strong lines of evidence support the link between obesity and diabetes. Generally, the progressive increase in BMI is linked to an increased risk of T2D (Colditz et al., 1995) and it has been demonstrated that also the distribution of fat plays a role in modifying this risk ((Klein et al., 2022). Indeed, individuals with a greater

accumulation of upper body fat (including abdominal subcutaneous and intra-abdominal fat), intrahepatic TG and pancreatic fat have a higher risk of developing T2D compared to those with a lower body (gluteo-femoral) fat phenotype (Klein et al., 2022).These results indicate that the site of fat storage, together with the amount of total fat, significantly can influence metabolic dysfunction and diabetes.

Diabetes occurs when insulin is no longer effective in promoting the normal uptake, storage, and utilization of glucose as energy in metabolic tissues like the liver and muscles. It is broadly classified as type 1 diabetes (T1D), which develops based on immune destruction of pancreatic beta cells; and T2D, which is associated with IR and defective insulin secretion insufficient to compensate for IR. Another type of diabetes is called gestational diabetes, which consists in hyperglycemia occurring during pregnancy (Gupta et al., 2024). However, there is growing evidence of significant overlap across the spectrum of T1D and T2D. Indeed, in T2D, the stress response induced by hyperglycemia and following IR are associated with a compromised β-cell function.

T1D, previously called "insulin-dependent diabetes" or "juvenile-onset diabetes," accounts for 5%–10% of all diabetes cases and is defined by the presence of one or more of autoimmune markers: islet cell autoantibodies and autoantibodies to GAD (GAD65), insulin, the tyrosine phosphatases IA-2 and IA-2β, and ZnT8 (American Diabetes, 2010).

The diagnosis of diabetes mellitus is established when the patient presents excessive hyperglycemia (blood glucose value of 200 mg/dL

or higher levels) established by using the following tests (American Diabetes, 2010):

- The oral glucose tolerance test (OGTT) which measures blood glucose after overnight fasting and followed by an oral glucose load. This test is based on the principle that following the oral administration of a high dose of glucose, the glycemia normally rises but then returns to the fasting levels thanks to insulin hypoglycemic action; while if insulin activity is compromised, the plasma glucose concentration takes longer than 2 hours to return to normal.
- Fasting glycemia test which directly measured blood glucose after a fasting period.

It has been demonstrated that managing obesity can delay the progression of prediabetes to T2D (American Diabetes Association Professional Practice, 2024).

T2D, referred to as "noninsulin-dependent diabetes" or "adult-onset diabetes," accounts for 90–95% of all diabetes. Notably, up to 85.2% of T2D patients are overweight or obese (Ruze et al., 2023).

An efficient function of pancreatic β cells is a key factor in determining whether individuals with obesity will develop T2D. In people with obesity who do not develop T2D, plasma insulin concentrations are typically higher than in lean individuals, but this increased secretion of insulin is able to counteract tissue resistance to insulin action, allowing a normal fasting blood glucose levels and good oral glucose tolerance. However, a consistent decrease in glycemic control occurs when β cell function progressively declines, leading to prediabetes and eventually T2D (Klein et al., 2022).

The term “diabesity” was coined by Sims et al., in 1973 to describe the very strong pathophysiological link between diabetes and excessive body weight. A plethora of pathophysiological processes underlying the progression of diabetes during obesity was proposed, along with the cellular and physiological mechanisms which link these two pathologies involving several key factors (Figure 2) (Al-Goblan et al., 2014).

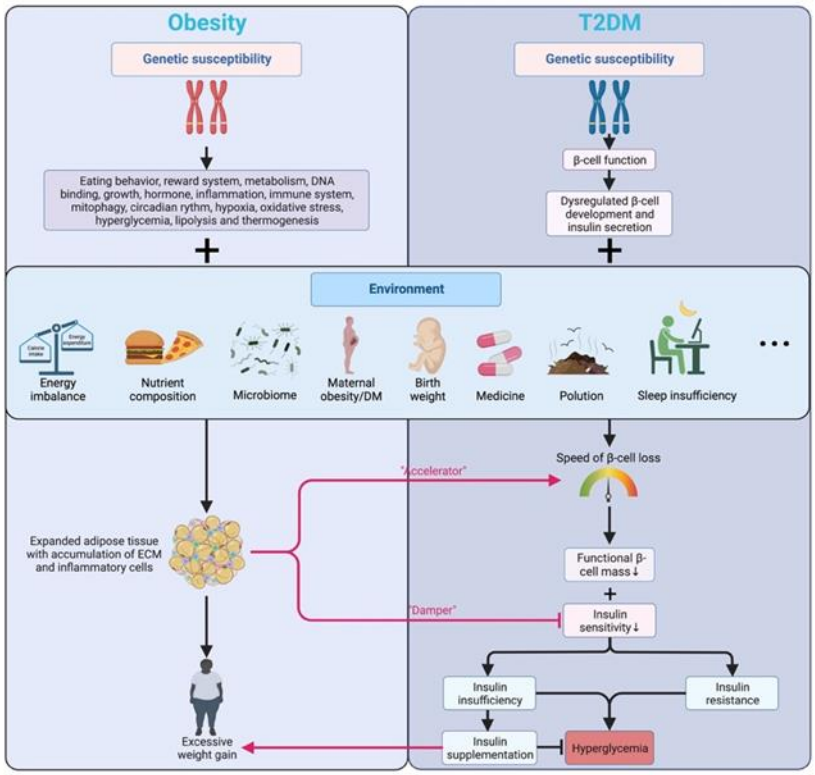


Figure 2. Obesity and T2D connections (Ruze R et al., Front Endocrinol (Lausanne). 2023; doi:10.3389/fendo.2023.1161521)

Firstly, obesity leads to alterations in β cell function, changes in AT biology, and multi-organ IR. These disruptions contribute to the impaired glucose metabolism and insulin sensitivity. However, these

adverse effects are often ameliorated through an adequate weight loss, which can restore normal β cell function, improve insulin sensitivity, and reduce the metabolic burden on multiple organs, highlighting the critical role of weight management in diabetes prevention and treatment.

Consistently, three main hypotheses about the development of diabesity have been described during these years (Ortega et al., 2020):

1) The "**inflammation hypothesis**" asserts that excessive body fat accumulation can induce a state of chronic inflammation characterized by the production of elevated circulating FFAs and adipocyte-derived cytokines which mediate IR and trigger pathological changes in insulin-sensitive tissues and β-cells.
2) The "**lipid overflow hypothesis**" suggests that adipocyte hypertrophy can cause lipid accumulation outside normal fat depots, depositing in tissues such as the liver, muscle, and pancreas, potentially impairing their functions.
3) The "**adipokine hypothesis**" refers to the loss of endocrine function of white adipocytes, which occurs during obesity, causing a dysfunctional secretion of endocrine factors and the metabolic impairment of insulin sensitive tissues.

All these changes contribute to a condition known as "lipotoxicity," triggering chronic inflammation and oxidative stress (Oguntibeju, 2019). This process is a key factor in the progression of diabesity and in the reduction of β-cell mass. Indeed, insulin-producing β cells are susceptible to elevated lipid levels, and preserving their mass and

function is a critical goal in managing diabetes under these metabolically stressful conditions (Son and Accili, 2023).

Given the low antioxidant capacity of human β cells, they are particularly vulnerable to reactive oxygen species (ROS) generated by increased mitochondrial activity in response to excess lipid overload. This makes β cells highly susceptible to oxidative damage, contributing to their dysfunction and loss, which are key features in the development and progression of T2D during obesity (Son and Accili, 2023).

Moreover, during obesity, the excessive intake of carbohydrates can increase IR and require more insulin to regulate blood glucose levels. This condition leads to persistent hyperglycemia and glucotoxicity. Once glucotoxicity sets in, it further impairs insulin secretion and action, creating a vicious cycle where IR and hyperglycemia reinforce each other (Martyn et al., 2008). Over time, this condition accelerates the progression of diabetes and its associated complications, such as neuropathy, retinopathy, and cardiovascular disease (Robertson et al., 2003).

This condition, where lipotoxicity and glucotoxicity act detrimentally and synergistically, can result in β-cell dysfunction and death. The primary mechanisms involved include oxidative stress, mitochondrial stress, and endoplasmic reticulum stress (Poitout and Robertson, 2008).

Therefore, an ideal treatment strategy for diabetes should include an optimal management of obesity, since weight loss improves glycemic control and reduces the need for glucose-lowering medications.

Indeed, decreasing body fat mass by inducing a negative energy balance, can ameliorate or normalize obesity-induced metabolic dysfunction and can even achieve diabetes remission if associated with an adequate restoration of β-cell function (Klein et al., 2022).

Even modest weight loss improves the sensitivity of AT and liver, suggesting that the therapeutic benefits of weight reduction is due, in part, from restoring homeostasis in these essential metabolic tissues. Furthermore, energy restriction and weight loss can partially restore insulin secretion in prediabetes and early-stage T2D. Unfortunately, reversing long-standing diabetes remains extremely challenging.

The importance of optimal management of diabesity is often overlooked by medical professionals when achieving adequate glycemic control which results in inappropriate management of other complications.

An appropriate selection of antidiabetic drugs is crucial in the management of diabesity, since several classes of anti-diabetic medications including sulphonylureas, thiazolidinediones and meglitinides are associated with the risk of weight gain and may potentially worsen diabesity. In addition, since diabesity is a multi-variant metabolic disorder, it is important to consider that a single drug or natural product may be insufficient to show the desired therapeutic effect.

1.2 Metabolic dysfunction-associated fatty liver disease (MAFLD)

Effective communication between the liver and AT is crucial for maintaining metabolic homeostasis. In obesity, this interaction is disrupted, triggering a cascade of metabolic complications.

Specifically, the excessive fat accumulation in AT can lead to increased circulating levels of FFAs and inflammatory markers, which negatively affect liver health and insulin sensitivity (Figure 3). This imbalance can create a vicious cycle, further exacerbating metabolic disorders (Ziolkowska et al., 2021).

The rise in obesity is driving a significant increase in the prevalence of NAFLD, which has reached epidemic levels. NAFLD is a progressive condition characterized by the accumulation of TGs, with more than 5% of hepatocytes containing lipid droplets (Semova and Biddinger, 2021). Its prevalence closely mirrors the rising rates of obesity and diabetes (Kim et al., 2021).

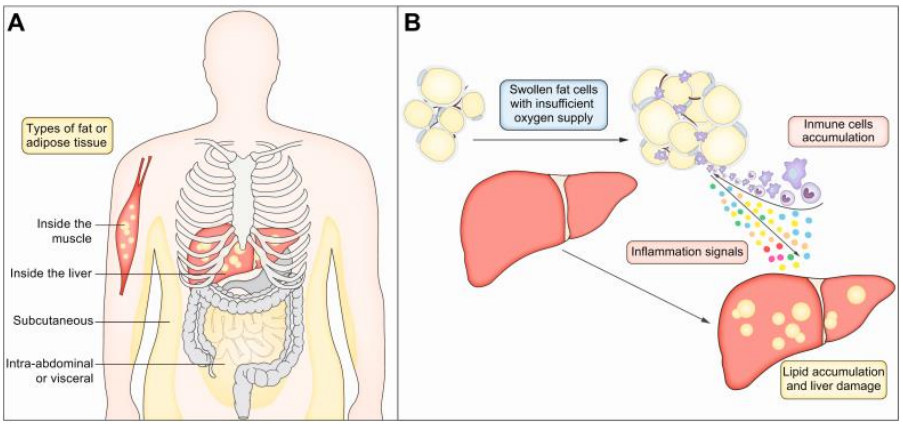


Figure 3. Fat localization in different tissues B) Fat accumulation in the liver during during obesity: Subcutaneous fat has a storage limit, so when fat tissue is overwhelmed and the adipocytes swell and become inflamed due to a lack of blood flow and oxygen, leading to lipidic accumulation in the liver and meta-inflammation (Francque et al., JHEP Rep. 2021; doi: 10.1016/j.jhepr.2021.100322)

NAFLD ranges from simple steatosis to NASH which is characterized by liver steatosis, lobular inflammation, and hepatocellular ballooning with or without fibrosis (Pouwels et al., 2022).

The inflammation and damage to hepatic cells caused by NASH can lead to serious complications. As the inflamed liver progresses to fibrosis, scar tissue forms, replacing healthy liver tissue. If untreated, this can advance to cirrhosis, characterized by extensive liver scarring (Figure 4).

Over time, this progression can significantly impair liver function and heighten the risk of severe life-threatening complications (Simon et al., 2023).

Various theories have been proposed to explain the progression of this complex disease. The initial "two hits hypothesis" suggested that the first hit involves hepatic lipid accumulation due to factors including an excessive high-fat diet consumption, sedentary lifestyle and IR.

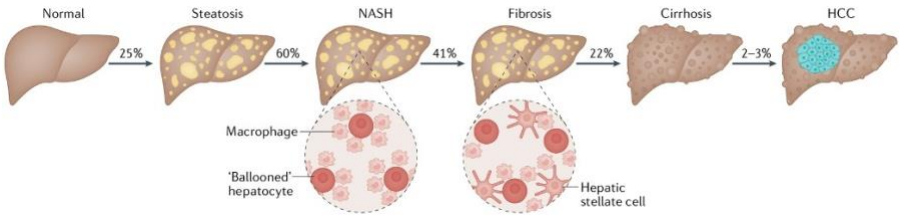


Figure 4. The pathological spectrum of NAFLD: Ferguson D. et al., Nat Rev Endocrinol. 2021; doi:10.1038/s41574-021-00507-z)

The second hit is then required to trigger inflammation and fibrosis (Paschos and Paletas, 2009). However, this model oversimplifies the disease's complexity, as multiple factors interact synergistically to drive its development and progression.

Consequently, a multiple-hit hypothesis has replaced the two-hit hypothesis. This theory considers more ‘’hits’’ acting together in predisposing to NAFLD. These insults include IR, hormones secreted

from the AT, nutritional factors, gut microbiota and genetic and epigenetic factors (Buzzetti et al., 2016).

The heterogeneity of this disease is closely due to hepatic cell plasticity, specifically to the ability of hepatic cells to change their identity or phenotype in response to environmental factors. During NAFLD progression, liver cells, including hepatocytes, cholangiocytes, Kupffer cells, hepatic stellate cells, and endothelial cells exhibit this plasticity, which enables them to adapt to external challenges such as FFA accumulation, inflammation, and oxidative stress. These adaptive responses play a crucial role in driving the onset and the progression of the disease (Park et al., 2023).

Over the past 20 years, numerous studies have clearly illustrated NAFLD as a metabolic disease, representing the hepatic manifestation of a systemic metabolic disorder. From 2020 on, experts in liver pathophysiology have proposed the term "metabolic-associated fatty liver disease" (MAFLD) as a new acronym which more accurately reflects the shared etiology of conditions linked to hepatic dysmetabolism (Eslam et al., 2020).

Initially, NAFLD was described as a condition of "exclusion," meaning that it was diagnosed only in the absence of other factors, such as viral hepatitis B or C, or alcohol consumption above a certain threshold. The new terminology shifts toward an "inclusion" approach, recognizing the disease as part of a broader metabolic disorder. This updated definition includes new diagnostic criteria, requiring the presence of hepatic steatosis along with at least one of the following conditions: obesity, diabetes, or metabolic dysregulation (Eslam et al., 2020).

Consequently, alcohol consumption is not referenced in the MAFLD acronym, as well as in NAFLD.

MAFLD represents a complex form of fatty liver disease influenced by various factors, including age, gender, ethnicity, diet, smoking, genetic predisposition, and metabolic status (Eslam et al., 2020). Patients with MAFLD frequently develop cardiovascular diseases, including hypertension, atherosclerosis, and heart and kidney diseases. For instance, 80–90% of individuals with obesity and 70–80% of those with T2D are commonly affected by MAFLD (Bellentani et al., 2010). Multiple factors contribute to the development and progression of MAFLD. Among others, genetic variants play a significant role in increasing susceptibility. A notable example is the PNPLA3(patatin-like phospholipase domain-containing protein 3) gene variant (rs738409; I148M), which is strongly linked to elevated hepatic fat levels and inflammation (Pirazzi et al., 2012). The I148M substitution results in a loss of function for PNPLA3, as shown in studies with knock-in mice, disrupting its ubiquitylation and proteasomal degradation, leading to accumulation and impaired TG mobilization from lipid droplets, further promoting liver fat accumulation and inflammation. Additionally, TM6SF2, primarily expressed in the intestine and liver, appears to modulate VLDL-TG secretion in the liver. Variants of the MBOAT7 gene, associated with alcohol-related cirrhosis, have also been linked to the development and severity of NAFLD (Mancina et al., 2016). Other genetic variations, including those in the glucokinase regulator (GCKR) gene locus, as well as variations in LYPLAL1, APOB, MTP, LPIN1, UCP2, IL28B, MERTK, ENPP1, IRS1, SOD2, and KLF6, have been implicated in

lipid metabolism, innate immunity, insulin signaling, oxidative stress, and fibrogenesis, all of which are relevant to the progression of NAFLD (Donaldson, 2004; Kanmani et al., 2020).

1.2.1 Alterations of Lipid Metabolism in the Liver

As previously assumed, MAFLD is characterized by hepatic steatosis, which arises from the excessive accumulation of FFAs into the liver (Utzschneider and Kahn, 2006). Specifically, lipid overload and the resulting positive energy balance characterizing obesity leads to fat redistribution among metabolic organs, such as the liver, heart, and skeletal muscle (Mann et al., 2024).

This condition develops when the influx of fatty acids (FA)s exceeds their removal via β-oxidation, lipolysis, and VLDL-TG secretion(Kim et al., 2021).

This FA accumulation mainly originates from dietary consumption or AT lipolysis, but FAs are also synthesized *de novo lipogenesis* (DNL) in the liver from the surplus of glucose (Kim et al., 2021).

Both insulin and glucose can activate key signaling pathways that drive lipogenesis, leading to the storage of energy as fat. Specifically, carbohydrate intake raises serum insulin levels, which enhances DNL, as well as carbohydrate metabolism increases the production of acetyl-coenzyme A (CoA), a crucial substrate for fatty acid synthesis, further contributing to fat accumulation.

The uptake of FFAs in cells primarily occurs through simple passive diffusion and is influenced by intracellular and extracellular concentrations. Additionally, this process can be regulated by transport proteins in response to hormonal stimuli (Hamilton et al., 2001).

The lipid uptake in the liver is highly regulated, in both physiological and pathological conditions, via the actions of liver fatty acid transport proteins (FATPs) and the cluster of differentiation (CD)36.

Fatty acid-binding protein 1 (FABP1) was originally referred to liver-FABP or L-FABP because is primarily expressed in the liver. It is responsible for shuttling FAs between different organelles and plays a crucial role in binding potentially toxic FFAs (Habibullah et al., 2024). Moreover, it promotes the oxidation of FAs or their storage as TGs, thereby supporting lipid metabolism and mitigating lipid-induced damage. This process leads to the maintenance of cellular lipid homeostasis and protects the liver from metabolic stress associated with MAFLD.

Consistently, a previous study found that higher plasma levels of FABP1 correlated with hepatic steatosis in human patients affected by MAFLD, suggesting how it may be involved in a compensatory mechanism to counteract hepatocellular steatosis (Wang et al., 2015). *In vivo* experiments demonstrated that the restoration of hepatic FABP1 through a fish oil diet led to a decrease in serum TGs and very-low-density lipoprotein (VLDL) cholesterol levels. This dietary intervention also resulted in an increase in high-density lipoprotein (HDL) cholesterol levels and it was associated with a reduction in the expression of inflammatory markers in the liver of diabetic rats. (Devarshi et al., 2013).

CD36 is the major receptor involved in long-chain FA transport and TGs storage and is expressed in several tissues such as liver and AT and different cells such as macrophages and monocytes (Rada et al., 2020). Increasing evidence indicates that CD36 not only acts as a FFA

transporter, but also regulates FFA oxidation, lipid synthesis, autophagy and inflammation in the liver (Samovski and Abumrad, 2019). Specifically, there are convincing *in vitro* and *in vivo* evidence that describe how CD36 can drive hepatic steatosis onset and might contribute to its progression to NASH (Feng et al., 2022).

The liver regulates the fatty acid metabolism through different molecular mechanisms, particularly DNL and the utilization of FA by FAO (Figure 5). When the balance between these pathways is altered, hepatic lipid accumulation begins, inducing a long-term activation of inflammatory and fibrotic pathways that can progress and worsen liver diseases.

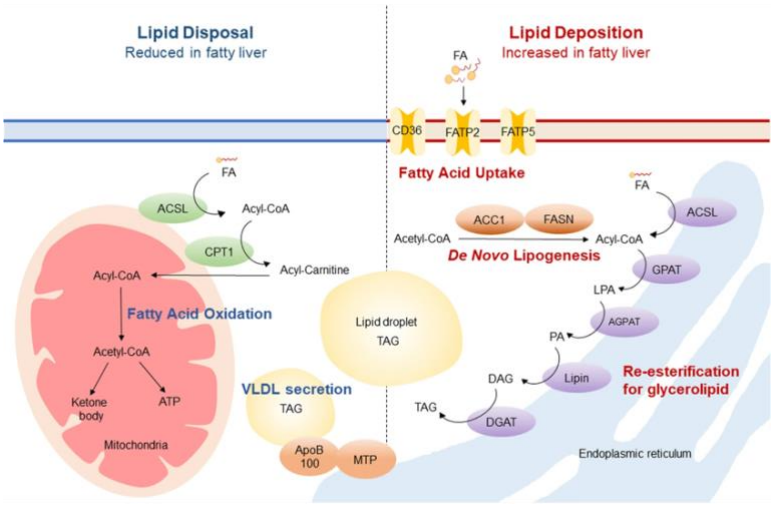


Figure 5. Lipid metabolism in the liver in lipid disposal and in the context of fatty liver disease (Huh JY et al., Exp Mol Med. 2021; doi:10.1038/s12276-021-00712-w)

DNL is the metabolic process through which the liver converts excess carbohydrates into FAs once the body's energy demands are met, storing the surplus as fat. Three key enzymes: acetyl-CoA carboxylase

(ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase-1 (SCD1) regulate the process of DNL in the liver. ACC catalyzes the conversion of acetyl-CoA to malonyl-CoA, which is subsequently transformed into palmitate (PA) by FAS. SCD1 facilitates the conversion of stearoyl-CoA and palmitoyl-CoA in oleic and palmitic acids, respectively. These newly synthesized FAs undergo various biological modifications, including desaturation, elongation, and esterification, before being stored as TGs or exported as VLDL.

FAO primarily occurs in the mitochondria, although the oxidation of very long-chain FAs starts in the peroxisomes before being completed in the mitochondria. The transport of FAs into the mitochondria is managed by carnitine palmitoyltransferase (CPT)1, located on the outer mitochondrial membrane. An upstream regulator of CPT1 is the PPARα, which, when activated, promotes the transcription of genes involved in FAO in the mitochondria including CPT1, peroxisomes (acyl-CoA oxidase, ACOX), and the cytochrome P450 system (Cyp4A family) (Zhuang et al., 2022).

In pathological conditions such as MAFLD, the excessive intake of carbohydrates, particularly sugars like fructose, stimulates DNL leading to increased fat (i.e. TGs) storage in the liver (Alfadda et al., 2022).

Liver mitochondria account for about 18% of the hepatocyte volume, playing a key role in the metabolic networks. Indeed, mitochondrial damage leads to the loss of membrane polarization, reducing the organelle's ability to effectively complete β-oxidation and energy metabolism, thereby exacerbating fatty acid accumulation (Karkucinska-Wieckowska et al., 2022). In MAFLD, the massive

release of ROS by mitochondria is primarily linked to the pathological increase of FAO caused by the excessive FFA overload. Briefly, when FFAs are converted into fatty acyl-CoAs in the cytoplasm and transferred to the mitochondria via CPT1, they undergo FAO, generating acetyl-CoA that enters the tricarboxylic acid cycle. Excessive activation of tricarboxylic acid cycle leads to an overflow of electrons through the electron transport chain. In normal conditions, electrons flow through cytochrome c oxidase, where they combine with oxygen and protons to form water (H_2O). Obesity can impair the electron transport chain, leading to electron leakage that activates molecular oxygen and generates reactive species, such as superoxide (O_2•) and hydrogen peroxide (H_2O_2) (Prasun et al., 2021).

Long-chain FAs are oxidized by peroxisomes more powerfully than mitochondria. However, peroxisomal β-oxidation produces H_2O_2, which is quickly transformed into the highly reactive hydroxyl radical (HO•). ROS trigger lipid peroxidation, targeting polyunsaturated FAs and generating reactive aldehyde products like 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) (Jomova et al., 2023). These highly reactive molecules diffuse into the extracellular space, exacerbating hepatic damage. In patients with NASH, there is a marked reduction in the activity of key antioxidant enzymes, including catalase, glutathione peroxidase, glutathione S-transferase, and superoxide dismutase, associated with diminished levels of ROS scavengers such as ascorbic acid, glutathione, α-tocopherol, ubiquinone, thioredoxin, and bilirubin. This compromised antioxidant response contributes to ROS accumulation and oxidative stress, and the following progression of liver damage (Chen et al., 2021).

Under physiological conditions, the nuclear factor erythroid 2-related factor 2 (NRF2)/ heme oxygenase-1(HO-1) axis is essential for resistance to oxidative stress, and upregulation of this axis significantly suppresses the expression of NLRP3, caspase-1, and inhibits inflammasome formation (Xu et al., 2018). Specifically, NRF2 plays a key role in the antioxidant and anti-inflammatory mechanisms, and it is primarily regulated by KEAP1, which facilitates its degradation via the polyubiquitin-proteasomal pathway. Under normal conditions, NRF-2 is bound to KEAP1, keeping it inactive. However, when NRF-2 is not bound to KEAP1, it translocates into the nucleus, where it binds to antioxidant response elements (AREs) in the DNA, promoting the transcription of detoxifying enzymes such as NADPH, glutathione peroxidase, ferritin, and HO-1 (Ulasov et al., 2022). Additionally, increased levels of NRF-2 can suppress the transcription of pro-inflammatory cytokines such as IL-6 and IL-1, thereby exerting an anti-inflammatory effect (Solano-Urrusquieta et al., 2020).

Several studies have reported that targeting NRF2 is crucial in the protection against NASH, since this factor enhances the expression of most antioxidant enzymes and down-regulates the expression of genes involved in fatty acid synthesis. In line with these findings, mice lacking Nfe2l2 (encoding NRF2) showed many NASH features, suggesting a key role of NRF2 in preventing NASH progression, not only due to the activation of antioxidant genes, but also by modulating fatty acid metabolism in hepatocytes (Rada et al., 2020).

Another central process, deeply altered during obesity and related-IR, is the TG synthesis, which involves the assembly of FAs (whether

produced through DNL or derived from dietary fats) into TGs, and then their storage in AT and/or in the liver.

In the context of IR, insulin is unable to effectively suppress the release of FAs from adipocytes, resulting in increased flux of FFAs to the liver that will serve as substrates for TG synthesis, promoting TG accumulation.

The excess of TGs provides substrates for the generation of lipotoxic molecules such as lysophosphatidic acid, lysophosphatidylcholine, lysophosphatidylinositol, diglycerides (also known as diacylglycerols or DAGs), and ceramides (Semova and Biddinger, 2021). Inhibiting diacylglycerol acetyltransferase DGAT2 enzyme, involved in TG synthesis, improves hepatic steatosis in MAFLD patients(Loomba et al., 2020).

1.2.2 Hepatic glucose dysmetabolism and insulin resistance

Insulin is released from pancreatic β cells predominately because of increased plasma glucose levels, but also in response to other monosaccharides, amino acids, and FAs (Fu et al., 2013).

During the fed state, insulin binds and activates its receptors promoting glucose uptake and its utilization in liver, muscle, and AT (Habegger, 2022) (Saltiel and Pessin, 2002). Otherwise, in fasting conditions, glucagon released from pancreatic α-cells stimulates glycogenolysis in the liver, to raise blood glucose levels. Glycogenolysis involves the breakdown of glycogen into glucose, which occurs also during energy-demanding situations, and is activated by the sympathetic nervous system.

In the liver, the binding of insulin to its receptor activates the tyrosine kinase function of the insulin receptor, leading to the activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and other downstream pathways. The stimulation of AKT pathway leads to the activation of glycogen synthase (GS), which converts glucose-6-phosphate into glycogen, promoting glycogen storage in the liver. Specifically, this activation leads to AKT-mediated phosphorylation and inactivation of Glycogen Synthase Kinase 3 (GSK-3), which normally suppresses GS activity through its phosphorylation. When GSK-3 is inactivated by AKT phosphorylation, glycogen synthase efficiently converts glucose-6-phosphate into glycogen. This process ensures the storage of glycogen in the liver, allowing the body to maintain energy reserves during periods of nutrient abundance (Taheri et al., 2024). GS is also a target of the mammalian target of rapamycin complex 1 (mTORC1), and sterol regulatory element-binding protein (SREBP) (Hagiwara et al., 2012).

Furthermore, insulin suppresses gluconeogenesis (the production of glucose) by inhibiting the expression and activity of key enzymes involved in the process: the activation of AKT/PI3K induces the phosphorylation and the consequent inactivation FOXO1, a transcription factor that promotes the expression of gluconeogenic enzymes like phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). When FOXO1 is inhibited, the transcription of these enzymes decreases, leading to reduced gluconeogenesis (Cheng and White, 2011). Moreover, insulin antagonizes the effects of glucagon, by increasing cyclic AMP (cAMP) levels and activating protein kinase A (PKA). Insulin lowers cAMP

levels, reducing the activity of PKA, which in turn reduces gluconeogenic enzyme activity (Habegger, 2022).

In summary, insulin activates PI3K/ AKT pathway, GSK-3, PP1, and GCK, upregulating glycogen synthesis and inhibiting its breakdown in the liver. Additionally, in response to insulin receptor activation, peripheral glucose uptake is primarily facilitated by the glucose transporter GLUT4. Upon insulin signaling, GLUT4 translocates from its intracellular storage sites to the plasma membrane, allowing enhanced glucose entry into the cells. This process is crucial for maintaining glucose homeostasis and supporting energy metabolism in tissues such as muscle and AT (Dugani and Klip, 2005).

In the context of IR, these tightly regulated mechanisms are disrupted, promoting liver fat deposition and contributing to metabolic dysfunction (Figure 1.2.4.1) (Pal and Mendez-Sanchez, 2023).

IR consists in a decreased ability of insulin to suppress glucose production in the liver and to enhance glucose utilization in AT and skeletal muscle (Figure 6). On these last tissues IR induces the reduction of glucose uptake from blood in the muscles and fat depots with increased efflux of FFAs; on the other hand, IR causes an uncontrolled production of hepatic glucose resulting from the impaired suppression of gluconeogenesis and glycogen synthesis (Erichsen et al., 2022).

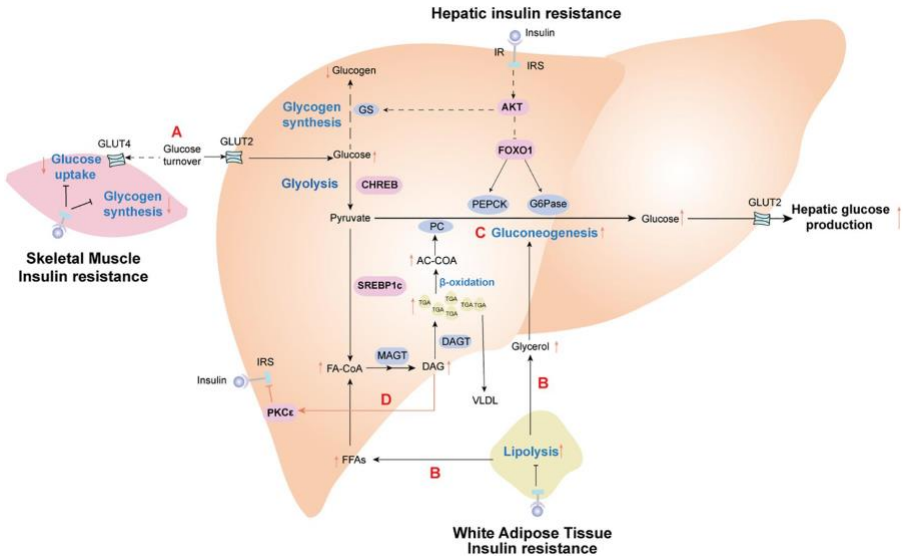


Figure 6. IR effect in the metabolic crosstalk between liver and peripheral tissue (Mu W, et al., Potential Nexus of Front Pharmacol. 2019; doi:10.3389/fphar.2018.01566)

Moreover, IR promotes AT dysfunction with the following altered production and secretion of adipokines and inflammatory cytokines (Pal and Mendez-Sanchez, 2023).

MAFLD is closely linked with both hepatic and AT IR, as well as decreased whole-body insulin sensitivity. Several studies showed that individuals with MAFLD exhibit a 45–50% reduction in insulin sensitivity, and impaired insulin-mediated suppression of endogenous glucose production, which is a key indicator of hepatic IR (Utzschneider and Kahn, 2006).

Research using tissue-specific insulin receptor knockout mice showed that knocking out the insulin receptor in the liver leads to significant metabolic dysfunction, while the loss of insulin receptors in muscle or AT does not result in altered glucose levels. These mice develop hyperglycemia, both in fasting and fed states, along with peripheral IR,

and hepatic steatosis, highlighting the essential role of hepatic insulin signaling in glucose and lipid metabolism (Biddinger and Kahn, 2006). As discussed above, muscle and AT's IR can exacerbate liver steatosis by reducing insulin's capability to suppress lipolysis, which increases the influx of FFAs into the liver. At the same time, hyperinsulinemia enhances DNL via the activation of sterol regulatory element-binding protein-1c (SREBP-1c) (Tong et al., 2016).

This combination promotes fat accumulation in the liver. The effectiveness of insulin-sensitizing agents like thiazolidinediones and metformin in reducing hepatic fat highlights the potential role of IR in the progression of NAFLD. Understanding how IR contributes to metabolic dysfunction is essential for developing targeted strategies to manage MAFLD in diabetic or obese patients. By addressing the underlying IR, treatment plans can more effectively reduce liver fat accumulation and improve overall metabolic health.

1.3 Adipose tissue: the changing color of fat

Until the 1940s, AT was widely considered as an inert tissue, primarily responsible for storing lipids. However, this perception changed in the 1990s with the discovery of leptin, which was shown to be released in response to changes in nutritional status (Rosen and Spiegelman, 2014). This finding demonstrated that AT functions as an endocrine organ, playing a pivotal role in regulating energy homeostasis. Indeed, AT is now considered a multi-depot organ with distinct anatomical structures, vascular networks, innervation and cellular composition (Cinti, 2012).

Fat represents the body's major fuel reserve, and acts as vital, transportable energy source, essential for survival during periods of food scarcity (Frayn, 2010). Due to its high energy density and hydrophobic characteristics, TGs are about five times more efficient as fuel compared to glycogen (Lu et al., 2014). Specifically, TGs are stored compactly as oil within adipocytes and when oxidized they generate approximately 9.3 kcal per gram. In contrast, glycogen is stored as a gel-like substance and produces only 4.1 kcal per gram (Klein et al., 2022).

Fat metabolism is tightly regulated through a complex interplay of energy status, adipokine secretion, and neuronal signals. However, this balance is severely disrupted in obesity, leading to metabolic dysfunction and related health problems.

The defining feature of the adipose organ consists in its ability to change, not only by expanding the size of each single cell (hypertrophy), but also increasing the number of resident cells (hyperplasia) (Rosen and Spiegelman, 2014). During obesity, its

excessive expansion causes significant alterations in AT microenvironments, leading to changes in adipokine secretion, local hypoxia, and altered fatty acid metabolism (Guerreiro et al., 2022).

Specifically, chronic overnutrition can induce a progressive decline in AT functions, particularly in its ability to store lipids efficiently.

In this context, adipocytes undergo significant changes, reaching a threshold beyond which their ability for size expansion becomes limited, leading to dysfunction and cell death (Sun et al., 2013).This dysfunction manifests as reduced insulin sensitivity, impaired lipolysis, disrupted fatty acid uptake, and altered adipokine secretion, all of which contribute to chronic low-grade inflammation and exacerbate metabolic disorders (Sun et al., 2013).

Furthermore, lipid-overload leads to mitochondrial damage and to the production of lipid peroxidation byproducts, which trigger an uncontrolled inflammatory response (Fromenty and Roden, 2023). This response results in systemic low-grade inflammation, contributing to the progression of obesity-related diseases, metabolic dysfunction and promoting conditions such as T2D and cardiovascular disease. Additionally, stromal vascular cells within AT, including immune cells, play essential roles in various adaptive processes like clearing dead adipocytes, adipogenesis, and angiogenesis. In obesity, these processes become dysregulated, leading to impaired remodeling of AT (Sakers et al., 2022).

All these phenomena modify the immune-endocrine profile of fat, sustaining the pathogenesis of obesity.

A deep knowledge of the functional anatomy and pathology of the AT is essential to understand these molecular mechanisms linking obesity and related disorders.

This organ is not merely a uniform compartment, but a dynamic ecosystem composed of different cell types whose interactions are crucial to its physiological and pathological functions. These cellular populations including adipocytes, macrophages, T cells, fibroblasts, endothelial cells, neutrophils, and B cells form an intricate network that regulates AT homeostasis (Sakers et al., 2022).

Each anatomical fat depot displays a unique pattern of functionality, cell composition and susceptibility to disease (Bjorndal et al., 2011).

AT has usually been classified into two types: white AT (WAT) and brown AT (BAT), easily distinguishable by their colors. The white and brown adipocytes constituting these depots exhibit distinct physiological characteristics, which result in specialized tissue functions (Figure 7). Precisely, WAT primarily functions as an energy storage reservoir, while BAT plays a crucial role in thermogenesis, generating heat by burning energy (Cypess et al., 2009). Interestingly, there is another type of AT depot called ‘’beige or brite’’ (brown-like-in-white) fat, which can express the uncoupling protein (UCP)1 when stimulated by cold exposure or β3-adrenoceptor agonists that mimic cold stress (Bostrom et al., 2012; Kotzbeck et al., 2018).

Patrick Seale, a developmental biologist at the University of Pennsylvania, highlights that it's misleading to label white and brown fat cells as simply "bad" or "good." While brown fat cells are often seen in a more positive light due to their role in burning energy and producing heat, white fat cells also play different crucial roles in our

body. They emphasize that white fat cells are important for protecting against metabolic diseases, storing lipids as a "safe home", avoiding their accumulation in other tissue like muscle and liver, in which they can otherwise be harmful (Sakers et al., 2022). Therefore, when they function properly, these cells help regulate energy balance and prevent metabolic diseases, such as IR or T2D.

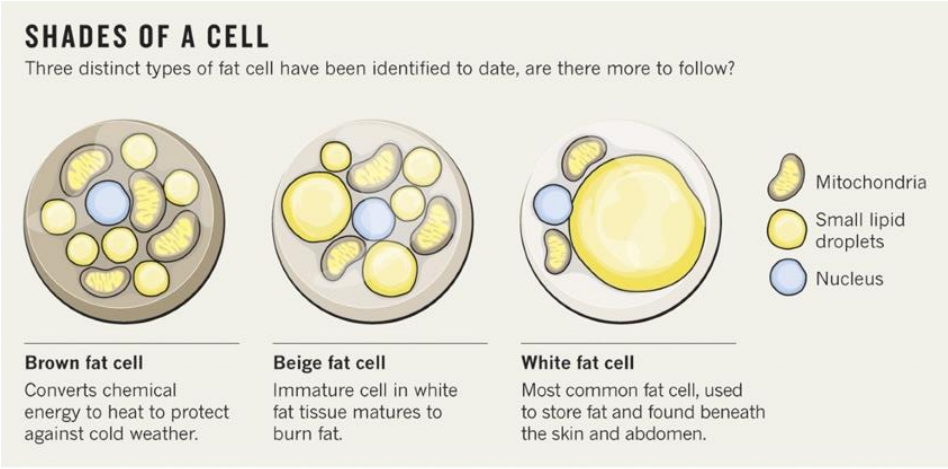


Figure 7. Shades of a fat cells (Cohen P, et al., Mol Biol Cell. 2016; doi:10.1091/mbc.E15-10-0749)

1.3.1 White Adipose Tissue (WAT)

The primary functions of WAT are:

a) the synthesis and storage of lipids derived from various substrates

b) the breakdown of TGs through lipolysis, which leads to the release of FAs.

Additionally, white adipocytes are capable of expressing and secreting numerous factors known as adipokines, including leptin and adiponectin, which exert autocrine, paracrine, and endocrine effects throughout the body (Bays et al., 2008; Cinti, 2009; Luo and Liu, 2016).

In addition to its primary functions WAT also provides mechanical support and isolation, protecting organs from trauma and cold, supporting reproductive organs, aiding immune defense in the intestine, and facilitating blood vessel dilation.

While human WAT is categorized into subcutaneous AT (ScWAT) and visceral AT (scVAT), smaller depots such as bone marrow AT, dermal white AT, and mammary white AT are being recognized as distinct entities with varied and specialized functions (Figure 8) (Mittal, 2019).

- ScWAT): This is the fat stored under the skin, serving as insulation, cushioning, and energy storage. It is often associated with metabolic health when present in moderate amounts.
- ScVAT): Found around internal organs, VAT is linked to various health risks, including T2D, metabolic syndrome and cardiovascular disease.
- Bone Marrow AT (BMAT): This type of AT plays a role in hematopoiesis (blood cell production) and has been involved in the regulation of energy and bone health (Suchacki et al., 2020).
- Dermal White AT (dWAT): Present in the skin, this depot may influence thermoregulation and wound healing (Chen et al., 2019).

WAT is predominantly composed of white adipocytes, which account for 35–75% of all cells. Light microscopy reveals that unilocular adipocytes, characteristic of WAT are typically large and characterized by a single, large lipid droplet that dominates the cytoplasm, displacing the nucleus to the periphery. Their diameter varies, ranging from 10–20 μm to 70–80 μm in mice, and approximately 30% larger in humans (Arner and Ryden, 2022).

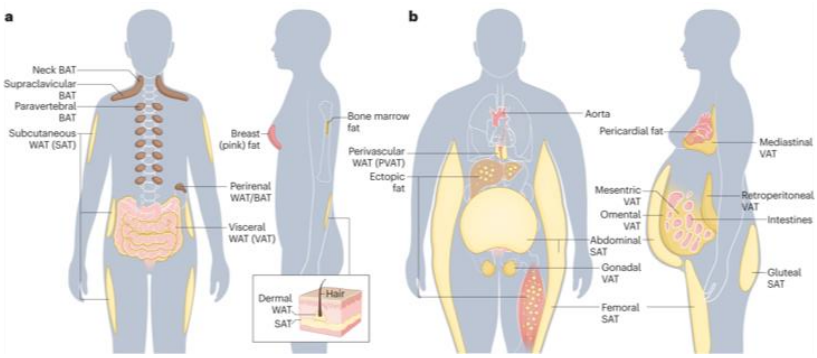


Figure 8. Human AT in individuals who are lean and individuals with obesity. Human AT can be categorized into subcutaneous AT (SAT), visceral AT (VAT), and brown AT (BAT). Lean individuals also have other types of AT, such as breast (pink) AT, bone marrow AT, and dermal white AT (WAT), which is distinct from SAT. In people with obesity, lipids begin to accumulate in organs like the liver and muscles, as well as in VAT depots, including the omental, mediastinal, retroperitoneal, epiploic, and mesenteric regions. Additionally, fat accumulates around blood vessels as perivascular AT (PVAT) and in pericardial areas around the heart.
(Hagberg CE et al., Nat Rev Mol Cell Biol. 2024: doi: 10.1038/s41580-024-00712-4)

While the remaining cell types include components of the stromal vascular fraction, such as fibroblasts, endothelial cells, blood cells, macrophages, pericytes, and preadipocytes, among others (Cinti, 2009). These non-adipocyte cells play key roles in supporting tissue structure, angiogenesis, and immune regulation within the WAT.

As an endocrine organ, WAT releases adipokines that signal its metabolic state to the brain and other organs, influencing metabolism, inflammation, and appetite regulation. This extensive role highlights the various physiological processes that are disrupted in obesity, leading to metabolic imbalances and related health issues. Leptin, one of the most studied adipokines, is secreted by WAT in response to food intake and helps inhibit appetite by modulating neural circuits in the brain. It acts on receptors in the lateral hypothalamus, particularly in

AGRP neurons to suppress hunger, and in POMC neurons in the medial hypothalamus to stimulate satiety (Obradovic et al., 2021). Although circulating leptin levels are elevated in obesity, hypothalamic leptin resistance contributes to worsening obesity by disrupting appetite control and lipid oxidation (Obradovic et al., 2021).

On the other hand, adiponectin promotes "healthy" AT expansion, preventing harmful ectopic fat accumulation (Stern et al., 2016). This adipokine, secreted abundantly by adipocytes, has anti-obesity and antidiabetic properties, alleviating IR by enhancing lipid oxidation and reducing inflammation. Adiponectin signals are initiated through two major receptors, AdipoR1 and AdipoR2, both of which activate AMPK, a key pathway in the anti-obesity and antidiabetic effects of adiponectin (Kubota et al., 2007).

1.3.2 Brown Adipose Tissue (BAT)

Multilocular adipocytes, characteristic of BAT, differ significantly from unilocular adipocytes found in WAT. Indeed, these cells are smaller than white adipocytes and present a central nucleus surrounded by numerous lipid droplets (Giralt and Villarroya, 2013).

The high mitochondrial density in brown adipocytes, densely packed with cristae, imparts BAT its characteristic brown color and is crucial for non-shivering thermogenesis thanks to the action of the UCP1 (Giralt and Villarroya, 2013). UCP1 facilitates a proton leak across the inner mitochondrial membrane, effectively "uncoupling" fuel oxidation from ATP synthesis, allowing energy to be dissipated as heat instead (Nedergaard et al., 2001).

BAT activation is stimulated by environmental factors such as cold exposure, dietary intake, and noradrenergic signals (Giordano et al., 2014). Noradrenergic innervation is essential for BAT function, where its activation induces heat production in a process called non-shivering thermogenesis. This thermogenic response occurs without muscular contractions and is driven by increased activity of the sympathetic nervous system, which innervates both BAT and skeletal muscle, contributing to body temperature regulation and energy balance (Blondin et al., 2020).

Brown fat is especially abundant in hibernating mammals and human newborns. In neonates, BAT accounts for about 5% of body mass and is primarily located along the upper half of the spine and around the shoulders, particularly in the interscapular area. This concentration of BAT is crucial for thermoregulation in newborns, who are more susceptible to cold and rely on non-shivering thermogenesis to maintain body temperature (Bienboire-Frosini et al., 2023).

Although BAT is more abundant in infants and animals, recent research utilizing positron emission tomography/computed tomography (PET/CT) scans has confirmed the presence of functional BAT in adult humans as well. Moreover, a large retrospective study has demonstrated significant seasonal variation in BAT activity (Au-Yong et al., 2009), with enhanced activation during colder months, highlighting its role in thermoregulation and suggesting its therapeutic potential for treating obesity by stimulating its activation.

BAT localization differs significantly across species. In mice, brown adipocytes are concentrated in distinct BAT depots, including the interscapular, subscapular, and cervical regions (Negroiu et al., 2024).

These depots differ in size and composition based on factors such as age, gender, and genetic background of the mouse strain. These variations influence BAT's thermogenic capacity, reflecting how both environmental and genetic factors modulate BAT's role in energy expenditure and heat production. Consequently, the efficiency of non-shivering thermogenesis and overall metabolic outcomes can differ between individual animals and species (Negroiu et al., 2024).

The activation of non-shivering thermogenesis in BAT is critically governed by the β-adrenergic signaling pathway.

In rodents, β3-adrenergic receptors are the most potent initiators of thermogenesis, but in humans, recent studies show that β2-adrenergic receptors are the main drivers of this process (Blondin et al., 2020). This evidence suggests that in humans, targeting β2 receptors could be more effective for activating BAT and promoting thermogenesis.

Thermogenic pathway induces the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a key regulator of mitochondrial biogenesis and energy metabolism, which subsequently enhances the expression of UCP1. UCP1 facilitates increased mitochondrial activity, promoting heat production and energy expenditure. Enhancing this crosstalk between β-adrenergic signaling, PGC-1α, and UCP1 could potentially offer new therapeutic strategies for metabolic disorders and obesity management in adults, targeting the body's natural mechanisms for energy dissipation (Tabuchi and Sul, 2021).

The scientific community had previously assumed that brown and white adipocytes shared the same developmental genetic pattern.

However, subsequent research revealed that brown adipocytes arise from a precursor shared with skeletal muscle cells, both deriving from Myf5+ precursors (Figure 9) (Seale et al., 2008).

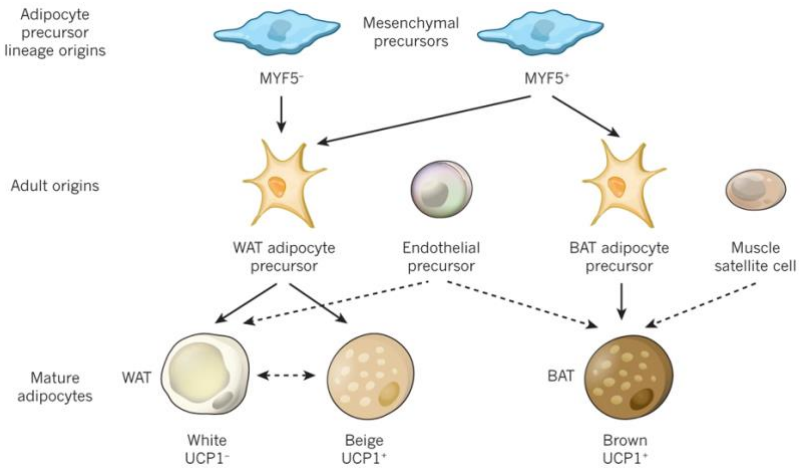


Figure 9. Origins of white, beige and brown adipocytes (Peirce V, et al., Nature. 2014; doi:10.1038/nature13477)

This developmental pathway is regulated by PRDM16, a transcription factor that controls the switch from myoblasts (muscle precursors) to brown fat cells (Seale et al., 2008). In contrast, white adipocytes do not share this skeletal muscle lineage.

Additionally, like WAT, BAT releases various signaling molecules known as "batokines," which include fibroblast growth factor 21 (FGF21), neuregulin 4, vascular endothelial growth factor (VEGF), and cytokines such as interleukin 6 (IL-6). Although the endocrine roles of batokines are not yet fully understood due to the relatively small amount of BAT present in humans, these molecules clearly have

paracrine and autocrine effects, influencing local tissue behavior and systemic metabolic processes.

1.3.3 Beige adipose tissue (BEIGE or BRITE)

This type of fat shares characteristics with both WAT and BAT, contributing to energy expenditure and thermogenesis under certain conditions. Beige adipocytes are a unique type of thermogenic adipocytes that resemble brown fat, characterized by their multilocular structure and UCP1 positivity.

Beige adipocytes are predominantly found in subcutaneous fat, but they can also be present in small amounts in visceral fat. However, the extent of their presence in visceral fat is generally less compared to subcutaneous fat (Luo and Liu, 2016). Their activation is induced by cold-induced stress or by a β3-adrenoceptor agonist stimulation, in a process known as ‘browning’ or ’beiging’ of WAT (Harms and Seale, 2013; Young et al., 1984)

Today, AT is understood to be a convertible and plastic organ, capable of undergoing transdifferentiation (Giordano et al., 2014).

AT plasticity allows its adaptation in response to various physiological conditions, enabling the tissue to switch between different functional states, such as white, brown, and beige adipocytes, thereby influencing energy metabolism and thermogenesis (Sakers et al., 2022).

The plasticity of AT is a fascinating area of research, especially in the context of developing strategies for obesity management and metabolic disorders. To date, beige adipocytes can develop within WAT depots through two key mechanisms (Ziqubu et al., 2023).

De novo beige adipogenesis: This involves the differentiation of progenitor cells (Myf5− progenitor cells) into new beige adipocytes.

Phenotypic conversion: Mature white adipocytes can convert into beige adipocytes by activating or reactivating the thermogenic program. This process is known as the "beigeing" of white AT, underscores the plasticity of AT and its ability to adapt to various metabolic demands and environmental stimuli (Wang et al., 2013).

The “beigeing” of white AT involves the rise of UCP1-positive cells in white fat depots, such as subcutaneous or inguinal fat pads (Nedergaard and Cannon, 2014). These new brown-like or beige adipocytes share characteristics with brown adipocytes, including the multilocular morphology due to the accumulation of lipid droplets, a higher mitochondrial content and elevated UCP1 expression (Figure 10).

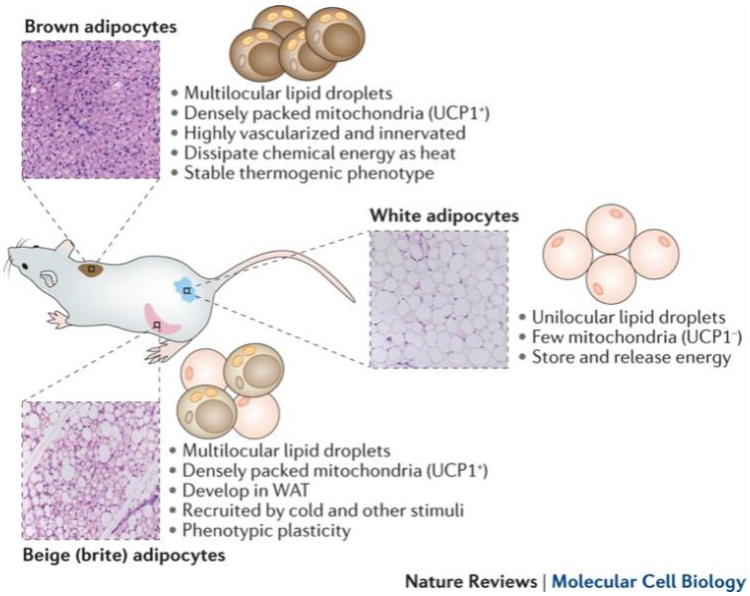


Figure 10. Brown, white and beige adipocytes (Wang et al., Nat Rev Mol Cell Biol. 2016; doi:10.1038/nrm.2016.96)

Notably, these modifications induced, for example, by cold exposure are reversible and can regress once the exposure ceases. This highlights the remarkable plasticity and adaptability of AT in response to environmental changes (Giordano et al., 2014) (Figure 11).

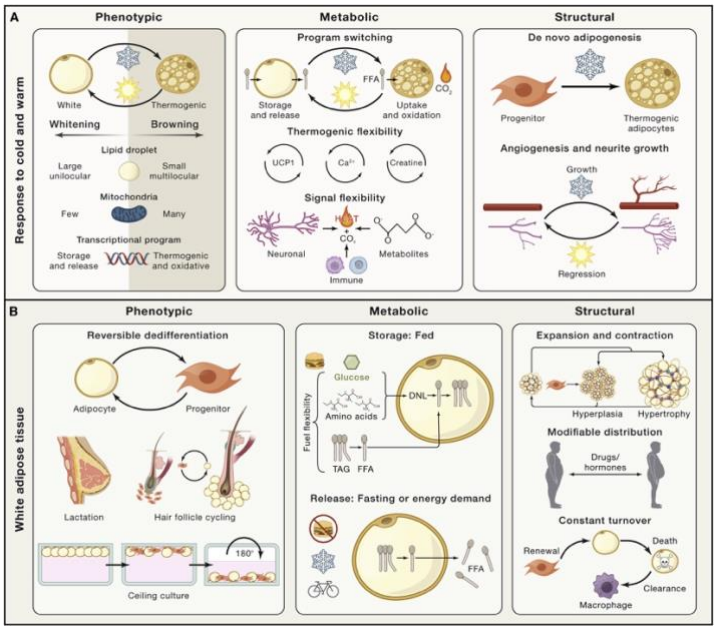


Figure 11. Adipose tissue plasticity (Sakers et al., Adipose-tissue plasticity in health and disease. Cell. 2022; doi:10.1016/j.cell.2021.12.016)

Currently, the mechanisms known to induce the ’beigeing’ of WAT in humans include cold exposure and β-adrenergic receptor stimulation. However, both methods pose challenges for human application due to the non-specific effects of β-adrenergic agonists and the difficulty humans have in tolerating prolonged cold conditions (Negroiu et al., 2024).

In recent years, researchers have explored the possibility of inducing beige adipocyte differentiation without relying on cold exposure or adrenergic stimulation.

Recent studies suggest promising answers: specific key genes, such as PRDM16 and Early B-cell Factor 2 EBF2, have been identified as playing crucial roles in programming brown adipocytes. The activation of these genes triggers a signal transduction cascade that ultimately leads to the overexpression of UCP1 and other thermogenic proteins (Schirinzi et al., 2023).

On the other hand, AT under certain conditions such as obesity can induce the 'whitening' of brown adipocytes (Kotzbeck et al., 2018). Indeed, it has been reported that housing mice in thermoneutral conditions, with or without a high-calorie diet, significantly reduces metabolic capacity and increases lipid accumulation in BAT, resulting in a 'white-like' phenotype, associated to a reduced sympathetic nervous system activity and a weakened thermogenic program, as evidenced by decreased levels of tyrosine hydroxylase and norepinephrine turnover, along with reduced mRNA expression of UCP1, PGC1α, and DIO2 (Maurer et al., 2021). Other evidence suggests that the whitening of BAT is associated with the recruitment of immune cells involved in pro-inflammatory responses and mitophagy (Ziqubu et al., 2023)(Ziqubu et al., 2023). The whitening of BAT is characterized by lipid accumulation, which arises from decreased substrate oxidation and mitochondrial loss, due to disruptions in the molecular mechanisms that regulate thermogenesis (Ziqubu et al., 2023).

Another important aspect of this tissue is its immune profile. AT is not only involved in energy storage but also plays a significant role in the immune response. It contains various immune cells, including macrophages, lymphocytes, and adipocytes, which contribute to inflammation and immune regulation (Grant and Dixit, 2015). In AT, there are two types of activated macrophages: M1 macrophages and M2 macrophages. In lean individuals, the majority of macrophages are M2-activated, which produce anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β). These M2 macrophages play a key role in promoting tissue repair and maintaining a balanced immune environment by reducing inflammation and supporting tissue homeostasis (Viola et al., 2019). In contrast, during obesity there is the recruitment of a significant number of M1 macrophages, which secrete pro-inflammatory cytokines, leading to AT inflammation and metabolic dysfunction. These M1 macrophages form a crown-like structure surrounding dead adipocytes (Murano et al., 2008).

Several studies have demonstrated that anti-inflammatory cytokines, particularly those associated with M2 activation, correlate with increased thermogenesis and browning (Omran and Christian, 2020). Conversely, M1 macrophages and their pro-inflammatory cytokines usually inhibit these processes (Yao et al., 2021).

For instance, the cytokine TNFα reduces the sensitivity of adipocytes to β-adrenergic stimulation, thereby inhibiting UCP1 gene expression, thermogenesis, and lipolysis (Omran and Christian, 2020). Moreover, *in vitro* studies with beige adipocytes exposed to various pro-inflammatory cytokines showed that TNFα and IL-1β moderately

inhibited adrenergic signaling when tested separately, while a combination of four cytokines (IL-1α, IL-1β, IL-6, and TNFα) resulted in a dramatic inhibition of thermogenesis and lipolysis (Yao et al., 2022).

1.4 Senescence and autophagy: different cellular responses to nutrient excess

Obesity is associated with a state of chronic low-grade inflammation, likely triggered by changes associated with nutrient excess, such as mitochondrial ROS production and endoplasmic reticulum stress (Wellen and Thompson, 2010).

While nutrient stress is typically considered in terms of how cells respond to a lack of essential nutrients for their needs, in the context of obesity, cells can also experience stress from nutrient overload. As a result, various mechanisms have evolved to enable cells to detect and adapt to elevated levels of intracellular metabolites (Wellen and Thompson, 2010).

Specifically, because of nutrient excess, cells can activate biological pathways associated with the chronic activation of adaptive stress mechanisms, including cellular senescence, autophagic flux dysfunction and oxidative stress (Figure 12)(Wellen and Thompson, 2010).

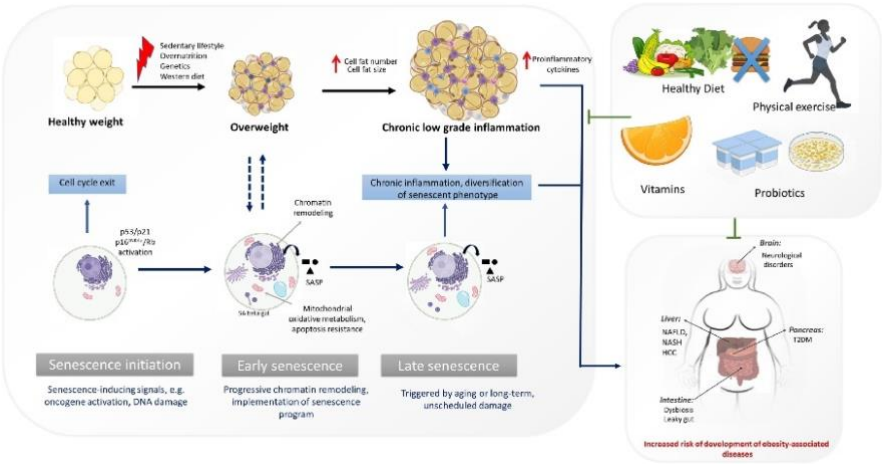


Figure 12. Nutrition and cellular senescence in obesity and related disorders (Rubio-Tomás et al., J Nutr Biochem. 2022. https://doi.org/10.1016/j.jnutbio.2021.108861)

Senescence is characterized by an irreversible cell cycle arrest triggered by various stresses including telomere dysfunction (Kumari and Jat, 2021), oxidative stress (Ogrodnik et al., 2019), inflammation (Jurk et al., 2014), intracellular accumulation of damage (Ogrodnik et al., 2019), and metabolic dysfunction (Wiley and Campisi, 2021), which is associated with macromolecular damage and altered metabolism. Senescent cells are characterized by the well-known 'senescence-associated secretory phenotype' (SASPs), by which they can alter the behavior of neighboring cells and induce inflammation and fibrosis (Wiley and Campisi, 2021). These cells also display telomere-associated DNA damage foci (Jurk et al., 2014), increased activity of lysosomal senescence-associated β-galactosidase (Dimri et al., 1995), chromatin changes (senescence associated heterochromatin foci, SAHF) and frequently increased expression of the cyclin-

dependent kinase inhibitor proteins, p16Ink4a and p21Cip1 (Narita, 2007).

DNA damage is the primary inducer of cellular senescence. Indeed, DNA double-strand breaks trigger the DNA-damage response (DDR) pathway, leading to elevated levels of phosphorylated histone H2AX and p53-binding protein 1 in the chromatin and this response also activates DDR signaling kinases, ultimately resulting in the activation of the p53/p21^CIP1 axis. The activation of P53 pathway can induce the expression of a cyclin-dependent kinase inhibitor, p21, which results in inhibition of cyclin-dependent kinase-2 (CDK2) activity and finally cell-cycle exit (Shreeya et al., 2023).

Several studies have shown a positive correlation between obesity and cellular senescence burden in organs such as AT, liver, and pancreatic-β-cells (Burton and Faragher, 2018). Several preclinical studies demonstrated that HFD feeding can induce senescence in multiple organs, mainly in visceral AT (Schafer et al., 2016; Xu et al., 2015) but also in other organs including the liver (Ogrodnik et al., 2019). Accordingly, recent studies have demonstrated that targeting cellular senescence, either genetically or pharmacologically, can mitigate obesity-related dysfunction (Narasimhan et al., 2022). Additionally, it has been demonstrated that cellular senescence induced by obesity can drive anxiety and impair neurogenesis in mice fed with an HFD (Narasimhan et al., 2022). The elimination of senescent cells using senolytic drugs or attenuating their effects with SASP inhibitors (senomorphics) has been shown to alleviate obesity-induced dysfunction in various tissues.

Another protective mechanism activated in response to stress is the autophagic pathway. Autophagy (from the Greek, auto ‘self’ and phagy ‘eating’) is a cellular process whereby a cell eats and digests its own components. This process is required for removing damaged molecules and subcellular elements, including nucleic acids, proteins, lipids, and organelles to promote homeostasis in response to stress (Gomez-Virgilio et al., 2022). Specifically, it is an evolutionarily conserved, lysosome-dependent catabolic mechanism that degrades and recycles damaged proteins and organelles, enabling the generation of ATP and the formation of new organelles (Galluzzi et al., 2014).

To date, there are three types of autophagy that have been classified based on the modality by which autophagosomes are delivered into lysosome: microautophagy, macroautophagy and chaperone-mediated autophagy.

Among these microautophagy is a process characterized by the direct invagination of lysosomal membranes, leading to the internalization of cytoplasmic materials into the lysosome (Kuchitsu and Taguchi, 2024). the chaperone-mediated autophagy is mediated by the interaction of the pentapeptide KFERQ motif to heat shock cognate 71 kDa protein (HSC70; also known as HSPA8), which then binds to a lysosomal complex, followed by the unfolding and translocation of the substrate into the lysosome for proteolysis (Kuchitsu and Taguchi, 2024).

In macroautophagy, a double-membraned autophagosome vesicle is formed to sequester cytosolic components, which subsequently fuse with lysosomes to create autolysosomes. Within these autolysosomes, the degradation of the sequestered cargo occurs (Kim and Lee, 2014).

Intracellular signaling pathways that activate the initiation of the autophagic process are triggered by various intracellular stresses and changes in nutrient status. The primary target of these pathways is the UNC-like autophagy-activating kinase 1 (ULK1) initiation complex, which is an upstream hub which integrates and relays the activities of mTORC1 and AMPK (Figure 13) (Feng et al., 2014).

AMPK is a master regulator of metabolism, and it stimulates autophagy by multiple mechanisms. Beyond inhibiting mTORC1, AMPK phosphorylates and activates ULK1 (Kim et al., 2011). The activated ULK1 complex recruits the beclin 1–VPS34 complex to the site of autophagosome formation. ATG14 and VPS15 may also play a role in regulating autophagosome formation through mTOR-independent pathways (Debnath et al., 2023).

Two ubiquitin-like conjugation systems involving ATG proteins regulate the elongation of the double-membraned autophagosome. The conjugation of ATG5 to ATG12 requires ATG7and ATG10 (Nakatogawa, 2013).

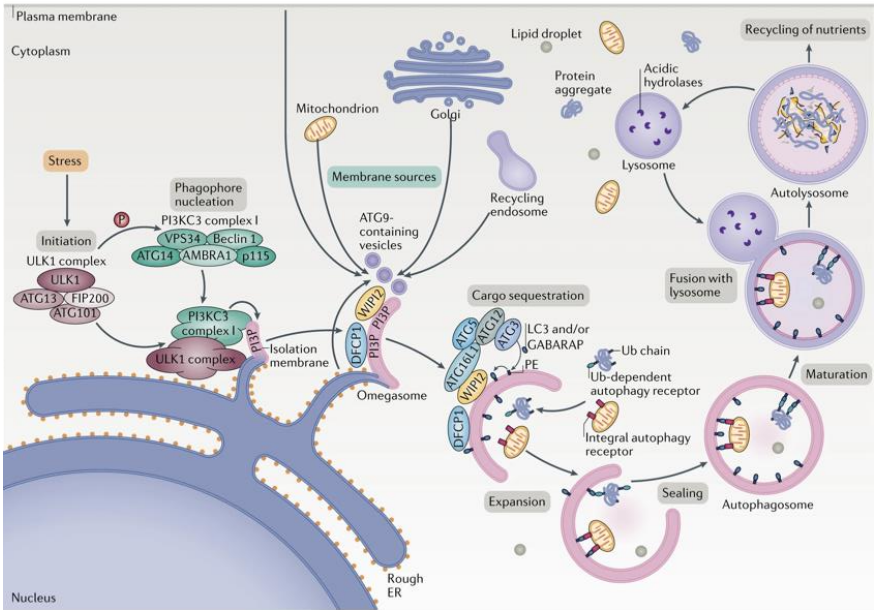


Figure 13. Overview of the autophagy process (Dikic et al., Nat Rev Mol Cell Biol. 2018; doi:10.1038/s41580-018-0003-4)

The ATG5–ATG12 conjugate forms a complex with ATG16, which possesses E3-like ubiquitin ligase activity and facilitates the conjugation of LC3-I (the conjugation of light chain 3) to phosphatidylethanolamine, producing LC3-II, crucial for the phagophore formation. Once the double-membraned autophagic vesicles (autophagosomes) are formed, they mature and fuse with lysosomes to create autophagolysosomes, where the sequestered cargo is degraded (Feng et al., 2014).

While basal autophagy is essential for maintaining cellular homeostasis, its alterations contribute to the pathogenesis of various human disorders, including obesity, diabetes, and aging (Choi et al., 2013).Notably, altered autophagy, whether enhanced or suppressed, has been observed in patients with obesity and in animal models of

genetically predisposed or diet-induced obesity (Juarez-Rojas et al., 2014; Kovsan et al., 2011).

Autophagy is very sensitive to changes in nutrient status, particularly high-fat and/or excess caloric dietary intake but the assessment of changes in autophagy that occur with obesity can be complicated as they depend on the duration and models of obesity used as well as on the tissue or cell types or simply the autophagy monitoring techniques used (Zhang et al., 2018).

For example, an increased autophagy and elevated expression of autophagy-related genes have been documented in the AT of individuals with obesity and in experimental animal models.

These data suggest a seemingly 'detrimental' role for autophagy in adiposity, although it may be compensatory. This concept is supported by clinical observations indicating that elevated autophagy in AT is associated with a high-risk obesogenic phenotype characterized by visceral fat accumulation and IR (Zhang et al., 2018). While chronic HFD intake in mice decreased the formation of autophagolysosomes in the liver (Koga et al., 2010). Consistently, mice with obesity, whether induced by a high-fat diet or genetically determined, exhibited suppressed autophagy in the liver, as demonstrated by decreased autophagosome formation, impaired lysosomal fusion, and reduced expression of lysosome-associated membrane glycoprotein 2 (LAMP2)(Yang et al., 2010).

Anyway, dysregulation in autophagy can exacerbate obesity-related complications, further contributing to the development of conditions like IR, cardiovascular disease, and metabolic syndrome (Zhang et al., 2018).

Notably, targeting these stress-related cellular responses, such as senescence or autophagy, to prevent or treat multiple age-related diseases, including metabolic disorders, has recently received considerable attention from researchers and clinicians.

1.4.1 Spotlight on mitophagy

A specific process of autophagy is mitophagy, an important mechanism of quality control, able to selectively remove dysfunctional or damaged mitochondria and maintain healthy mitochondria population. A deficiency in this system can trigger ROS production, inflammation and cell death (Schofield and Schafer, 2021).

When mitochondria are damaged under stress conditions, such as hypoxia or the accumulation of misfolded proteins, their mitochondrial permeability transition pore opens. This leads to mitochondrial depolarization and activates mitophagy-associated proteins, then damaged mitochondria are enclosed by autophagosomes and transferred to lysosomes, where they fuse to form mature mitochondrial autolysosomes and then damaged mitochondria are degraded by lysosomal enzymes (Lu et al., 2023).

Mitophagy is regulated by two key proteins: PINK1 (PTEN-induced putative kinase 1) and PARKIN, an E3-ubiquitin ligase. PINK1 is initially directed to healthy, polarized mitochondria, where it is released into the cytosol, and it gets ubiquitinated and degraded. While, in damaged, depolarized mitochondria, PINK1 accumulates on the outer membrane, activating its kinase activity through auto-phosphorylation. Then, Activated PINK1 phosphorylates and stabilizes PARKIN. Once activated, PARKIN ubiquitinates various

targets on the mitochondrial outer membrane, such as mitofusin, VDAC, and the pro-apoptotic factor BAK, as well as cytosolic proteins. The polyubiquitinated substrates recruit LC3 adapters. LC3 then transports dysfunctional mitochondria into autophagosomes, where ultimately occurs in autolysosomes to complete mitophagy (Tang et al., 2024). Metabolic diseases such as obesity, IR, T2D, NAFLD, and heart disease are associated with abnormal mitochondrial function, resulting in impaired β-oxidation and increased oxidative stress. Several studies indicated how mitophagy improvement (enhancing PINK1-PARKIN pathway) could represent way to treat obesity related disorders, including hepatic steatosis (Miao et al., 2023). For example, Hoshino and colleagues demonstrate that PINK1/Parkin pathway-derived mitophagy protects pancreatic β-cells and improves glucose intolerance in T2D patients (Su et al., 2021). Additionally, in white adipocytes, Parkin promotes mitochondrial homeostasis by balancing mitophagy and Pgc1α-mediated mitochondrial biogenesis suggesting a potential therapeutic target in adipocytes to combat obesity and obesity-associated disorders (Moore et al., 2022).

2. N-ACYLETHANOLAMINES: NEW LIPIDIC PLAYERS IN METABOLISM

NAEs are a family of endogenous lipids with different functions that received attention for the first time in 1957 when it was discovered the anti-inflammatory activity of PEA in guinea pigs (Keppel Hesselink et al., 2013). They are composed of fatty acid attached to an ethanolamine via an amide bond and are classified according to the length of their acyl chain and its degree of saturation.

PEA was isolated from soybeans, peanuts, and egg yolk, in 1940 by Coburn and Moore, two American researchers who demonstrated that a diet enriched with egg yolk prevented the onset of rheumatic fever in the pediatric population. Formerly in 1993, the Nobel Prize Rita Levi Montalcini with a group of researchers described the anti-inflammatory and neuroprotective role of PEA (Aloe et al. 1993). Their studies clarified the mechanism by which NAEs could be considered endogenous autacoid molecules, regulating mast cell migration during inflammatory processes. Subsequently, numerous other studies have investigated and demonstrated the role of these molecules in other biological systems as well (Aloe et al., 1993).

These bioactive lipids are synthesized 'on demand' from membrane glycerophospholipids (Tsuboi et al., 2013; Tsuboi et al., 2018) and act through various receptors, exerting a wide range of pharmacological effects (Alhouayek and Muccioli, 2014), as they are involved in inflammation, food intake, pain and neural control (Mattace Raso et al., 2014a).

They are ethanolamides of various saturated, monounsaturated, and polyunsaturated FAs (Figure 14). For example, the endocannabinoid

arachidonoylethanolamide (or Anandamide, AEA) is the ethanolamide of arachidonic acid and together with 2-arachidonoylglycerol (2-AG) primarily interact with the CB1 and CB2 cannabinoid receptors ; (Devane et al., 1992; Mechoulam et al., 1995). Additionally, they can interact with the transient receptor potential vanilloid 1 (TRPV1) receptor (Zygmunt et al., 1999).

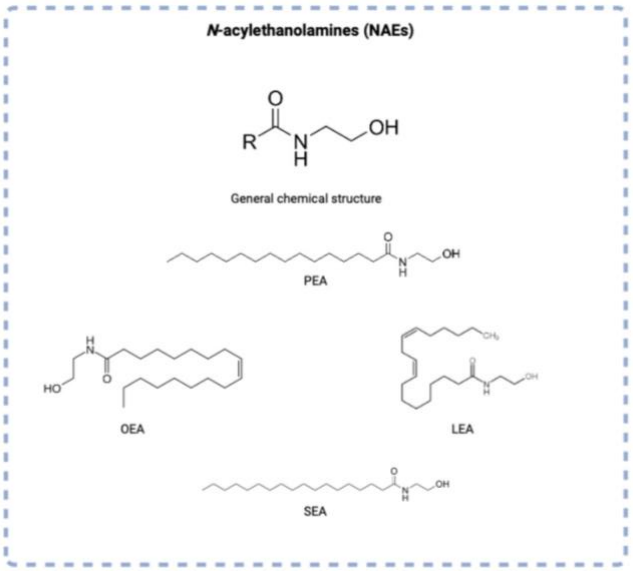


Figure 14. Chemical structures of NAEs.

The ethanolamide of oleic acid, the N-oleoylethanolamine (OEA), is the ligand for several targets, including G protein-coupled receptors (GPCR) (Godlewski et al., 2009), TRPV1 (Ahern, 2003) and PPAR-α (Guzman et al., 2004).While PEA, the ethanolamide of palmitic acid (PA), despite its discovery has been more than half a century ago ((Keppel Hesselink et al., 2013), has been recognized as a PPAR-α agonist many years later (Lo Verme et al., 2005), along with its

characterization as a ligand for the orphan G protein-coupled receptor (GPR)55 (Ryberg et al., 2007).

Initially, its structural analogy with other endocannabinoids suggested that CB2 was involved in the effects underlying its pharmacological activity. However, subsequent studies clarified that PEA has a low affinity for the CB2 receptor and that only 2-arachidonoylglycerol (AG) acts as the intrinsic endogenous ligand for the cannabinoid receptor CB2 (Sugiura et al., 2006). In contrast, PEA binds more specifically to the nuclear receptor PPARα, even at low concentrations (Lo Verme et al., 2005). To date, SEA and LEA received less attention compared to aforementioned NAE family members (Mock et al., 2023). However, LEA exhibits similar bioactivities as OEA through PPAR-α (Syed et al., 2012) or GPR119 activation (Diep et al., 2011).

Although SEA and PEA differ by two methylene groups in chain length, SEA did not present affinity for PPAR-α (Lo Verme et al., 2005). Nevertheless, SEA showed affinity for GPR119 and shares several bioactivities with PEA (Dalle Carbonare et al., 2008). Importantly, non-endocannabinoid NAEs such as PEA, OEA, LEA e SEA are much more abundant than AEA in most animal tissues (Tsuboi et al., 2018).

The total levels of NAEs in tissues are typically around a few nmol/g. In the brain, the main NAE species are PEA, SEA, and OEA, while in the other tissues, such as the intestine, LEA is among the most abundant species (Bernal-Chico et al., 2023). The absence of LEA in the brain is due to the low percentage of linoleic acid in brain lipids, unlike other tissues where it is much more prevalent.

To date, few studies have examined the impact of the type and amount of dietary fat on endogenous NAE levels in the various tissues.

It has been suggested that high-fat diets can influence endocannabinoid levels regardless of their fatty acid composition, and the exposure to dietary fats has been shown to increase endocannabinoid production in rodents (DiPatrizio et al., 2011).

Indeed, HFD feeding led to a gradual increase in 2-AG levels, as well as in AEA levels, coinciding with increased expression of its synthesizing enzymes: the diacylglycerol lipase alpha (Daglα) and N-Acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (Nape-pld), respectively (Kuipers et al., 2018). While Jones et al. demonstrated an inverse association of plasma AEA concentrations with body fat percentage. This correlation is atypical, as AEA is typically linked to increased adiposity. Different published studies found a moderate negative correlation between body fat percentage and plasma OEA concentrations (Diep et al., 2011).

2.1 Biosynthesis and metabolism

Unlike classical neurotransmitters, which are stored in secretory vescicles and released from them, NAEs are produced ’on demand’ in response to various stimuli. This process occurs near the phospholipid bilayer, where glycerophospholipids are converted by a membrane phospholipase (Tsuboi et al., 2018).

Although NAEs have widely divergent functions, they share common biosynthetic and degradation mechanisms that have been described in various tissues, particularly in neurons. It is generally accepted that, in animal tissues, NAEs are synthesized through the classical

‘transacylation-phosphodiesterase pathway’. This pathway consists of two key enzymatic reactions involving membrane glycerophospholipids and phospholipid precursors. The first reaction, catalyzed by N-acyltransferase, converts membrane precursors into N-acylphosphatidylethanolamine (NAPE) in a process that relies on calcium and cyclic AMP. The second reaction involves the hydrolysis of NAPE by NAPE-hydrolyzing phospholipase D (NAPE-PLD), which generates specific NAEs) and phosphatidic acid (Figure 15). This hydrolysis can result in either membrane depolarization or the activation of G-protein-coupled receptors.

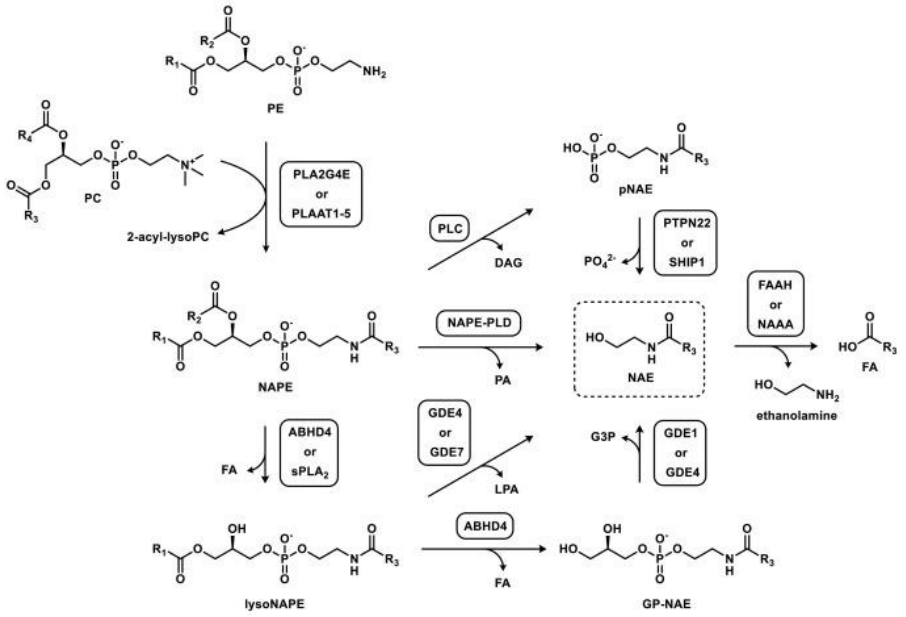


Figure 15. Biosynthesis of NAEs (Mock ED et al., Prog Lipid Res. 2023 Jan; doi: 10.1016/j.plipres.2022.101194)

For example, PEA is synthesized from palmitic acid (C16:0), the most common fatty acid in mammals, in two steps:

1) The calcium-cAMP-dependent transfer of PA from phosphatidylcholine (PC) to phosphatidylethanolamine (PE) by N-acyltransferase (NAT), resulting in the formation of N-acylphosphatidylethanolamine (NAPE)
2) The cleavage of membrane-bound N-acylphosphatidylethanolamine (NAPE) and the formation of PEA occur through the action of a specific phospholipase D, known as NAPE-phospholipase (NAPE-PLD) (Lo Verme et al., 2005).

Recent studies have revealed that the synthetic pathways for NAEs are more complex than previously assumed. Several enzymes involved in this traditional biosynthetic pathway remain poorly characterized. This intricacy indicates that additional regulatory mechanisms and pathways may contribute to their synthesis and function (Tsuboi et al., 2013). OEA synthesis occurs in the intestine and its production involves multiple biochemical reactions that utilize oleic acid and phosphatidylethanolamine as substrates.

After performing their function, NAEs are rapidly metabolized and inactivated. The inactivation of NAEs occurs via enzymatic hydrolysis into FFAs and ethanolamine by specific hydrolases. In particular, the fatty acid amide hydrolase (FAAH), located in the endoplasmic reticulum, has a high affinity for AE) (Ahn et al., 2009), while the N-acylethanolamine-hydrolyzing acid amidase (NAAA), a lysosomal enzyme abundant in macrophages, preferentially hydrolyzes PEA (Ueda et al., 2013).

FAAH, first cloned in 1996, is classified as a membrane-bound serine hydrolase within the amidase signature family and exhibits higher

catalytic activity at neutral and alkaline pH levels This enzyme is widely distributed across various tissues, with particularly high expression in the brain and liver. FAAH-deficient mice show elevated levels of several NAEs, including AEA, highlighting FAAH's essential role in their degradation (Ahn et al., 2009). The development of specific FAAH inhibitors holds promise as potential therapeutic agents for treating conditions such as pain, depression, and anxiety (Ahn et al., 2009). NAAA plays a crucial role in the metabolism of NAEs, impacting various physiological processes. It is a lysosomal enzyme that specifically hydrolyzes NAEs, but only in an acidic pH environment. NAAA is widely present in various tissues, particularly in macrophages and the prostate. Unlike FAAH, which preferentially targets anandamide, NAAA's best substrate *in vitro* is PEA. Consistent with the anti-inflammatory properties of PEA, the use of specific NAAA inhibitors has been shown to suppress inflammatory responses in rodent models by increasing local PEA levels (Tsuboi et al., 2018).
The formation and turnover of NAEs are typically slower, occurring over the course of minutes, compared to many other lipid-derived signaling molecules like diacylglycerol and 2-arachidonoylglycerol, which can be generated in a matter of seconds (Zou and Kumar, 2018). In addition to the traditional hydrolytic routes, polyunsaturated NAEs also serve as substrates for oxidative enzymes such as lipoxygenases (LOX), cyclooxygenases (COX), and cytochrome P450 enzymes. The resulting oxygenated products exhibit structural similarities to well-studied eicosanoids but have distinct signaling activities, highlighting the crossover between endocannabinoid and eicosanoid signaling pathways (Mock et al., 2023).

Considering the pharmacological effects of NAEs, many are administered exogenously for the treatment of certain disorders, including pain and obesity. However, their administration must overcome solubility challenges, as seen in the case of PEA(Valenza et al., 2022).

Specifically, PEA can be administered exogenously; however, due to its lipophilic nature, it is virtually insoluble in water and only sparingly soluble in most aqueous solvents, with a log partition coefficient exceeding 5. As a result, the oral absorption of PEA is quite complex, and most studies focus on micronization, a process that reduces the particle size of PEA to enhance its surface area for improved absorption (Impellizzeri et al., 2014).

2.2 Physiological and pharmacological effects of NAEs

NAEs are known as "Autacoid Local Injury Antagonism Amides" (ALIAmides) to emphasize their local autacoid mechanism, which counteracts tissue damage caused by inflammation and other factors. Their involvement in inflammation is supported by the accumulation of these lipid mediators in tissues during inflammatory states, as well as their effects on mast cell activation (Aloe et al., 1993). As previously mentioned, these non-endocannabinoid NAEs exert biological activities through various receptors, including CB1, TRPV1, GPR119, and PPARα (Tsuboi et al., 2018).

NAEs of saturated and monounsaturated FAs are classified as non-canonical endocannabinoids because don’t exhibit ligand activity for CB1 and CB2 receptors. Anyway, extensive studies have identified AEA as a partial agonist at both CB1 and CB2 receptors (Reggio,

2010), while OEA activates PPARα with high potency (Fu et al., 2003).

Although PEA was initially considered a potential agonist of the CB2 receptor, it has been demonstrated to be inactive in binding to both CB1 and CB2 receptors. Instead, PEA binds to PPARα, but with lower affinity compared to OEA (Mock et al., 2023).

For many years, the functional roles of OEA were largely unknown until a pivotal study by Rodríguez de Fonseca et al. in 2001 identified it as an anorexic lipid mediator linked to feeding behavior. The researchers found that OEA biosynthesis significantly decreased in the small intestine during periods of food deprivation, while levels in the brain remained unchanged. Conversely, feeding stimulated OEA production in the intestines, highlighting its regulatory role in appetite control (Piomelli, 2013). When administered, OEA promotes satiety and leads to a reduction in body weight gain. This molecule binds with high affinity to the PPARα receptor, and studies have shown that its anorexic effects are absent in mice lacking PPARα, indicating that this receptor is crucial for mediating OEA's appetite-suppressing actions (Fu et al., 2003).

Furthermore, OEA exhibited neuroprotective properties in *in vivo* models of Parkinson's disease and notably, it was effective in reducing levodopa-induced dyskinesias in a murine model of PD induced by 6-OHDA striatal lesion (Gonzalez-Aparicio and Moratalla, 2014).

OEA also acts as a cytoprotective agent in hepatocytes, influencing various biological processes associated with NAFLD, including lipid metabolism, inflammation, and oxidative stress (Tutunchi et al., 2019). Additionally, OEA also showed a nephroprotective effect, mitigating

folic acid-induced kidney injury in mice through a mechanism dependent on PPAR-α. Specifically, the authors described OEA effects in improving kidney function and attenuating tubular epithelial injury. This effect of OEA was related to PPAR-α activation since OEA failed to exert its beneficial activity in FA-insulted PPAR-α-/- mice (Comella et al., 2024).

Over the years, PEA has been shown to exert numerous pharmacological effects, including neuroprotective (Lambert et al., 2001) and antinociceptive effects (Calignano et al., 1998). In various experimental models of inflammation, both *in vitro* and *in vivo*, a reduction of PEA levels aggravated inflammatory processes (Zhou et al., 2008), while anti-inflammatory treatments restored its levels (Jhaveri et al., 2008). Early on, PEA was considered a CB2 receptor agonist, as pharmacological blockade of the CB2 receptor using antagonists partially reduced PEA's anti-inflammatory and analgesic effects (Calignano et al., 1998). Specifically, the CB2 antagonist SR144528 inhibited PEA's analgesic effect but not its antiperistaltic effect (Capasso et al., 2001). Conflicting data led to the theory of the 'entourage effect' (Jonsson et al., 2001), where PEA enhances the activity of endogenous CB receptor agonists, amplifying their actions on other targets, such as TRPV1 channels (also known as vanilloid receptors type 1) (De Petrocellis et al., 2001). Consistently, with its antinociceptive effects, in an animal model of chronic constriction injury-induced neuropathy, PEA supplementation significantly reduced pain and improved neuronal function by activating PPAR-α and reducing neuro-inflammation (Di Cesare Mannelli et al., 2013). This effect was further supported by clinical studies demonstrating

PEA's efficacy in reducing pain and improving the quality of life for over different patients with various painful conditions (Scuteri et al., 2022). Furthermore, PEA has shown an antidepressant effect in a murine model of obesity induced by HFD. Specifically, during obesity, increased levels of circulating FFAs and pro-inflammatory cytokines cross the blood-brain barrier and reach the hypothalamus, initiating an inflammatory process that extends to the hippocampus and cortex (Guillemot-Legris and Muccioli, 2017). This results in a state of chronic inflammation that contributes to behavioral alterations, changes in neurotransmitter concentrations, and disruptions in signal transduction (Miller and Spencer, 2014). In this context, Lama et al. (2019) demonstrated the antidepressant and antianhedonic effects of PEA treatment in HFD mice highlighting PEA's ability to modulate monoaminergic neurotransmission and enhance synaptic plasticity. Specifically, PEA was able to increase dopamine levels and serotonin levels, while reducing the concentration of 5-hydroxyindoleacetic acid in the prefrontal cortex, the main metabolite of serotonin. These experimental findings correlate with a reduction of anhedonic and depressive behaviors observed in HFD animals, as well as a restoration of brain-derived neurotrophic factor (BDNF)/TrkB/CREB signaling pathway, an effect also demonstrated by chronic treatments with various antidepressant drugs (Lama et al., 2021). Moreover, Pirozzi et al. recently demonstrated how PEA exerted a protective effect in long-term HFD-induced intestinal damage in mice, modulating gut microbiota composition and restoring tryptophan-derived metabolites altered by HFD, suggesting its role in decreasing obesity-related disorders (Pirozzi et al., 2023).

LEA is the most abundant NAE found in the diet, particularly in foods like wheat-based products (coffee powder, cocoa, carrots, margarine, eggs, and legumes., coffee powder, cocoa, carrots, margarine, eggs, and legumes). This linoleic acid derived NAE is present in the plasma and gut with concentrations much higher than those of other polyunsaturated NAEs (Mock et al., 2023)(Mock et al 2022).

LEA showed an anti-inflammatory effect in murine macrophages, suppressing the LPS-induced expression of pro-inflammatory cytokines, such as tumor necrosis factor-a (TNF-a), interleukin (IL)-1b, and IL-6 (Ishida et al., 2013). Furthermore, a preclinical study showed that LEA reduced feeding in rodents, and this effect ceased when the PPAR-α receptor was blocked. This suggests that LEA may play a role like OEA in regulating appetite and feeding behavior through PPAR-α activation (Diep et al., 2011).

While SEA, the ethanolamide of stearic acid, received comparatively less attention than others. However, recent studies have shown that it possesses neuroprotective effects against LPS-induced neuroinflammation in mice. Additionally, SEA levels were found to increase in the rat brain following ischemia, suggesting its involvement in modulating brain damage (Kasatkina et al., 2020).

2.3 How N-acylethanolamines work against obesity

As previously demonstrated, certain NAEs function as agonists of PPAR-α, a pivotal receptor in controlling lipid metabolism, specifically in regulating the expression of some genes involved in mitochondrial FFA β-oxidation (Todisco et al., 2022).

Several studies have focused on the potential role of NAEs in regulating obesity, investigating their effects on metabolic dysregulation and fat accumulation.

PPAR-α receptor is a part of the nuclear receptor superfamily, playing a crucial role in regulating food intake and lipid metabolism, thereby balancing energy homeostasis. Consistently, it is the main target of hypolipidemic drugs, including fibrate, promoting fatty acid catabolism and enhancing their oxidation (Todisco et al., 2022).

The roles of AEA and OEA in feeding behavior are increasingly well documented, as numerous animal studies have highlighted the contrasting role of these two molecules: AEA exhibits orexigenic (appetite-stimulating) properties, while OEA has anorexigenic (appetite-suppressing) effects (Mock et al., 2023). These findings highlight their distinct roles in regulating food intake and energy balance. Anandamide has been associated with increased food intake and fat accumulation through its interaction with the endocannabinoid system, particularly CB1 receptors (Kurtov et al., 2024).

Consistently, obesity deeply increases AEA levels and CB1 receptors expression, inducing overeating and increasing fatty acid synthesis (Osei-Hyiaman et al., 2005).

While the anorectic effect of OEA was first recognized by Rodríguez de Fonseca et al. in 2001, who demonstrated that OEA selectively reduces food intake through mechanisms distinct from the interactions between AEA and CB receptors (Rodriguez de Fonseca et al., 2001). Specifically, food intake stimulated NAT activity, enhancing OEA biosynthesis in the small intestine. The new synthesized OEA activates local sensory fibers, inducing the inhibition of eating to brain regions

suc h as the nucleus of the solitary tract and the paraventricular nucleus. In *ad libitum*-fed rats, OEA administration delays the start of eating without altering meal size, indicating that OEA's hypophagic effect is mainly due to a prolonged duration of satiety (Brown et al., 2017).

However, two primary pathways contribute to the hypophagic effect of OEA: The first pathway involves oxytocin, a neuropeptide that is key in emotional responses and metabolic regulation. Notably, administering OEA intraperitoneally has been found to enhance oxytocin expression in neurons within the paraventricular and supraoptic nuclei of the hypothalamus, areas essential for appetite regulation and energy balance. Indeed, the intracerebroventricular infusion of the antagonist of oxytocin receptor (L-368,899) stopped the satiety effect of OEA, suggesting that activation of oxytocin signaling in the CNS is essential for OEA’s appetite-suppressing effects(Gaetani et al., 2010).

The second mechanism is linked to the brain histamine production, indeed Provensi et al., 2014, demonstrated that the ablation of the histamine-synthesizing enzyme histidine decarboxylase reduced the anorectic effect of OEA (Provensi et al., 2014).

Besides acting as a satiety signal, OEA also plays a crucial regulatory role in lipid metabolism in both genetical and nutritional models of obesity in rodents. These findings suggest that OEA promotes the expression of genes encoding for proteins that are involved in lipid catabolism and energy balance both in lean rats and Zucker rats, including CD36, L-FABP, UCP-2 (Fu et al., 2005; Guzman et al., 2004).

Additionally, it has been demonstrated OEA protective role in lessening fatty liver disease of mice with obesity, specifically in reducing oxidative stress and modulating the expression of transcriptional factors involved in the control of lipid metabolism such as ACC and FAS (Giudetti et al., 2021).

Moreover, clinical studies demonstrated that OEA treatment significantly decreased serum levels of TG, alanine aminotransferase (ALT), aspartate aminotransferase (AST) while increased serum levels of HDL, and improved appetite sensations in patients with NAFLD (Tutunchi et al., 2020).

On the other hand, several studies indicated the role of PEA in reducing the chronic low-grade inflammation associated with metabolic disorders, named meta-inflammation (Mattace Raso et al., 2014b). This latter effect suggested its potential role in improving metabolic health compromised during obesity.

Mattace Raso and collaborators demonstrated that in a mouse model of ovariectomy-induced obesity, PEA reduced body weight, fat mass, and restored hypothalamic sensitivity to leptin, a protein hormone that contributes to metabolic homeostasis by regulating food intake and energy expenditure. Notably, in SH-SY5Y cells, a human immortalized neuroblastoma cell line, PEA directly and significantly induced the phosphorylation of STAT3, the transcriptional factor downstream of leptin receptor activation.

Recently, it has been demonstrated that PEA reduces obesity-related hepatic dysmetabolism and improves mitochondrial bioenergetics, both *in vivo* and *in vitro* (Annunziata et al., 2020). In this study, the authors found that chronic administration of PEA for 7 weeks in mice

with HFD- induced obesity improved the metabolic profile of obese animals, reducing hepatic lipid accumulation, IR and increasing energy expenditure. Mechanistically, similar results were obtained in an *in vitro* model of lipid accumulation and IR in HepG2 cells challenged with PA. The authors highlighted that the treatment with PEA reduced hepatic lipid accumulation in insulin-resistant cells, clarifying the central role of AMPK in the metabolic effects underlying PEA activity (Annunziata et al., 2020)- Then in another study, the supplementation of ultra-micronized PEA increased the 'beigeing' of white adipocytes, along with enhancing thermogenic markers and leptin signaling in HFD-induced obesity, by activating PPAR-α (Annunziata et al., 2022). Moreover, given the crucial role of gut microbiome dysfunction in the progression of obesity (Liu et al., 2021), ultra-micronized PEA was found to improve the gut microbiome by enhancing PPAR-α activity and associated tryptophan metabolism (Pirozzi et al., 2023).

Consistently, with its hepatoprotective effects, PEA administration has been shown to reduce the activation of hepatic Kupffer and stellate cells in an *in vitro* study and in an animal model of carbon tetrachloride-induced liver fibrosis. Specifically, PEA significantly decreased the overall rate of hepatic fibrosis and the hepatic type I collagen deposition (Ohara et al., 2018).

Concerning the effect of SEA and LEA in obesity, there are very few studies exploring its role in modulating obesity-related disorders. Specifically, it has been observed that the systemic administration of SEA is associated to an anorexic action in vivo, without PPARα activation. In addition, when orally administered, SEA is accompanied with downregulation of liver SCD-1 mRNA expression, involved in

the synthesis of monounsaturated FAs (Terrazzino et al., 2004). SCD-1 has been recently proposed as a molecular target for the treatment of obesity, since SCD-1 deficient mice showed increased energy expenditure, reduced body adiposity and are resistant to diet-induced obesity (Dobrzyn and Ntambi, 2005).

Furthermore, SEA ameliorated fatty acid composition in pancreatic lipid fractions and modulated the major acyl-Co-A desaturases in IR rats (Onopchenko et al., 2015).

While LEA is usually described as an anorectic NAE, but an analysis of overall published data reveals that this effect is restricted in time, lasting around one hour. Its exogenous administration normalized the metabolic and inflammatory disorders caused by prolonged exposure to HFDs. However, subchronic treatment with LEA did not lead to a reduction in food intake or liver steatosis (Tovar et al., 2023).

3. POLYPHENOLIC COMPOUNDS

Polyphenols are the main class of antioxidants present in diet, and their effects represent the source of the well-recognized health benefits associated with the consumption of fruits and vegetables. Over the past decade, there has been a marked increase in interest regarding polyphenols, largely attributed to their well-established antioxidant properties and their widespread occurrence in various dietary sources as well as their potential protective effects against a range of diseases, including cancer, cardiovascular disorders and neurodegenerative conditions (Grabska-Kobylecka et al., 2023; Rana et al., 2022). The polyphenolic compounds are characterized by multiple hydroxylic groups attached to an aromatic ring. They have been found in high plants as secondary metabolites that play crucial roles in plant defense against ultraviolet radiation and pathogen attack. Polyphenols can be categorized into different groups based on the number of phenolic rings present in their structure and on the elements connecting these rings (Rana et al., 2022), as shown in Figure 16.

Polyphenols are commonly categorized as flavonoids, characterized by a C6-C3-C6 structure, and non- flavonoids.

Flavonoids generally present in foods are anthocyanins, flavonols, flavan-3-ols, flavones, isoflavones, flavanones, and stilbenes. They can react with one-electron oxidants. This oxidation helps inhibit the formation of free radicals within biological systems and is considered the main mechanism by which polyphenols function as therapeutic agents (Rana et al., 2022).

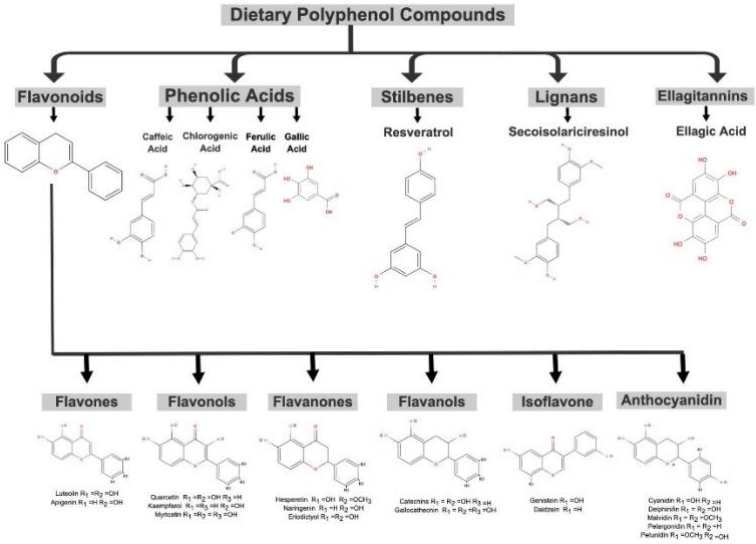


Figure 16. Different classes of polyphenolic compounds (Rosales et al., Front Nutr. 2023 doi: 10.3389/fnut.2023.1144677)

Their ability allows them to interact with metal ions, such as Fe^{2+}, which can catalyze the generation of free radicals. By chelating these metal ions, polyphenols can inhibit radical formation and mitigate oxidative stress (Lakey-Beitia et al., 2021).

However, several other molecular mechanisms have been identified that contribute to their benefic effects on health, including the regulation of lipid metabolism and the modulation of inflammatory mediators' synthesis (Cory et al., 2018). The health effects of polyphenols depend on the amount consumed and on their bioavailability. Since they are stored in plants as glycoside and non-glycosylated conjugates, the nature of the moiety can influence their later bioavailability in humans (Makarewicz et al., 2021).

Research indicates that colonic bacteria are active in the absorption and metabolism of a wide array of flavonoids, as well as other phenolic and

polyphenolic compounds, significantly affecting their bioavailability in systemic circulation (Makarewicz et al., 2021).

Furthermore, they remain in the colon for extended periods and their presence exerts direct effects on colonic health and the gut microbiota (Di Lorenzo et al., 2021).

Polyphenols, including resveratrol, and curcumin, have been associated with anti-obesogenic effects, enhancing FAO and inhibiting lipogenesis in ATs. In addition, these polyphenols exhibit anti-inflammatory properties, which may be particularly beneficial in addressing obesity-related inflammation (Wang et al., 2014).

3.1 The flavonoid rutin

Flavonoids are characterized by numerous phenolic structures and are widely present in numerous natural sources such as fruits, vegetables, grains, bark, roots, stems, flowers, tea, and wine (Panche et al., 2016). They share a common structural framework comprising two aromatic rings connected by three carbon atoms that form an oxygenated heterocycle and are categorized into six subclasses based on the type of heterocycle present: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols, which include catechins and proanthocyanidins.

Several studies have reported different properties of flavonoids, including anti-inflammatory, antidiabetic, antioxidant, antibacterial, antiviral, antiallergic, cytotoxic, and anticancer effects (Abdou et al., 2022; Hasnat et al., 2024). Apigenin, hesperetin, myricetin and quercetin are the most studied compounds in this class.

Their structure, the amount of hydroxyl groups and the degree of polymerization affect the bioavailability, metabolism, and biological activity of flavonoids. Specifically, these compounds are primarily absorbed in the small intestine, where they frequently undergo metabolism by phase II enzymes before entering systemic circulation. An important role in their pharmacokinetics has been attributed to gut microbiota, since some flavonoids are absorbed in the small intestine and then moves into the large intestine, where colonic microbiota further degrade the deconjugated metabolites and aglycones into more readily absorbable forms, such as phenolic acids. This microbial action significantly enhances their bioavailability contributing to their potential benefits (Thilakarathna and Rupasinghe, 2013). For example, research on the pharmacokinetics of quercetin, a well-studied flavonol, indicates that in humans, it has very low oral bioavailability because of its extensive metabolism. However, conjugated forms of quercetin, which occur naturally, exhibit higher bioavailability compared to free forms (Li et al., 2016).

Experimental evidence revealed that dietary intake of flavonoids has a positive effect on cardiovascular diseases, including systemic arterial hypertension and coronary artery disease (Ciumarnean et al., 2020).

For example, quercentin administration has a positive effect on myocardial damage, linked to an antioxidant and anti-inflammatory activity and are confirmed a beneficial effect also on myocardial fibrosis and mitochondrial dysfunction (Kozlowska and Szostak-Wegierek, 2022). Anyway, flavonoids may play a role in cardiovascular health also inhibiting the generation of toxic lipid products as well as the oxidation of cholesterol, which can reduce LDL

levels and subsequently lower the risk of cardiovascular disease (Hernaez et al., 2015).

Another flavonol is rutin (Rut) (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) which is abundantly found in plants, such as passionflower, buckwheat, tea, and apple and possesses various potential pharmacological properties, including anti-diabetic and vasorelaxant effects (Ganeshpurkar et al., 2017).

The name ‘rutin’ comes from the plant Ruta graveolens, which also contains Rut. Chemically, it is a glycoside composed of the flavonolic aglycone quercetin and the disaccharide rutinose (Figure 17) and it demonstrated a variety of pharmacological activities, including antioxidant, cardioprotective effects, antidiabetic, vasoprotective, anticarcinogenic, neuroprotective (Ganeshpurkar and Saluja, 2017) (Fernandes et al., 2010; Javed et al., 2012)

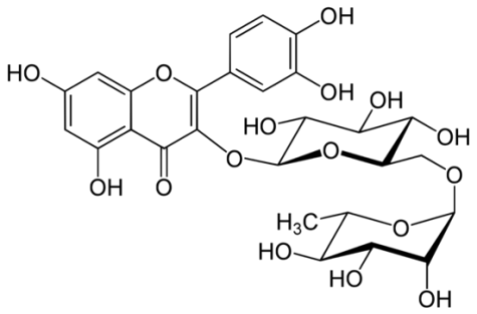


Figure 17. Rutin chemical structure

3.1.1 Antioxidant and metabolic effects of rutin

Concerning its effects on obesity and related disorders, it has been demonstrated that Rut can mitigate oxidative stress in tissues and cells exposed to lipid challenges and reduce the expression of key lipolytic and lipogenic genes. Previous data by Xin e coll. (2004), highlights Rut

ability to lower TG and cholesterol levels in a high-fat diet model, which could have significant implications for managing conditions such as hyperlipidemia and fatty liver disease. This protective effect underscores the importance of flavonoids in dietary strategies aimed at improving cardiovascular health(Ciumarnean et al., 2020).

Moreover, previous studies highlighted the potential effects of Rut on diabetes. Specifically, Rut improved glycemic control in rats with streptozotocin-induced diabetes, likely by stimulating glucose utilization in extrahepatic tissues (Fernandes et al., 2010). Additionally, Rut has been found to reduce glucose absorption in the small intestine by inhibiting α-glucosidase and α-amylase, enzymes involved in carbohydrate digestion (Ghorbani, 2017). Furthermore, its role in alleviating diabetic complications, including diabetic nephropathy, has been demonstrated (Ghorbani, 2017).

Moreover, Rut enhances energy expenditure by activating BAT thermogenic function and promoting the 'beigeing' of scWAT in mouse models of genetic obesity (Db/Db leptin receptor-deficient) and in diet-induced obesity (DIO) models (Yuan et al., 2017). *In vitro* studies further support these findings, indicating that Rut may also induce browning in 3T3-L1 adipocytes, as evidenced by increased mRNA levels of Prdm16 and elevated protein levels of PGC1α. Additionally, Rut also reverses adipocyte dysfunction by restoring intracellular lipid accumulation and inhibiting TNF-α-induced lipolysis. Finally, Rut improves disorders of glycol-lipid metabolism, reduces hepatic steatosis, enhances BAT activity, promotes the browning of iWAT, and increases the content of short-chain FAs (SCFAs) in the colon (Cheng et al., 2021).

3.2 Hydroxytyrosol as an olive oil-derivated polyphenol

While thirty different phenolic compounds have been identified in olive oil, the most abundant ones are HT, tyrosol, and oleuropein (Figure 18).

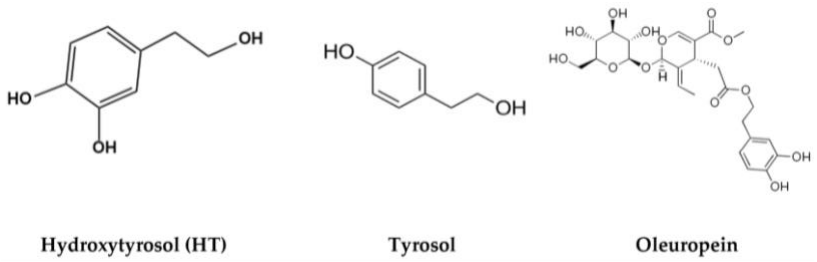


Figure 18. Chemical structures of HT, tyrosol, and oleuropein.

HT is a powerful phenolic phytochemical recognized for its significant antioxidant properties. This compound is primarily sourced from olive leaves and olive oil which is an essential constituent of the Mediterranean diet and has been associated with numerous health benefits, including anti-inflammatory effects, cardiovascular protection, and potential neuroprotective properties (Bilal et al., 2021). This diet is characterized by a high intake of polyphenol and has been linked to some beneficial health outcomes, including the promotion of cardiovascular health and cognitive performance in aging populations (Ross et al., 2024). However, its application is limited because of its hydrophilic properties, which complicate both extraction from aqueous solutions and solubilization in lipid environments (Bertelli et al., 2020).

It is widely recognized that the consumption of extra virgin olive oil has a beneficial effect on human health, thanks to its well-known antioxidant effect, useful for the prevention of cardiovascular and metabolic diseases (De Santis et al., 2019). In addition to the well-known antioxidant activity, several studies have shown that in animal models of NAFLD, olive oil can reduce hepatic lipid accumulation by increasing the secretion of VLDL which are the lipoproteins responsible for transporting TG from the liver to other tissues (Lama et al., 2017).

HT, the major metabolite of oleuropein, is a phenolic alcohol, able to exert a wide range of biological, antioxidant, anti-inflammatory, cardioprotective, antitumor, antimicrobial and neuroprotective effects (Sun et al., 2014) (Reyes et al., 2017). His chemical formula is C8H10O3 and his structure is identical to tyrosol except for an extra hydroxyl group in meta-position in the aromatic ring. It is derived from hydrolysis of oleuropein during maturation of olives (Santos et al., 2012), is stable in the free form and penetrates readily into tissues. HT and its metabolites accumulate mainly in the liver, kidneys, and brain at nutritionally relevant doses, then. is metabolized into HT 3′–O–sulphate and is recovered in the urine (Karkovic Markovic et al., 2019). Oleuropein, the precursor of HT, exhibits antioxidant activity that can mitigate hepatocellular steatosis induced by FFAs in HepG2 cells. This effect is evident by a reduction in both the quantity and size of lipid droplets, as well as a decrease in TG accumulation (Hur et al., 2012). *In vitro* studies indicate that, in addition to its antioxidant properties, HT can also enhance endothelial function (Vijakumaran et al., 2023). This improvement in endothelial dysfunction suggests potential

cardiovascular benefits, playing a crucial role in maintaining vascular health and regulating blood flow. Additionally, both *in vivo* and *in vitro* studies indicate that high doses of HT confer cardiovascular protection by enhancing lipid profiles, minimizing plaque formation, and reducing inflammation (Noguera-Navarro et al., 2023). Regarding its neuroprotective effects, they seem to be related to a reduction in oxidative and nitrosative stresses and brain inflammation in rats with T2D (Reyes et al., 2017). Additionally, elegant experiments using Caco-2 cells treated with a range of phenolic compounds (including HT) alongside LPS-induced inflammation revealed a significant reduction in nitric oxide release following HT treatment, which inhibited the expression of inducible nitric oxide synthase (iNOS) (Serreli et al., 2021).

3.2.1. Antioxidant and metabolic effects of HT

Evidence indicates that HT, through its antioxidant properties, influences glucose and lipid homeostasis and may provide protective effects against diabetes (Vlavcheski et al., 2019).

Specifically, several studies suggest that HT exhibits insulin-like effects on insulin target cells, including adipocytes, hepatocytes, and muscle cells, proving a significant anti-diabetic property in animal models of T2D (Vlavcheski et al., 2019).

Notably, in 2011, the European Food Safety Authority approved the use of HT (5 mg/day) and its derivatives, recognizing their capacity to protect against oxidative damage and inflammation, as well as to reduce the risk of cardiovascular disease and IR (Gaforio et al., 2019).

HT acts as a free radical scavenger and increases the gene expression of detoxifying and antioxidant defense systems in the liver, through the activation of the Nrf2 factor (Wen et al., 2024). Pirozzi et al., 2016 demonstrated the protective effect of HT in limiting metabolic alterations and liver inflammation occurring in HFD-induced steatosis in rats. They demonstrated that HT significantly enhanced metabolic disorders induced by obesity and counteracted early inflammatory events responsible for IR and steatosis. Specifically, HT reduced hepatic nitrosative /oxidative stress, and restored glucose homeostasis through the activation of AKT pathway.

At cellular level, HT has been shown to enhance glucose uptake in both muscle and adipose cells and to activate the energy sensor AMPK and its downstream effector ACC in fat and liver cells (Priore et al., 2014). Other *in vitro* studies confirmed the hypolipidemic and anti-inflammatory effect of HT, modulating the activity of key enzymes involved in fatty acid metabolism including ACC and FAS (Vlavcheski et al., 2019).

Tabernero et al., 2014 examined body and tissue weights, lipid profiles, redox status, and various biochemical, hormonal, and inflammatory biomarkers in rats fed a HT acetate diet compared to a cholesterol-rich diet (Tabernero et al., 2014). Their results showed that plasma levels of total cholesterol, LDL cholesterol, glucose, insulin, and leptin, as well as MDA in serum, significantly increased in the cholesterol-rich diet group compared to the HT-AC diet group. These findings confirm the metabolic effects of HT, particularly enhanced by its hydrophobic derivatives.

These compounds have attracted considerable attention because of their potential anti-inflammatory effects, antioxidant activity, and their ability to modulate signaling cascades and transcriptional networks. This multifaceted impact places them as promising candidates for therapeutic interventions in various inflammatory and oxidative stress-related conditions, including obesity.

4. INNOVATIVE THERAPEUTIC APPROACHES: TARGETING LIVER-ADIPOSE TISSUE AXIS

Liver and AT represent a crucial metabolic circuit in maintaining body homeostasis, particularly in the context of lipid metabolism and energy storage.

In physiological conditions, these two tissues cooperate to sustain metabolic homeostasis by secreting adipokines and growth factors. This metabolic interaction between AT and the liver is shaped not only by the caloric intake but also by the caloric balance from many macronutrient including carbohydrates and fats.

Anyway, the function of liver-adipose axis is influenced by many factors and mechanisms involved in obesity.

Specifically, during obesity, although insulin levels are very high, adipocytes fail in responding to the anabolic effects of this hormone, causing the spillover of FFAs and glycerol from AT to the liver. However, when lipid overload exceeds the liver's capacity for TG deposition, certain lipid metabolites, such as ceramide and diacylglycerol, accumulate in the liver, inducing toxic effects and contributing to IR and metabolic stress.

This phenomenon appears to be exacerbated by the rise in pro-inflammatory cytokines associated with obesity. These changes in nutrient flux along the adipose-liver axis play a critical role in the development and progression of fatty liver disease (Figure 19).

Several human and animal studies provided evidence that targeting AT may protect against hepatic disorders, including MAFLD, rather than focusing solely on the liver itself.

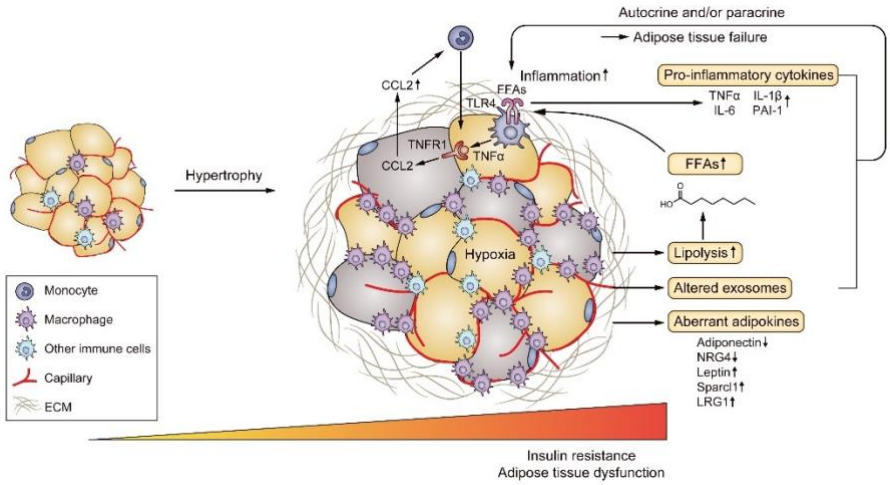


Figure 19. Crosstalk between AT and the liver in obesity promotes the development of NAFLD (Lee et al., J Hepatol. 2023; doi:10.1016/j.jhep.2023.01.024)

The crucial role of AT in regulating hepatic lipid homeostasis is further highlighted by the observation that lipodystrophy (a condition characterized by AT deficiency) lead to the development of fatty liver (Safar Zadeh et al., 2013).

To date, targeting the liver-adipose axis has emerged as a potential therapeutic strategy for the treatment of obesity and its associated metabolic dysfunctions.

4.1 NORMAST3®

Given the multiple underlying mechanisms of diabesity, MAFLD and their intricate interrelations, targeting a single feature could be insufficient, and oversimplistic. A more effective approach to fine-tuning this complex pathological process can only be achieved through multitarget therapies that exert synergistic and convergent effects in the management of diabesity.

The first formulation analyzed during this PhD project is NORMAST3 (NORM3), which consists of an association of NAEs and phenolic compounds. Specifically, PEA is co-micronized with Rut and associated with HT (with a ratio of 10:2:0.5 PEA: Rut: HT), three antioxidant compounds present in lower concentrations than those that are effective not used in combination as reported in the literature. Specifically, NORM3 is a nutritional supplement designed to a company (EPITECH group) to evaluate the potential synergistic effects of these compounds on hepatic damage associated with diabetes, by leveraging their combined antioxidant properties (Melini et al., 2024).

4.2 OLALIAMID®

Given the beneficial effects of each individual NAEs, their combination appears promising in the context of developing multitarget therapies for metabolic disorders (Melini et al., 2024).

To this purpose, the second formulation here analyzed consists of a mixture of NAEs derived from olive oil, specifically through the esterification of the FFAs contained in olive oil into their corresponding ethanolamides. This mixture contains a higher percentage of OEA and lower amounts of LEA, PEA, and SEA, preserving the natural ratio of the respective FAs—oleic, linoleic, palmitic, and stearic acids—found in vergin olive oil.

Although the primary health benefits of olive oil are attributed to its content of polyphenols and vitamins (such as vitamin E and vitamin K) (Finicelli et al., 2021). Indeed, in terms of nutritional composition, olive oil is primarily composed of FAs (98–99%), predominantly

monounsaturated FAs (MUFA), such as oleic acid and palmitoleic acid (55.3–86.5%), followed by polyunsaturated FAs (PUFA), including linoleic and linolenic acids (3.5–21%), and saturated FAs (8–25.1%), such as myristic, palmitic, and stearic acids (Schwingshackl and Hoffmann, 2014).

Given the lack of scientific evidence regarding the effects of NAE combinations on obesity and its associated metabolic disorders, we investigated the impact of this formulation on AT-liver axis to manage a complex pathology such as obesity.

5. MATERIAL AND METHODS

5.1 Preparation of NORMAST 3® and OLALIAMID®

5.1.1 NORMAST 3®

PEA-Rut (PEA-Rut) 5:1 homogeneously premixed powder was micronized by standard air jet-mill technology (Remington's Pharmaceutical Sciences Handbook, Mack Pub. Co., N.Y., USA, 22nd edition). Regular and reproducible particle size > 90% in the 2.0-10-micron range was achieved. The micronized powder was mixed with 20% HT to reach the final w/w ratio of NORM3, i.e., 10:2:0.5, PEA:Rut:HT, respectively.

5.1.2 OLALIAMID®

The examined formulation, named OLALIAMID® (OLA), provided by Epitech group S.p.A (Padova, Italy) is a mixture of NAEs in same ratio of the fatty acids naturally found in olive oil. Indeed, it was obtained from European olive oil by direct uncatalyzed aminolysis in the absence of solvent. Briefly, the oil was reacted with an approximative stoichiometric amount of 2-aminoethanol at 140 °C for 4 hours to achieve complete conversion of esterified fatty acids into corresponding ethanolamides. The reaction was conducted under a nitrogen atmosphere to protect unsaturated fatty acids from oxidation. The mixture was finally dried under vacuum and obtained as a waxy solid at room temperature (RT), with the following composition: OEA 65%, LEA 12%, PEA 10%, SEA 2%, and glycerol 8%, acylglycerols and minor components.

5.2 HFD-induced obesity: in vivo procedures and treatments

5.2.1 Animals and in vivo procedures

Young male C57Bl/6J mice (6 weeks of age, Charles River, Wilmington, MA, USA) were housed in stainless steel cages at 22 ± 1°C with a 12:12 h light-dark cycle. Mice were randomly divided into two experimental groups based on their body weight: the first group of animals received a standard chow diet (STD, Mucedola srl, Milan, Italy) (n= 12); the second group fed with an HFD (Research Diets Inc, NJ, USA, D12451) (n= 24 animals) for 12 weeks. The standard diet contained 17% fat without sucrose, while HFD had 45% of energy derived from fat, 20% from proteins, and 35% from carbohydrates. After 12 weeks, diabesity was established by evaluating i) the significant weight gain (STD= 30,19 g ± 0,24 and HFD= 42,85 g ± 1,04****, p< 0,0001 *vs* STD) and ii) the fasting hyperglycemia of HFD animals compared with STD group (STD= 110,2 mg/dL ± 8,49 and HFD= 164,3 mg/dL ± 9,33***, p< 0,001 *vs* STD). At the 12th week when obesity was fully blown, HFD obese mice were divided into three groups with no significant differences in body weight mean ± S.E.M. at the beginning of treatment period: HFD, HFD mice treated with OLA (HFD+OLA, 10 mg/kg /die per os by gavage) and, in another set of experiments, a group of HFD animals treated with NORM3 (PEA 10 mg/kg/die-rutin 2 mg/kg/*die*, HT 0,5 mg/kg/*die* per os) from week 12 up to week 19. OLA and NORM3 (synthesized and provided by Epitech group S.p.A Padova, Italy) were suspended in carboxymethyl cellulose (1.5%). At the end of the 7th week of treatment, fat mass was evaluated by bioelectrical impedance analysis as previously described (Rutter et al., 1998) and then modified for mice by Mollica et al.

(2017). Briefly, a weak electric current flows through the mouse body, and the voltage is measured to calculate the body's impedance (resistance and reactance). Then, mice were anaesthetized by enflurane followed by intracardiac puncture and cervical dislocation. Serum, interscapular brown adipose tissue (iBAT), subcutaneous white adipose tissue (scWAT), and liver were collected, frozen, and stored at −80°C for the following determinations. Animal care and experimental procedures were carried out in compliance with international and national law and policies and approved by the local animal care office (Centro Servizi Veterinari, University of Naples Federico II) and the Italian Ministry of Health under protocol no. 320/2021-PR for OLA and no. 140/2022-PR for NORM3. Animal studies were performed in compliance with the Italian D.L. (No. 26 of 4 March 2014) of the Italian Ministry of Health and the EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines 2.0, and the Basel declaration, including the 3Rs concept.

5.2.2 Metabolic tests: Glucose, insulin, and pyruvate tolerance tests

At the end of the experimental period, oral glucose, insulin, and pyruvate tolerance tests (OGTT, ITT, and PTT) were performed (at least 6 animals for each group). For the OGTT and PTT, mice were fasted for 16 h, whereas for the ITT, mice were starved for 6 h.

Briefly, after fasting blood glucose measurement, animals received an aqueous solution of glucose (1 g/kg, cat. number G8270, Sigma-Aldrich, Milan, Italy) by oral gavage or intraperitoneal injection of pyruvate (2 g/kg i.p., cat. number P2256, Sigma-Aldrich, Milan, Italy) or insulin (0.75 IU/kg, Humulin R, cat. number HI210, Ely Lilly,

Indianapolis, IN, USA). Glucose levels were measured in tail vein blood at different selected time points (0, 30, 60, 90, and 120 min for OGTT; 0, 15, 30, 60, and 120 min for ITT and PTT) using a blood glucometer (One Touch Verio). The area under the curve (AUC) was calculated as an integrated and cumulative measurement of glycemia from time 0 up to 120 min for each animal.

5.3 Biochemical determinations and histological analysis

5.3.1 Serum analysis

At the end of the experimental time, blood was collected by intracardiac puncture from all experimental groups of mice. Serum samples were obtained by centrifugation at 2500 rpm at 4 ◦C for 12 min and then stored at 80 °C. Serum insulin, leptin, ghrelin, resistin, PAI-1, glucagon, GLP1, and GIP concentrations were measured using Bio-Plex Pro Mouse Diabetes 8-Plex Assay kit (cat. number 171F7001M, Bio-Rad Laboratories, Milan, Italy) according to the manufacturer's instructions.

Furthermore, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), cholesterol, TGs, adiponectin (cat. numbers AL146, AS101, LD3842, CH200, TR210, respectively, Randox Laboratories ltd., Crumlin, UK), and pro-inflammatory cytokines as interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1 and interferon (IFN)-γ levels (cat. numbers MLB00C, M100B, M6000B, MTA00B, MJE00B, and MIF00, respectively, R&D Systems, Inc., Minneapolis, MN) were measured by colorimetric enzymatic method using commercially available ELISA kits. Then, we

calculated the Homeostasis Model Assessment (HOMA-IR) using the formula (HOMA = fasting glucose (mmol/L) × fasting insulin (μU/mL)/22.5).

5.3.2 Liver histological evaluations

Livers were removed, weighed, fixed in neutered buffered formalin, and embedded in paraffin. Ten-micrometer sections were stained with hematoxylin-eosin (H&E) for morphological assessment. Livers were subjected to blinded histomorphometric analysis and analyzed by the same pathologist to perform a semi-quantitative evaluation. Steatosis was scored on a scale of 0 (no steatosis), 1 (mild, 1<30% of affected hepatocytes), 2 (moderate, 30-70% of affected hepatocytes), and 3 (severe, >70% of affected hepatocytes).

5.4 *Ex vivo* analysis on tissues

5.4.1 Western blot analysis

Total protein lysates, obtained by scWAT, liver or cell homogenization, were subjected to SDS-PAGE as previously described (Pirozzi et al., 2023). The blot was performed by Trans-Blot Turbo transfer system (cat. number 704150, Bio-Rad Laboratories, Segrate, Milan, Italy) at room temperature for 240 mA for 60 min. The obtained membrane filter was then blocked with 1X phosphate buffer solution (PBS) and 5% nonfat dried milk for 60 min at room temperature and probed with anti-phospho-insulin receptor (InsR)β mouse polyclonal antibody (dilution 1:1000, cat. number 44809G, Thermo Fisher Scientific, cat. number: 32109, Rodano (MI), Italy), anti-InsRβ rabbit monoclonal antibody (dilution 1:1000, cat. number

3025), anti- phosphatidylinositol 3 kinase (PI3K) rabbit monoclonal antibody (dilution 1:1000, cat. number 4249S), anti-phospho-AKT (Ser473) rabbit monoclonal antibody (dilution 1:1000, cat. number 4060), anti-AKT (pan) mouse monoclonal antibody (dilution 1:1000, cat. number 4691), anti-phospho-AMPKα rabbit monoclonal antibody (dilution 1:1000, cat. number 2535), and anti-AMPKα mouse monoclonal antibody (dilution 1:1000, cat. number 2793) (Cell Signaling Technology, Inc., Beverly, MA, USA), anti-CPT1L rabbit polyclonal antibody (dilution 1:500, cat. number A30939, Bioworld Technology, Inc), anti-cyclooxygenase (COX)-2 mouse monoclonal antibody (dilution 1:200, cat. number), anti- NFκB p65 subunit rabbit monoclonal antibody (dilution 1:200, cat. number sc8008) (Santa Cruz Biotechnology, Inc, USA), anti-glucose transporter (GLUT)-4 rabbit monoclonal antibody (diluition 1:1000, cat. number 2213T) (Novus Biologicals Europe, United Kingdom), anti LC3B (diluition 1:1000 cat. number 2775S), anti SQSTM1/p62 (diluition 1:1000 cat. number 5114), β-Actin mouse polyclonal antibody (dilution 1:2000, cat. number A5441, Sigma-Aldrich, Milan, Italy) and GAPDH rabbit polyclonal antibody (diluition 1:2000 and cat no. 2118) (Cell Signaling Technology, Inc., Beverly, MA, USA) was used as housekeeping to ensure equal sample loading. The signals were visualized with the ECL system (Pierce, Thermo Fisher Scientific, cat. number 32109, Rodano (MI), Italy) for chemiluminescence assay (ChemiDoc Imaging Systems, cat. number 12003153, Bio-Rad Laboratories, Segrate, Milan, Italy).

5.4.2 Semi-quantitative Real-Time PCR analysis

Total RNA isolated from scWAT and iBAT was extracted using RNeasy lipid tissue kit (Qiagen, Hilden, Germany) while total RNA isolated from the liver or HepG2 cells and ear fibroblasts was extracted using PureZOL™ RNA Isolation Reagent (cat. number: 7326890, Bio-Rad Laboratories, Segrate, Milan, Italy) following the extraction kit's protocol for RNA (cat. number FC140955N, NucleoSpin®, Macherey-Nagel GmbH & Co, Düren, Germany). cDNA was obtained using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA; 4374966) from 1 or 3 µg total RNA, depending on the tissue. RT-PCRs were performed with a Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories, Milan, Italy). The RT-PCR conditions were previously described (Lama et al., 2021). Each sample contained 500 ng cDNA in 2X QuantiTect SYBR Green PCR Master Mix (cat. number 204145, Qiagen, Hilden, Germany) and primers pairs to amplify phospho-enol pyruvate carboxy kinase (*Pck1*, QT00153013), glucose-6-phosphatase (*G6pc*, QT00114625), PPAR-α (*Ppara*, QT00137984), cluster of differentiation (CD)36 (*Cd36*, cat. number), fatty acid synthase (*Fasn*, QT00149290), IL-1β (*Il1b*, mouse QT010485355, human QT00021385), TNF-α (*Tnf*, QT00104006), nuclear factor erythroid 2-related factor 2 (NRF-2 or *Nfe2l2*, mouse QT00095270, human QT00027384), NAD(P)H quinone dehydrogenase 1 (*Nqo1*, QT00094367), and heme oxygenase-1 (HO-1 or *Hmox1*, QT00159915) (Qiagen, Hilden, Germany), growth differentiation factor (GDF)15 (*Gdf15*, QT00124481), IL-10 (*Il10, QT00106169*), adiponectin (*Adipoq, QT01048047*), PR-domain containing

(PRDM)16 (*Prdm16*, *QT00148127*), uncoupling protein (UCP)1 (*Ucp1*, QT00097300), PPAR-γ (*Pparg*, QT00100296), PGC-1α (*Ppargc1a*, QT02524242), cell death-inducing DFFA-like effector A or CIDEA (*Cidea*, QT00095249), cytochrome C oxidase subunit VIIIb or COX8b (*Cox8b*, QT00100366), fatty acid synthase (FASN) (*Fasn*, QT00149240), SREBP-1c (*Srebf1*, *QT00167055*), cluster of differentiation (CD)36 (*Cd36*, QT01058253), p21(*Cdkn1a*, QT01752562) (Qiagen, Hilden, Germany), p16 (*Cdkn2a*, QT00252595) and IL-6 (*Il6*, QT00098875) in a final volume of 100 µL. The relative amount of each studied mRNA was normalized to GAPDH for liver (*Gapdh*, mouse QT01658692, human QT00079247), to βACTIN (*Actb* QT00095242) for iBAT, HepG2 and ear fibroblasts and (Rn18s, QT02448075) (Qiagen, Hilden, Germany) for scWAT as housekeeping genes (Qiagen, Hilden, Germany) as a housekeeping gene, and data were analyzed according to the $2^{-\Delta\Delta Ct}$ method.

5.4.3 ROS production

ROS assay was performed as previously reported (Pirozzi et al., 2020) in both liver and HepG2 cells. To quantify ROS production, an appropriate volume of freshly prepared liver homogenate was diluted in 100 mM potassium phosphate buffer (pH 7.4) and incubated with 5 µM dichloro-fluorescein diacetate (DCFDA) (cat. number 35845, Sigma-Aldrich, Milan, Italy) in dimethyl sulfoxide (DMSO, cat. number D5879, Sigma-Aldrich, Milan, Italy) for 15 min at 37 ◦C. The dye-loaded samples were centrifuged at 12,500× g per 10 min at 4 ◦C, and then 5 mL of 100 mM potassium phosphate buffer (pH 7.4) was added to the pellet and incubated for 60 min at 37 ◦C. The fluorescence

was measured using the Promega GloMax® Explorer Microplate Reader (cat. number GM3560, Milan, Italy) at 488 nm for excitation and 525 nm for emission wavelengths. ROS were quantified from the dichloro-fluorescein standard curve in DMSO (0-1 mM).

5.5 Purification and quantification of OEA and PEA

The extraction, purification, and the subsequent quantification of OEA and PEA from livers were performed as previously described (Di Marzo et al., 2001). Briefly, tissues were homogenized and extracted with a chloroform/methanol/Tris-HCl 50 mM pH 7.5 (2:1:1, v/v) mixture containing internal standards. The lipid extract was pre-purified by open-bed chromatography on silica columns, eluted with rising concentrations of methanol in chloroform. After extraction and purification, the OEA and PEA fractions were analyzed using isotope-dilution liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC-APCI-MS) with a Shimadzu HPLC system (LC-10ADVP) coupled to a Shimadzu quadrupole MS via an APCI interface, as described previously (Di Marzo et al., 2001). The amounts of OEA and PEA were expressed as picomoles per milligram of extracted lipids, per milligram of tissue weight.

5.6 Cell culture and treatments

5.6.1 In vitro model of oxidative damage

Human HepG2 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 complete medium (cat. number 11875093, Gibco Thermo Fisher Scientific, USA) at 37°C with 5% CO_2. Cells were seeded in P6-well plates (5,0 x 10^5 cells/well) and

starved for 6 h in 5% FBS medium. Cells were pre-treated with PEA-Rut (1 μM) and HT (50 nM) alone or in combination in the same ratio among the components contained in NORM3, as well as their vehicle for 6 h, before H_2O_2 (30% cat. number H1009, Sigma-Aldrich, Milan, Italy) challenge (400 μM for 4 hours) to obtain oxidative and inflammatory damage.

5.6.2 In vitro models of senescence

1) **Doxorubicin (DOXO)-induced cellular senescence**: SK-MEL-103 (human melanoma cells obtained from ATCC) were cultured in standard DMEM (Gibco), supplemented with 10% heat inactivated FBS (Gibco) and 1% antibiotics (penicillin/streptomycin 100 U/mL, Gibco). Senescence was induced by treatment with the DNA-damage inducing agent doxorubicin (DOXO) for 7 days to induce cell replication arrest and DNA damage.
2) **Palmitate (PA)-induced cellular senescence:** Ear fibroblasts were isolated from C57Bl/6J mice following the protocol described in previous studies (Khan et al., 2016). To induce senescence, ear fibroblasts ($8x10^4$ cells per plate) were treated with 100 μM palmitic acid (PA) for 7 days.

5.6.3 In vitro model of autophagic process alteration

To investigate the role of NAEs in modulating the autophagic process during metabolic alterations, HepG2 cells were first treated with 100 μM PA for 24 hours. After 1 hour of PA challenge, the cells were treated with different NAEs (PEA 3 μM, OEA 3 μM, LEA 3 μM, SEA 3 μM) for 24 hours, followed by a 3-hour treatment with bafilomycin,

used as an inhibitor of autophagosome-lysosome fusion, to determine the activity of autophagic flux.

5.6.4 Cell viability after PA challenge

HepG2 cells (15 × 10^3 per well) were plated in 96-well plates and treated with the compounds the following day. Cell viability was then assessed the day after stimulation with NAEs and PA, using the CellTiter-Glo 3D Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

5.7 Senescence-associated β-galactosidase assay

Staining was performed as previously described in (Dimri et al., 1995). In brief, cells were washed with phosphate buffered saline (PBS) and fixed in a 0.2% glutaraldehyde solution. After fixation, the cells were washed again with PBS and stained with a β-galactosidase (β-Gal) solution, prepared by dissolving 1 mg/mL of 5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside (X-Gal; #203782, Sigma-Aldrich) in dimethylformamide at pH 6. The cells were incubated overnight at 37°C, washed with PBS, and then imaged using a brightfield microscope.

5.8 Flow Cytometry

5.8.1 ROS detection

Assessment of ROS levels by flow cytometry was performed using the dichloro-fluorescein diacetate probe (DCFDA) (cat. number 35845, Sigma-Aldrich, Milan, Italy), in dimethyl sulfoxide (DMSO, cat. number D5879, Sigma-Aldrich, Milan, Italy) according to

manufacturer instruction. Briefly, after the challenge with DOXO 50 nM for 7 days (Med Chem Express, 100 nM), cells were harvested, centrifuged at 1200 rpm for 5 minutes, washed twice with PBS and then incubated for 30 minutes with DCFA and with the viability dye DAPI (#D9542, Sigma) at 5 µg/mL (cat. number 35845, Sigma-Aldrich, Milan, Italy) for 30′ at room temperature (RT) and analyzed with a Cytoflex (Beckman Coulter) flow cytometer. Data were analyzed using FlowJo software v.10.9.0.

5.8.2 Mitophagy assessment through mKEIMA assay

The mKeima assay can be used for FACS analysis to provide a thorough representation of mitophagy. This assay utilizes the unique properties of the Keima fluorophore, which exhibits bimodal excitation profiles depending on pH, with distinct responses under neutral and acidic conditions. Mitophagy was assessed following the protocol used by (Cheng et al., 2021; Wang, 2020). In brief, we utilized a stable SK-MEL cell lines expressing mito-mKeima, which were obtained through a lentiviral transfection followed by long-term antibiotic selection.

In this assay, the fluorescent protein mKeima exhibits a shift in its excitation wavelength depending on the pH of its environment. During mitophagy, mitochondria move from the neutral pH environment of the cytoplasm to the acidic environment of the lysosome. This pH-dependent shift allows the detection of mitophagy using flow cytometry.

5.8.3 Cell proliferation by flow cytometry

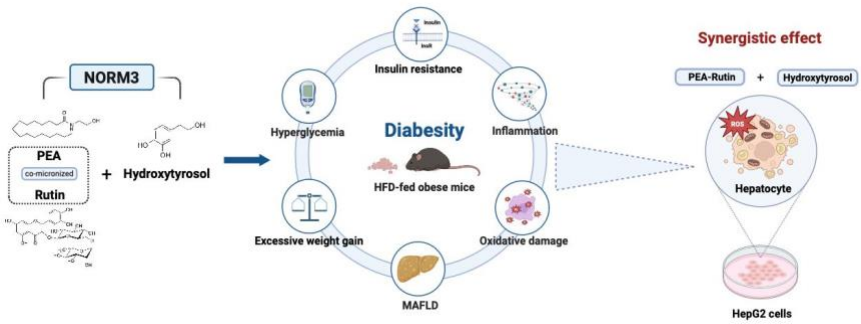
Assessment of cellular proliferation by flow cytometry was performed using the Ki-67 antibody (cat. number # 652404 Abcam), which is a nuclear protein only present in proliferating cells. Briefly, after the challenge with PA for 7 days, ear fibroblasts were harvested, centrifuged at 1200 rpm for 5′ and washed twice with PBS. Then cells were incubated for 10 minutes with the viability dye Live/Dead at 5 µg/mL (cat. number 35845, Sigma-Aldrich, Milan, Italy) at RT and then cells are fixed and incubated with Ki67 in permeabilization buffer for 30 minutes, then cells were washed in FACS buffer and analyzed with a Cytoflex (Beckman Coulter) flow cytometer. Data were analyzed using FlowJo software v.10.9.0.

5.9 Statistical analysis

All data shown are presented as mean value ± SEM. Shapiro-Wilk normality test was performed on all analyzed data. Statistical analysis was performed by one-way or two-way ANOVA (minimum n= 6 animals in each group), depending on different analyses, followed by Bonferroni post-hoc for multiple comparisons. Differences among groups were considered significant at values of $p < 0.05$. Analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

6. RESULTS

6.1 CO-MICRONIZED PALMITOYLETHANOLAMIDE AND RUTIN ASSOCIATED WITH HYDROXYTYROSOL RECOVER DIABESITY-INDUCED HEPATIC DYSFUNCTION IN MICE: *IN VITRO* INSIGHTS INTO THE SYNERGISTIC EFFECT



Graphical abstract 1

6.1.1 Effect of NORM3 on body weight and glucose homeostasis of obese mice

First, NORM3 macroscopically relieved the obese phenotype in mice (Figure 1.1A), decreasing the body weight compared with the HFD group starting from the 3rd week of treatment (Figure 1.1B), as also confirmed by the area under the curve (AUC) of the body weight measured up to the 7th week (Figure 1.1B, upper panel). Consistently, NORM3 treatment reduced fat mass and liver weight increased by HFD feeding (Figure 1.1C and D) and lessened IR, reducing glucose and insulin levels and improving HOMA-IR (Figure 1.1E-G).

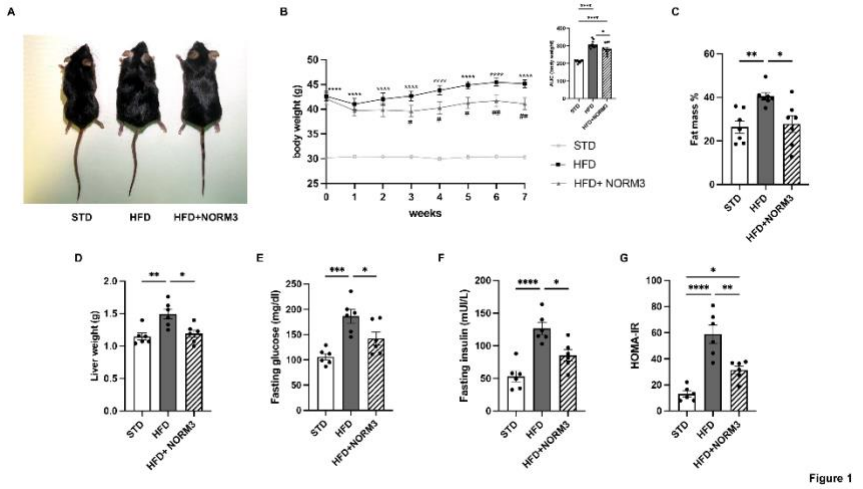


Figure 1.1 NORM3 reduces body weight and metabolic parameters altered in HFD-induced obesity. Body weight and respective AUC were measured throughout the experimental period (n=11 each group) (A), fat mass was assessed by bioelectrical impedance analysis at the end of 7th week of treatment (n=7 each group) (B). Liver weight (D), fasting glucose and insulin (E,F), and HOMA-IR (G) were determined after sacrifice (n= 5-7 each group). Data are presented as mean ± SEM. *p <0.05, **p <0,01, ***p <0,001, and ****p <0,0001 vs STD or HFD; #p <0.05 and ##p <0,01, vs HFD.

Furthermore, NORM3 improved blood glucose homeostasis and insulin sensitivity as assessed by OGTT and ITT (Figure 1.2A and B). Indeed, NORM3 limited the rise of glycemia at each analyzed time point and the relative AUC and enhanced the response to insulin action as shown by the decrease of blood glucose as shown by ITT. The lowering effect of glycemia by NORM3 was also observed in HFD-fed animals during PTT (Figure 1.2C), suggesting its effectiveness in reducing gluconeogenesis.

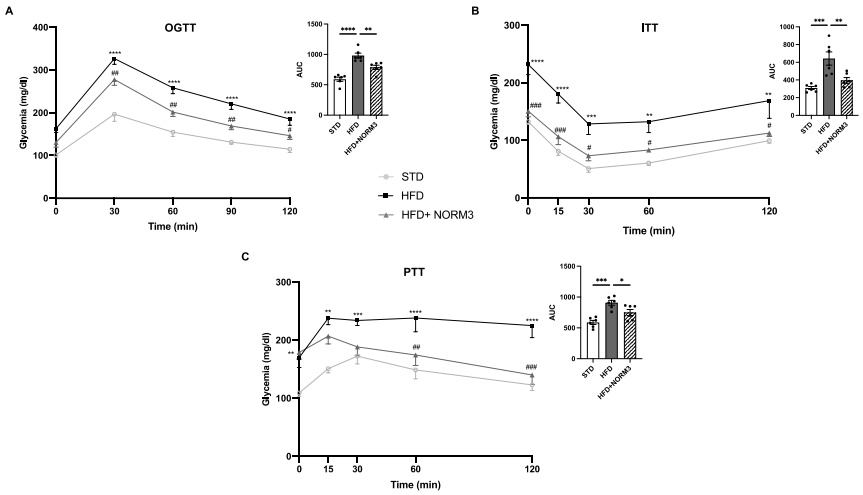


Figure 1.2. Effect of NORM3 on glucose homeostasis compromised by HFD feeding. OGTT (A) and intraperitoneal insulin (B) and pyruvate (C) tolerance tests (ITT and PTT) were performed in different sets of animals from all groups of mice (n=6). Data are presented as mean ± SEM. **p <0,01, ***p <0,001, and ****p <0,0001 vs STD or HFD; #p <0.05, ##p <0,01, ###p <0,001 vs HFD.

6.1.2 Effect of NORM3 treatment on serum biochemical and hormonal profile

The evaluations of serum biochemical markers of liver function and inflammation showed a protective effect of NORM3 in obese mice (Table 1.1). Indeed, NORM3 lowered HFD-induced elevation in systemic levels of ALT, AST, ALP, TGs, cholesterol, LDH, and the pro-inflammatory mediators (TNF-α, IL-6, and MCP-1), while enhancing the anti-inflammatory IL-10.

Table 1. Effect of NORM3 on serum hepatic and inflammatory parameters in HFD-fed mice

Serum Parameters	STD	HFD	HFD + NORM3
ALT (U/l)	64.17 ± 3.79	131.40 ± 6.37****	108.30 ± 5.41$^{\#}$ ****
AST (U/l)	63.83 ± 5.25	117.40 ± 5.63****	86.33 ± 2.75$^{\#\#}$ **
ALP (U/l)	150.20 ± 4.93	265.60 ± 4.33****	197.00 ± 3.65$^{\#\#\#\#}$ ****
Triglycerides (mg/dl)	110.00 ± 3.29	216.80 ± 5.05****	173.20 ± 5.42$^{\#\#\#\#}$ ****
Cholesterol (mg/dl)	136.80 ± 3.36	259.20 ± 5.68****	190.70 ± 6.39$^{\#\#\#\#}$ ****
LDH (U/L)	68.33 ± 3.13	134.20 ± 7.40****	82.20 ± 2.75$^{\#\#\#\#}$ ***
TNF-α (ng/ml)	1.04 ± 0,06	3.67 ± 0.15****	1.53 ± 0.09$^{\#\#\#\#}$ *
IL-6 (pg/ml)	22.00 ± 0.09	42.60 ± 4.14****	26.17 ± 0.94$^{\#\#\#}$
MCP-1 (pg/ml)	29.83 ± 1.17	64.20 ± 1.93****	39.00 ± 1.88$^{\#\#\#\#}$ **
IL-10 (ng/ml)	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.01$^{\#\#}$

Data are presented as mean ± SEM of all animals from different groups (n=5-7 each group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs STD; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001, $^{\#\#\#\#}$p < 0.0001 vs HFD.

Moreover, NORM3 restored serum glucagon and resistin to STD levels and induced a reduction trend of PAI-1, a physiological inhibitor of plasminogen activators, compared with untreated HFD, all factors involved in IR occurring in diabesity. Other serum hormones, including GIP, GLP1, and ghrelin, did not significantly change across groups (Table 1.2).

Table 2. Effect of NORM3 treatment on serum hormonal profile of obese animals

Serum Parameters	STD	HFD	HFD + NORM3
Glucagon (ng/ml)	0,51 ± 0.04	0,72 ± 0.03**	0,55 ± 0,03$^{\#}$
Resistin (ng/ml)	52.26 ± 4.79	99.02 ± 11.24**	61.75 ± 4.63$^{\#\#}$
PAI-1 (ng/ml)	1.14 ± 0.10	3.04 ± 0.57**	1.83 ± 0.16
GIP (ng/ml)	3.18 ± 0.16	2.70 ± 0.28	3.48 ± 0.22
GLP-1 (ng/ml)	0.08 ± 0.02	0.06 ± 0.01	0,09 ± 0.01
Ghrelin (ng/m)l	1,88 ± 0,24	3,58 ± 0,68	3,74 ± 0,55

Data are presented as mean ± SEM of all animals from different groups (n=6 each group). **p < 0.01 vs STD; $^{\#}$p < 0.05, $^{\#\#}$p

6.1.3 Effect of NORM3 on hepatic IR, glucose and lipid dysfunction

Then, we explore the effect of NORM3 treatment on hepatic glucose homeostasis, demonstrating its capability to improve insulin signaling altered by HFD. Indeed, NORM3 increased the phosphorylation of insulin receptor (InsR) and the activation of the PI3K/AKT pathway (Figure 1.3A-C), compared with the HFD group. Moreover, NORM3 significantly decreased HFD-altered transcription of *Pck1* and *G6pc*, the two main enzymes regulating critical steps in gluconeogenesis (Figure 1.3D and E).

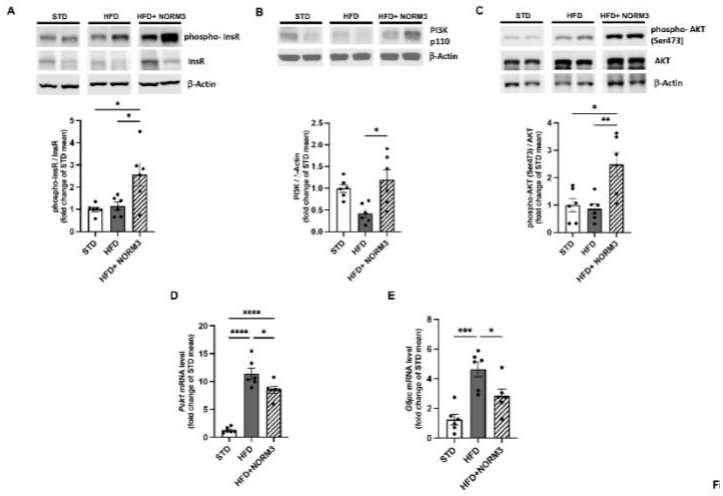


Figure 1.3. NORM3 improves hepatic insulin signalling and gluconeogenesis altered by HFD. Cropped images of the phospho-InsR/InsR (A), PI3K (B), and phospho-AK/AKT (C) were obtained by Western blot analysis in the livers of all animals (n=6 each group). The transcription of enzymes catalyzing hepatic gluconeogenesis Pck1 (D) and G6pc (E) was measured by RT-PCR (n=6 each group). Data are presented as mean ± SEM. *p <0.05, **p <0,01**,** ***p <0,001, and ****p <0,0001 vs STD or HFD.

The effect of NORM3 on the hepatic lipid dysmetabolism induced by HFD was also investigated. Seven-week administration of NORM3 significantly increased the phosphorylation of AMPK compared to

HFD group (Figure 1.4A); this kinase is a crucial energy sensor involved in glucose and lipid metabolism. NORM3 also restored the protein expression of CPT1 to STD levels (Figure 1.4B) and consistently increased HFD-altered expression of *Ppara* (Figure 1.4C) and reduced *Cd36* and *Fasn*, both critical markers of steatosis (Figure 1.4D, E).

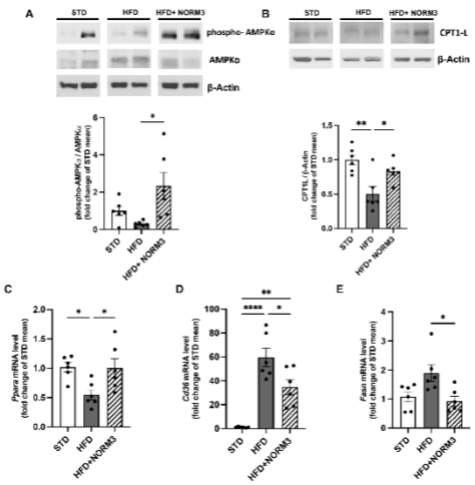


Figure 1.4. NORM3 dampens the alteration of lipid metabolism in liver of HFD-fed mice. Cropped images of Western blot for phospho-AMPK/AMPK (A) and CPT1-L (B) were shown (n=6 each group). The mRNA expression of Ppara (C), Cd36 (D), and Fasn (E) was evaluated by RT-PCR (n=6 each group). Data are presented as mean ± SEM. *p <0.05, **p <0,01, and ****p <0,0001 vs STD or HFD.

6.1.4 Effect of NORM3 on liver morphology and inflammation of HFD-fed mice

In HFD mice, liver morphological evaluations revealed moderate to severely degenerated hepatocytes with optically empty cytoplasm containing small and large, rounded vacuoles that delocalize the nucleus to the periphery, all features typical of micro- and macro-vacuolar steatosis, compared with the STD liver (Figure 1.5A and B).

Notably, NORM3 treatment counteracted hepatocyte morphological alteration induced by HFD. Based on the well-known anti-inflammatory activity of NORM3 components (PEA-Rut and HT), we demonstrated the marked protective effect of their association in reducing the transcription of pro-inflammatory mediators, such as IL-1β and TNF-α (Figure 1.5C and D), as well as the protein expression of COX-2 enzyme and the nuclear translocation of p65 NFκB (Figure 1.5E and F), all parameters altered by HFD.

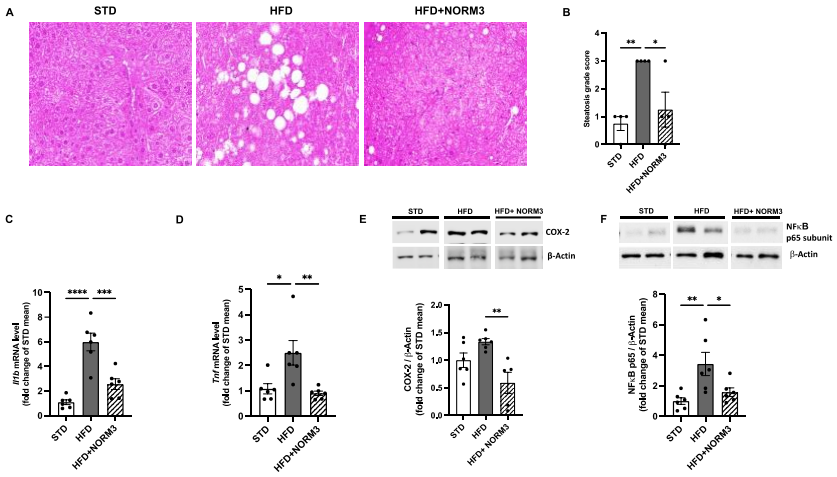


Figure 1.5. Effect of NORM3 on liver morphology and inflammation of obese mice. Representative paraffin-embedded sections of the liver were stained with H&E (A), and the degree of steatosis (B) was examined in a double-blinded manner (n= 4 each group). Hepatic pro-inflammatory Il1b (C) and Tnf (D) were evaluated by RT-PCR (n= 6 each group); the protein expression of COX-2 (E) and NFκB p65 subunit (F) was also assessed (n=6 each group). Data are presented as mean ± SEM. *p <0.05, **p <0,01, ***p <0,001 vs STD or HFD.

6.1.5 Antioxidant activity of NORM3 in the liver of obese mice and in vitro evidence for the synergistic effect of its single components

NORM3 counteracted hepatic oxidative stress caused by HFD, reducing ROS production (Figure 1.6A) and stimulating the hepatic

detoxifying system as shown by the increased mRNAs of antioxidant NRF2 (*Nfe2l2*), NQO1 (*Nqo1*), and HO1 (*Hmox1*) compared to HFD mice (Figure 1.6B-D). To investigate the synergistic effect of NORM3 components, HepG2 cells were treated with PEA-Rut and HT, alone or in combination, at the same rate and proportional concentrations used in the examined formulation in vivo. H_2O_2 challenge was used to mimic the detrimental conditions (oxidative stress and associated inflammation), characterizing liver damage related to IR. In our experimental conditions, these compounds, used alone or in combination, did not modify cell viability (data not shown).

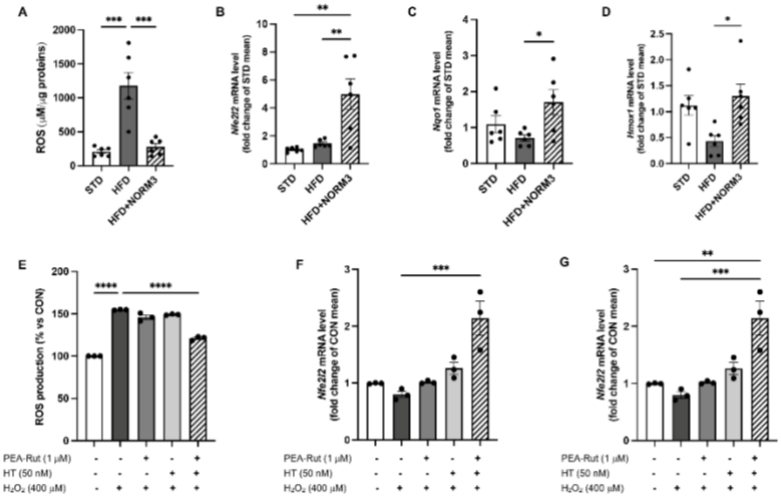
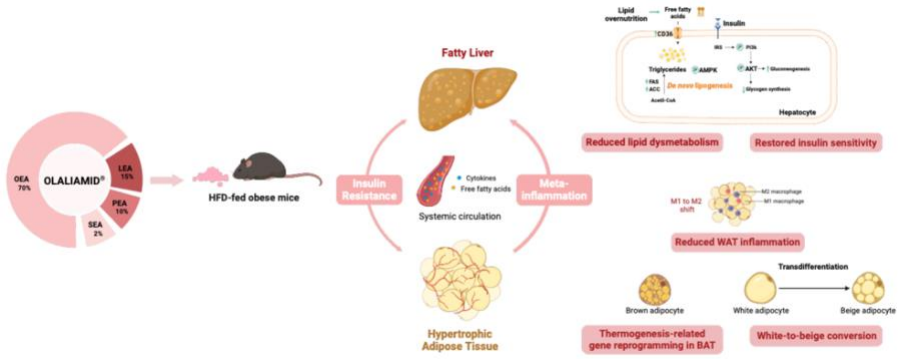


Figure 1.6. NORM3 counteracts hepatic oxidative stress in vivo and in vitro: synergistic effects of its single components. ROS production was evaluated by spectrofluorimetric analysis in both liver of obese mice (n=6 each group) (A) and HepG2 cells treated with PEA-Rut and HT (for 24 hours), alone or in combination, and challenged with H2O2 (for 4 hours) (E). Detoxifying gene expression of Nfe2l2 (B, F), Nqo1 (C), and Hmox1 (D), as well as inflammatory Il1b (G), were also determined by RT-PCR analysis (n= 6 each group). In vitro data are presented as means ± SEM from three different experiments in duplicate. *p <0.05, **p <0,01, ***p <0,001, ****p <0,0001 vs STD or HFD or vs untreated control cells.

Notably, PEA-Rut or HT did not arise any significant protective effect when used alone after H_2O_2 challenge, while only the combination PEA-Rut + HT was able to reduce cellular ROS amount (Figure 1.6E), to increase the mRNAs of detoxifying factor *Nfe2l2* and to reduce the inflammatory cytokine *Il1b* (Figure 1.6F and G).

6.2 TARGETING LIVER AND ADIPOSE TISSUE IN OBESE MICE: EFFECTS OF A N-ACYLETHANOLAMINE MIXTURE ON INSULIN RESISTANCE AND ADIPOCYTE REPROGRAMMING



Graphical abstract 2

6.2.1 OLA limits obesity progression and improves glucose tolerance in HFD-fed mice

The treatment with OLA limited the excessive weight gain due to HFD intake, starting from the 2nd week to the end of the experimental time, as also shown by the area under curve (AUC) (Figure 2.1A). Moreover, impedance analysis revealed a reduction in fat mass of OLA-treated HFD mice compared with HFD (Figure 2.1B). To investigate the effect of OLA on metabolic alteration induced by HFD and the consequent inability to control blood glucose levels in obese mice, we performed the OGTT at the 19th week. Obese mice showed hyperglycemia that was limited by OLA at 60, 90, 120 minutes after the oral glucose load, as confirmed by AUC (Figure 2.1C). Consistently, 7-week treatment with OLA reduced HOMA-IR index (Figure 2.1D), a key marker of IR, and leptin/ adiponectin ratio, a proxy of adipocyte dysfunction (Figure 2.1E).

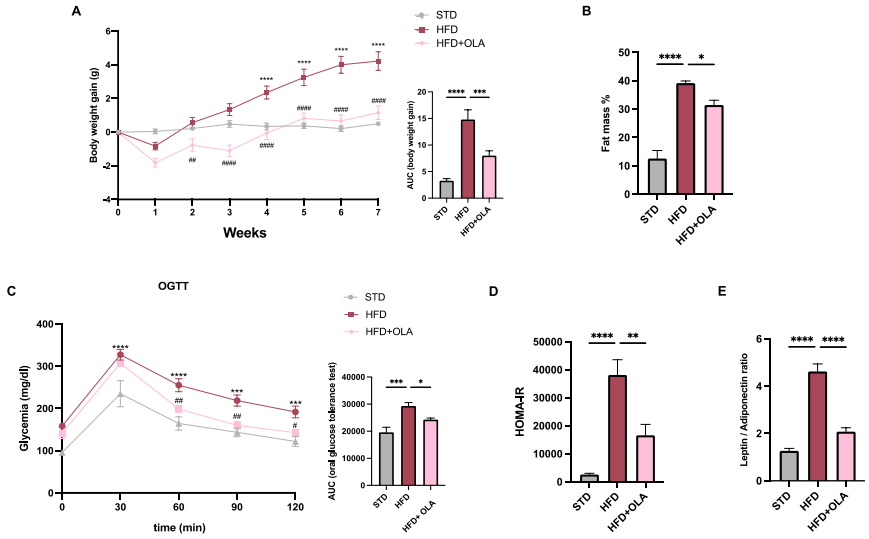


Figure 2.1. Effects of OLA on metabolic parameters altered in HFD-induced obesity. Body weight gain and respective AUC were measured throughout the experimental period (A) (n=12 each group), as well as fat mass before sacrifice (B) (n=7 each group). Moreover, the effect of OLA on IR was evaluated in vivo by OGTT (C) (n= 5-6 each group). and ex vivo by HOMA-IR index (D) (n= 6 each group). Leptin/adiponectin ratio was also measured in serum of all mice (E) (n=5 each group). Data are presented as mean ± SEM. *p < 0.05, **, p <0,01, *** p<0,001, and ****p<0,0001 vs STD or HFD; #p < 0.05, ## p <0,01, and ####p<0,0001 vs HFD.

6.2.2 Effect of OLA on serum biochemical, hormonal, and inflammatory parameters in obese mice

As known, HFD feeding causes a profound alteration in systemic lipid profile. We showed that the elevated serum cholesterol and TG levels in obese mice were significantly decreased by OLA (Figure 2.2A and B), that also improved hepatic function as shown by the reduction of transaminases (ALT and AST) and LDH (Figure 2.2C-E).

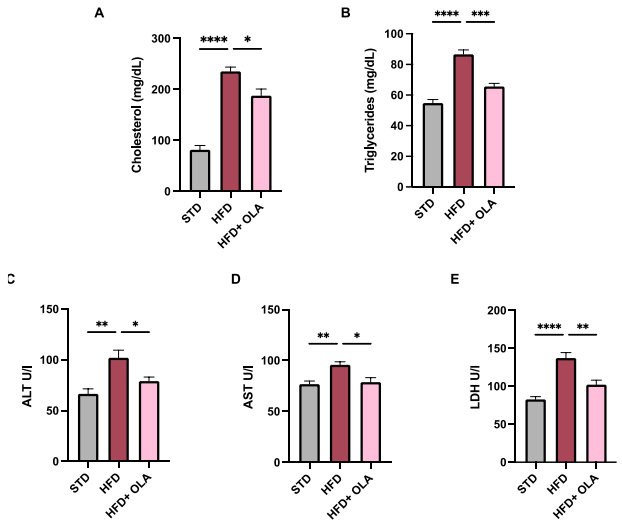


Figure 2.2. OLA normalizes serum lipid and hepatic biomarkers altered by HFD. Serum lipid profile, including cholesterol (A), and TGs (B), and hepatic and inflammatory markers, such as ALT (C), AST (D) and LDH (E) were evaluated by ELISA assay (n=5 each group). Data are presented as mean ± SEM. *p < 0.05, ** p <0,01, *** p<0,001, and ****p<0,0001.

As reported in Table 2.1, OLA affected the glucose hormone profile, modulating insulin and glucagon levels. OLA reduced leptin and PAI-1 and induced a trend of reduction in resistin levels; conversely, the anti-inflammatory adiponectin was increased. Ghrelin and incretins GLP-1 and GIP were not changed by OLA treatment. Finally, OLA counteracted meta-inflammation evoked by HFD, reducing IL-1β, TNF-α, IFN-γ, LPS, and MCP-1 in serum.

Serum Parameters	STD	HFD	HFD+OLA
Insulin ng/ml	2,24 ± 0,41	5,77 ± 0,41***	3,44 ± 0,66$^{\#}$
Glucagon ng/ml	0,51 ± 0,04	0,68 ± 0,05	0,51 ± 0,04$^{\#}$
PAI-1 ng/ml	1,17 ± 0,08	2,73 ± 0,62*	1,07 ± 0,31$^{\#}$
Resistin ng/ml	64,81 ± 5.79	106,40 ± 8,94**	88,04 ± 6,78
Leptin ng/µl	8,82 ± 0,73	18,40 ± 0,87****	12,80 ± 0,97$^{\#\#}$
Adiponectin µg/ml	6,96 ± 0,28	4,02 ± 0,18****	6,24 ± 0,30$^{\#\#\#}$
Ghrelin ng/ml	2,87 ± 0,50	3,23 ± 0,68	2,74 ± 0,41
GIP ng/ml	3,54 ± 0,30	2,40 ± 0,30	3,32 ± 0,36
GLP1 ng/ml	0,07 ± 0,01	0,06 ± 0,01	0,09 ± 0,01
TNF-α ng/ml	0,14 ± 0,01	2,00 ± 0,19****	1,36 ± 0,07$^{\#\#}$
IL-1β, pg/ml	58,40 ± 3,59	105,40 ± 5,73****	79,40 ± 5,59$^{\#}$
IFN-γ µg/ml	0,16 ± 0,01	0,31 ± 0,03***	0,18 ± 0,01$^{\#\#}$
MCP-1 (pg/ml)	25,80± 1,88	50,60± 3,44****	34,80± 2,20$^{\#\#}$
LPS (EU/ml)	0,48± 0,03	1,37± 0,08****	0,90± 0,03$^{\#\#\#\#}$

Data are presented as mean ± SEM of all animals from different groups (n=5-7 each group). *p < 0.05, **p < 0,01, ***p < 0,001, ****p < 0.0001 vs STD; #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001 vs HFD.

Table 2.1 Effect of OLA treatment on serum hormonal and inflammatory profile in HFD-fed mice

6.2.3 Effect of OLA on hepatic insulin sensitivity and lipid dysmetabolism of HFD-fed animals

OLA treatment increased the phosphorylation of AKT, the critical downstream kinase involved in the insulin signaling pathway, and the protein expression of phospho-AMPK, the master regulator of cellular and tissue energy homeostasis (Figure 2.3A and B). In obesity, hepatic lipid dysmetabolism occurs due to the increased DNL and altered fatty acid oxidation. OLA decreased gene expression of CD36, a fatty acid transporter and key marker of steatosis, strongly upregulated in obese mice (Figure 2.3C). Consistently, OLA limited hepatic lipogenesis decreasing FAS and PPAR-γ transcription (Figure 2.3D and E) and inducing a decreasing trend of SREBP1c (Figure 2.3F). Moreover,

OLA reduced the mRNAs of the nutritional stress-related hormone GDF15 in liver of obese mice (Figure 2.3G).

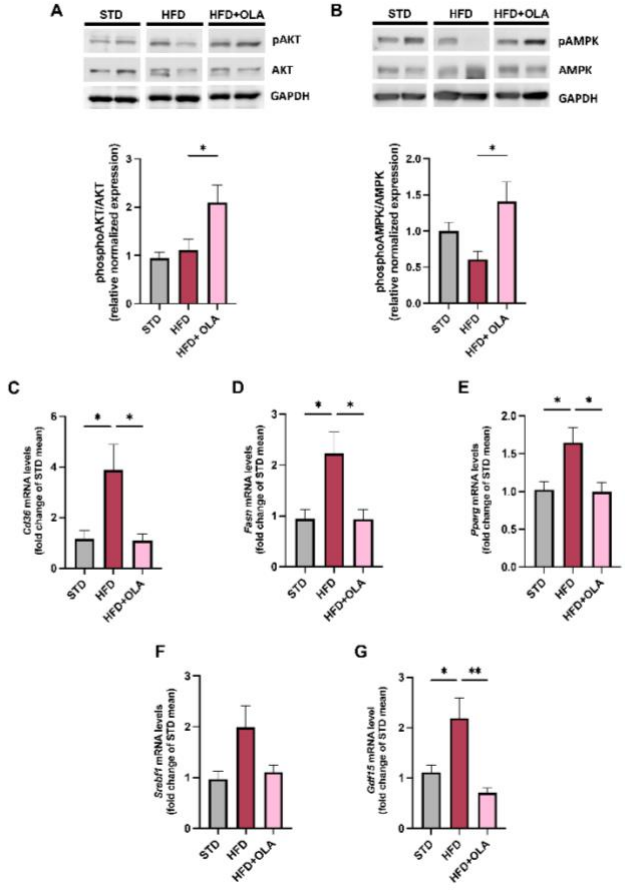


Figure 2.3. OLA counteracts hepatic lipid dysmetabolism in HFD-fed obese mice. The phosphorylation of AKT (A) and AMPK (B) was assessed by Western blot analysis in liver of all mice (n=7-8 each group). Furthermore, the hepatic mRNA expression of Cd36 (C), Fasn (D), Pparg (E), Srebf1 (F), and Gdf15 (G) was also quantified by RT-PCR (n=5-6 each group). Data are presented as mean ± SEM. *p < 0.05, and ** p <0,01.

6.2.4 OLA reprograms genes involved in iBAT thermogenesis of obese mice

Here, we investigated the effect of OLA in restoring the thermogenesis impaired in HFD-fed mice. Brown adipocytes use FFAs and glucose as fuels, combusting macronutrients to generate heat; BAT dysfunction contributes to the development of obesity. OLA increased the transcription of BAT-associated genes, namely *Prdm16*, which induces the thermogenic genes program (Figure 2.4A), and UCP1 or thermogenin, a mitochondrial protein that uncouples oxidative phosphorylation from ATP synthesis, allowing the dissipation of the proton gradient as heat (Figure 2.4B). OLA treatment also induced the transcription of *Pparg*, *Ppargc1a*, *Cidea*, and *Cox8b*, all genes involved in the regulation of BAT thermogenesis (Figure 2.4C-F). Finally, OLA reduced the mRNAs of IL-1β and GDF15 in brown adipocytes of obese mice (Figure 2.4G, H).

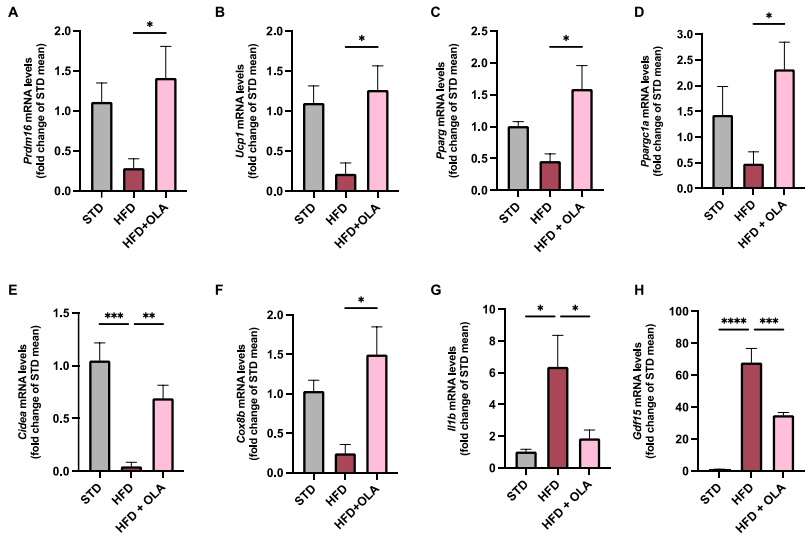


Figure 2.4. Effect of OLA treatment on thermogenesis and inflammation in iBAT of obese mice. The transcription of thermogenic genes, such as Prdm16 (A), Ucp1 (B), Pparg (C), Ppargc1a (D), Cidea (E), and Cox8b (F) was determined in

iBAT of all groups by RT-PCR (n=5-6 each group). The mRNA expression of the batokine Il1b (G) and the inflammation-related hormone Gdf15 (H) was also evaluated (n=5-6 each group). Data are presented as mean ± SEM (n=). *p < 0.05, ** p <0,01, *** p<0,001, and ****p<0,0001.

6.2.5 OLA induces beigeing of white adipocytes in HFD-fed mice

In scWAT of obese animals, OLA induced the mRNA expression of PPAR-γ and PGC-1α (Figure 2.5A and B), resulting in the increase of PRDM16, a key marker of the thermogenic activity and brown-like function (Figure 2.5C). Consistently, OLA increased the expression of UCP1, responsible for non-shivering thermogenesis (Figure 2.5D).

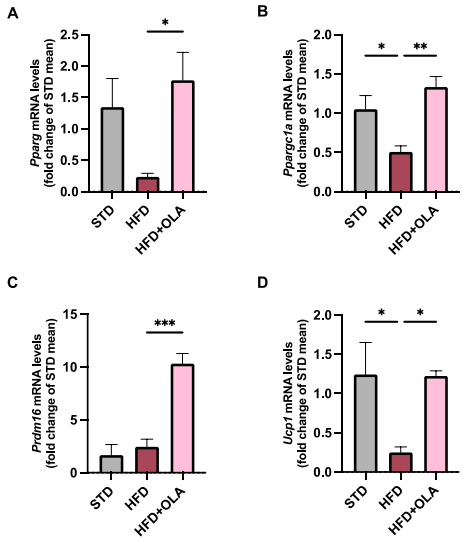


Figure 2.5. OLA induces white-to-beige fat reprogramming in scWAT of HFD mice. The mRNAs of *Pparg* (A), *Ppargc1a* (B), *Prdm16* (C) and *Ucp1* (D) in scWAT of HFD-fed mice were measured by RT-PCR (n=5-6 each group). Data are presented as mean ± SEM. *p < 0.05, ** p <0,01, and ***p<0,001.

6.2.6 Anti-inflammatory effect of OLA in scWAT of obese mice

AT expansion in obesity causes chronic tissue inflammation and the polarization of AT macrophages into the pro-inflammatory M1

phenotype; conversely, beigeing of WAT is associated to an increase of M2 macrophages. Here, OLA reduced the expression of inflammatory mediators, including TNF-α and IL-1β and increased the transcription of anti-inflammatory IL-10 (Figure 2.6A-C). OLA also increased the expression of adiponectin, an adipokine secreted by WAT and able to attenuate the inflammation response due to obesity-driven metabolic changes (Figure 2.6D). Notably, the restored expression of GLUT-4 in scWAT of obese mice by OLA (Figure 2.6E) clearly shows its capability to improve insulin sensitivity by the converging effects on inflammation and remodeling of AT.

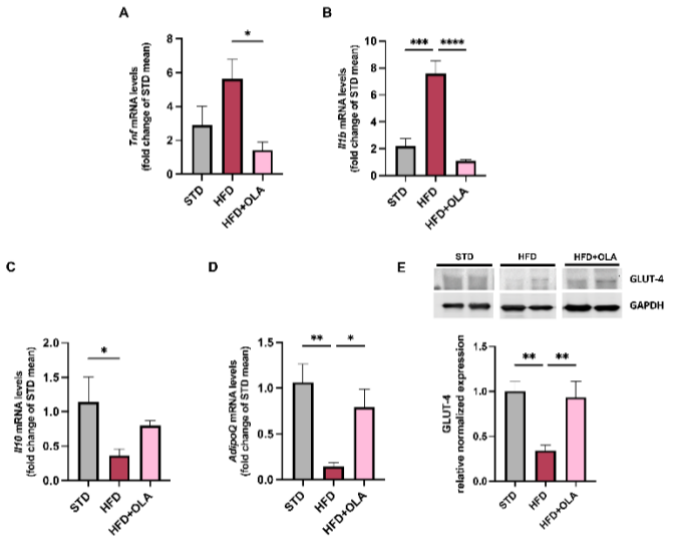


Figure 2.6. Anti-inflammatory effect of OLA in scWAT of HFD-induced insulin resistant mice. The modulation of Tnf (A), Il1b (B), Il10 (C) and Adipoq (D) mRNAs by OLA in scWAT of obese mice was shown (n=5-6 each group). Moreover, the protein expression of GLUT-4 (E) was evaluated by Western blot (n=6-7 each group). Data are presented as mean ± SEM. *p < 0.05, ** p <0,01, *** p<0,001, and ****p<0,0001.

6.3 THE IMPACT OF NAEs ON CELLULAR PATHWAYS ALTERED BY METABOLIC STRESS

Data obtained at the "Cellular Stress in Health and Disease" Lab, Department of Cellular and Molecular Biology, Karolinska Institute, Stockholm, Sweden.

6.3.1 NAEs induce autophagy in HepG2 cells, in presence or not of metabolic damage

First, we examined the possible beneficial effect of NAEs on autophagic pathway in hepatocytes. Our findings revealed that, in physiological conditions, NAEs activated autophagy, evidenced by a significant increase in LC3-II protein expression (Figure 3.1A, C), a key marker for autophagosomes, which is formed by the conjugation of cytosolic LC3-I with phosphatidylethanolamine (PE) on the surface of nascent autophagosomes.

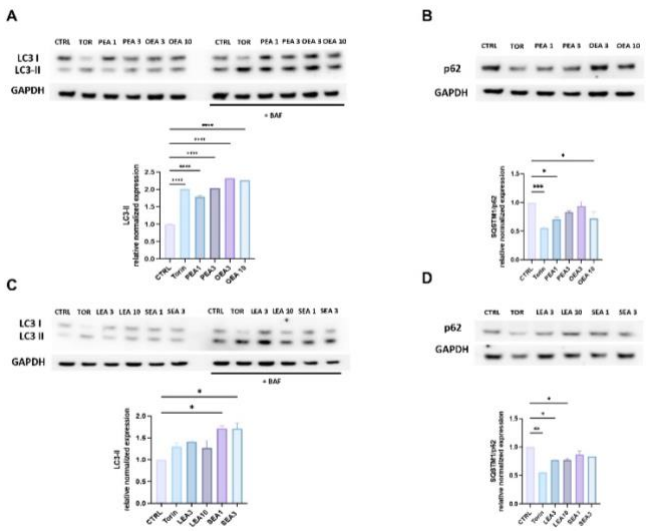


Figure 3.1. NAEs induced autophagy in hepatocarcinoma HepG2 cell line; A) PEA and OEA increased protein expression of LC3II and B) p62/SQSTM1, C) LEA and SEA increased protein expression of LC3-II and D) p62/SQSTM1. All data are shown as mean ± S.E.M. *p < 0.05., **p<0,01, ***p<0,001, ****p<0,0001.

This effect was more pronounced in the presence of bafilomycin, and there was also a decrease in p62/SQSTM1 (sequestosome 1), a protein involved in the proteasomal degradation of ubiquitinated proteins (Figure 3.1B, D).

Next, we investigated the impact of chronic exposure to saturated FAs on HepG2 cells. A 24-hour exposure to PA mimicked the pathological conditions associated with metabolic stress, resulting in reduced cell viability and the activation of the protective autophagic pathway. Specifically, a viability assay (CellTiter-Glo® Luminescent Cell Viability Assay) demonstrated a 50% reduction in the number of viable cells, which was fully restored by NAEs treatment (Figure 3.2A). Interestingly, we noticed that PA increased the expression of the conjugated form of LC3-II in hepatocytes, suggesting the activation of the autophagic pathway, probably as a protective response to lipotoxic stress. This aligns with the concept that autophagy functions as a pro-survival mechanism, protecting cells from various types of cellular stress. In this context, NAEs further induced autophagy, enhancing LC3-II expression (Figure 3.2B and C), suggesting an improvement in the cellular defense mechanisms.

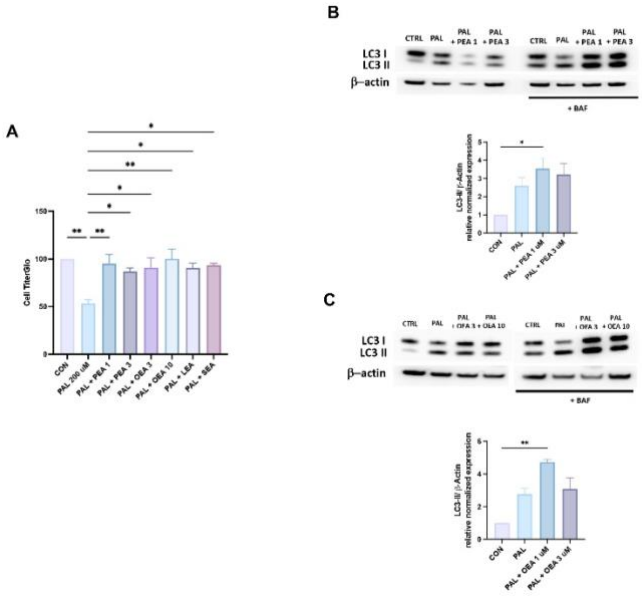


Figure 3.2. Autophagy induction by NAEs in PAL-treated HepG2 cell line; A) Effect of NAEs on PA-induced cytotoxicity, B) increasing LC3-II protein expression, C) PEA and OEA increased autophagic protective response reducing p62 protein expression. All data are shown as mean ± S.E.M. *p < 0.05., **p<0,01.

6.3.2 NAEs effect on mitophagy

We further explored NAEs effect on the selective autophagy of mitochondria (mitophagy) in SK-Mel-103 cells treated with NAEs for 24 hours, using Flow Cytometry. Our results showed that only PEA and LEA increased the mKeima ratio, shifting the emission towards wavelengths associated with acidic pH, as demonstrated by ratio upper/lower in Figure 3.3A. While no differences have been observed in OEA and SEA treated cells, suggesting a different regulation of mitophagic pathway in this cell line.

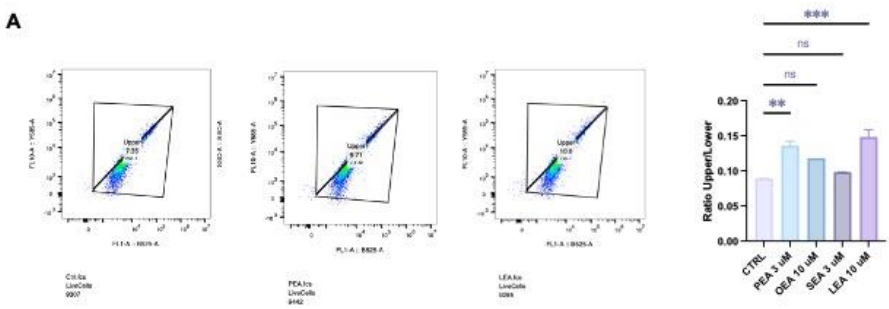


Figure 3.3. Effect of NAEs on mitophagy; A) FACS analysis of mKeima fluorescence: PEA and LEA increased mKeima emission toward specific wavelengths of acid pH. All data are shown as mean ± S.E.M. **p<0,01, ***p<0,001.

6.3.3 Effect of PEA on doxorubicin- / PA-induced senescence

In order to investigate the potential senolytic effects of NAEs, we employed a well-established model of senescence induced by DNA damage, using SK-MEL-103 cells challenged with 50 nM doxorubicin (DOXO) for 7 days. On the 6th day, the cells were treated with PEA for 24 hours. As indicated by the β-Galactosidase assay, there was no significant difference in the percentage of SA-β-gal-positive cells between PEA-treated and untreated senescent cells (Figure 3.4A).

We assessed the effect of PEA on oxidative stress associated with cellular senescence. Specifically, we measured ROS production in senescent SK-MEL-103 cells using the fluorescent probe DCFH-DA. Our results showed that DOXO-treated cells exhibited elevated ROS levels, which were significantly reduced following treatment with 3 μM PEA (Figure 3.4B). These findings suggest that PEA alleviates oxidative stress linked to senescence, without significantly affecting the senescent phenotype in this cell line.

We also established another in vitro model of senescence induced by metabolic stress, using PA in ear fibroblasts. It is well-known that

dietary FAs can act as pro-inflammatory stimuli, and growing evidence showed that senescence may be triggered by altered metabolic states (Inoue et al., 2017).

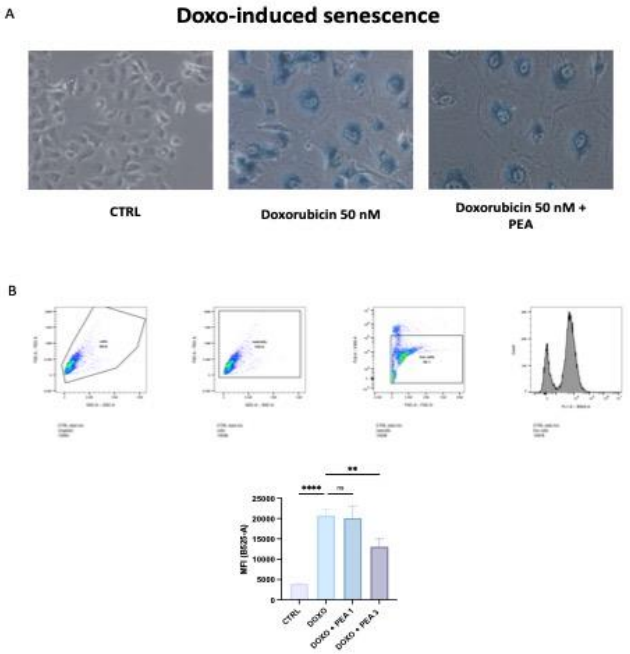


Figure 3.4. NAEs senolytic activity in DOXO-challenged cells A) β-gal staining DOXO-induced senescence on SK-MEL-103 cell line B) Measurement of ROS production using flow cytometry. All data are shown as mean ± S.E.M **p<0,01, ****p<0,0001.

First, we challenged ear fibroblasts with a high dose of PA (100 μM) for 7 days, followed by a β-Gal assay to measure the amount of active senescence-associated β-galactosidase (SA-β-gal) expressed in the cells. The PA challenge significantly increased the percentage of SA-β-gal-positive cells compared to the control group, as shown by the quantification of both the area and intensity of positive cells (Figure 3.5A).

To further confirm our model of senescence induced by a lipid overload, we evaluated the expression of KI67, a marker of cell proliferation, using flow cytometry (Figure 3.5B). PA-challenged cells exhibited a significant reduction in KI67 expression, confirming their senescent phenotype. Lastly, to analyze the effects of our compounds on senescence, we treated the cells with PEA for 24 hours following 6 days of PA challenge. We performed Real-Time qPCR to evaluate the modulation of senescence markers and SASP (senescence-associated secretory phenotype) gene expression. Our results indicated that PA-treated cells showed an increased transcription of tumor suppressor genes that can cause cell cycle arrest in senescent cells. In this context, PEA reduced the mRNA expression of p21 as well as Il-6, a pro-inflammatory cytokine linked to SASP production (Figure 3.5 C-E).

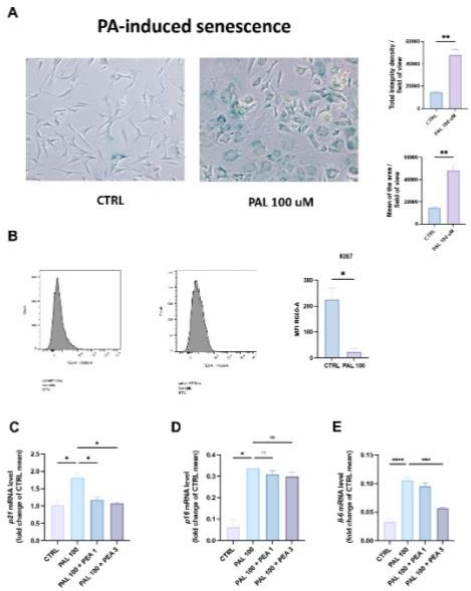


Figure 3.5. NAEs senolytic activity in PAL-challenged cells A) β-gal staining of PA-induced senescence B) KI67 expression C-E) Gene expression of SASPs and tumor suppressors.

6.4 CHANGES IN NAE LEVELS IN LIVER OF HFD FED MICE

Data obtained at Biomolecular Chemistry Institute (ICB), National Council of Research (CNR), Pozzuoli (NA), Italy.

To investigate the role of each NAE in HFD-induced obesity and related alteration of lipidic profile, we measured the levels of OEA and PEA in the liver tissue of all experimental groups using liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC-APCI-MS). Our results demonstrated a significant decrease in both AEA and OEA levels in the HFD group (Figure 4.1 A and C), whereas PEA amount was markedly elevated in obese mice (Figure 4.1 D). No significant alterations were observed in the levels of 2-arachidonoylglycerol (2-AG) between the different experimental groups (Figure 4.1 B).

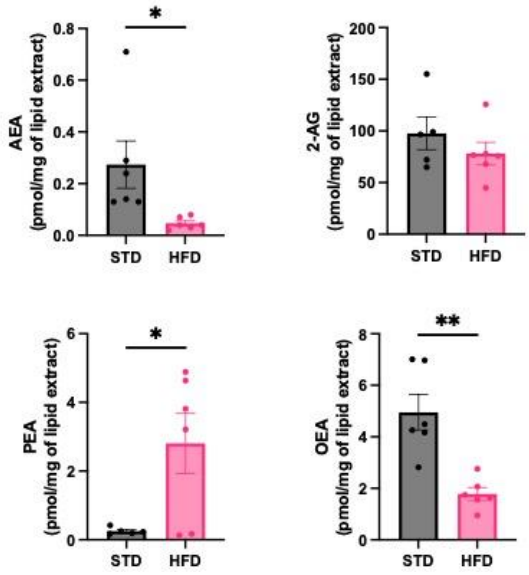


Figure 4.1. NAE levels in livers of STD and HFD mice. All data are shown as mean ± S.E.M. *p < 0.05, **p<0,01.

7. DISCUSSIONS

7.1 CO-MICRONIZED PALMITOYLETHANOLAMIDE AND RUTIN ASSOCIATED WITH HYDROXYTYROSOL RECOVER DIABESITY-INDUCED HEPATIC DYSFUNCTION IN MICE: *IN VITRO* INSIGHTS INTO THE SYNERGISTIC EFFECT

Given the therapeutic drawbacks and side effects of drugs for treating diabesity and related NAFLD or MAFLD, the exploration of new therapeutic natural compounds is needed. Therefore, we investigated the effects of NORM3, a nutritional supplement containing co-micronized PEA-Rut associated with HT, in a mouse model of diabesity induced by HFD feeding. The beneficial effects of hepatic glucose and lipid dysmetabolism, inflammation, and oxidative stress are at- tributed to the pleiotropic mode-of-action of NORM3 due to its chemical composition, which is rich in different bioactive compounds. As an insulin-sensitive organ, the liver plays a pivotal role in regulating metabolic homeostasis. In response to metabolic dysfunctions, hepatic IR is one of the early-developed pathological features, and improving IR has excellent potential for treating hyperglycemia and lipid storage in the liver (Norton et al., 2022). We report that the treatment of NORM3 significantly reduced body weight, fat mass, and IR in obese mice, conceivably due to the additive/synergistic effect of PEA-Rut with HT. Indeed, our previous data showed that PEA alone, at a higher dose (30 mg/kg) and later in time, counteracts metabolic inflexibility and hepatic mitochondrial dysfunction induced in mice by HFD, acting as a critical sensitizer of the AMPK enzyme (Annunziata et al., 2020). PEA also improved adipose tissue plasticity by restoring lipid

metabolism and promoting the white-to-brown conversion of adipocytes in obese mice (Annunziata et al., 2020).

Hepatoprotective properties and antidiabetic effects of the flavonoid Rut (used in a wide range of doses 5–100mg/kg) were previously identified (Ghorbani, 2017) and revealed to be the result of PPAR-α and antioxidant enzyme increased expression in hepatocytes (Liu et al., 2017).

While, HT (10–20mg/ kg) limited chronic inflammation and IR and reduced hepatic steatosis by the lessening of endoplasmic reticulum stress (Wang et al., 2018), nitrosative and/or oxidative stress (Vlavcheski et al., 2019), and intestinal barrier permeability (Pirozzi et al., 2016) in obese mice or rats.

The relevant outcome of this study was the demonstration that NORM3 can improve insulin sensitivity and glucose tolerance, an effect that is not entirely due to the observed reduction in body weight but also to the beneficial effects on glucose homeostasis.

Here, we first showed that NORM3 decreased HOMA-IR in HFD-fed mice and interestingly, the increased glucagon secretion by HFD was also normalized by NORM3. It is well known that glucagon hypersecretion is predictive of a worsening of glucose tolerance in diabetes (Honzawa et al., 2019). Recently, the aetiology and mechanisms of IR have been reviewed (James et al., 2021). Genetic and biochemical studies advocate a pivotal role for adipose tissue in the development of IR, conceivably by releasing lipids and other circulating factors, among which pro-inflammatory cytokines promote IR in other insulin sensitive organs, including the liver. When AT storage ability is overwhelmed, autocrine and endocrine functions of

adipose tissues are also altered. The subsequent accumulation of ectopic fat leads to lipotoxicity, promoting low-grade inflammation and IR in the liver (Rada et al., 2020). As recently reviewed, tissue-specific IR can provoke systemic IR (James et al., 2021). Indeed, several animal studies have supported the concept that metabolic or signalling alterations in one tissue can have a systemic impact and influence insulin action and its effects in other organs, as evidenced by observations in humans (Gancheva et al., 2018).

Chronic tissue inflammation considered a key feature of diabesity, is observed in insulin-target tissues, contributing to systemic meta-inflammation underlying the interplay between immunologic processes and metabolic dysfunctions (Rohm et al., 2022). In our experimental conditions, NORM3 significantly decreased serum inflammatory cytokines (TNF-α, MCP-1 IL-6) and increased the anti-inflammatory IL10. Interestingly, NORM3 also reduced resistin, a pro-inflammatory adipokine that links obesity to diabetes (Tripathi et al., 2020). Resistin induces a pro-inflammatory response involved in the development and advancement of hepatocellular carcinoma in individuals with diabetes (Abdalla, 2023).

In diabesity, the inflammatory process perturbs the intracellular concentration of several intermediates, leading to defects in cell responsiveness to insulin. Such intermediates may cause IR by inhibiting one or more of the downstream components in the insulin signalling cascade (James et al., 2021). Among others, insulin function is regulated by the activation of the insulin receptor/PI3K/AKT signalling pathway and the modulation of redox balance (Lennicke and Cocheme, 2021). In IR, a derailment in the PI3K/AKT/GLUT-4

signalling occurs in several organs, including the liver (Gao et al., 2019). Literature data indicate that the phosphorylation of AKT is also involved in the translocation of intracellular vesicles containing glucose transporter proteins to the plasma membrane, facilitating glucose utilization by tissues (Gao et al., 2019; Manning and Cantley, 2007). Here, NORM3 lessened glucose in- tolerance and IR, as shown by OGTT and ITT. Indeed, NORM3 increased the phosphorylation of insulin receptors and activated the PI3K/AKT pathway, improving hormone signalling in the liver of obese mice.

Another hallmark of IR is the inability to suppress hepatic glucose output, mainly due to a sustained elevation of gluconeogenesis (James et al., 2021). In this context, NORM3 downregulates hepatic gluconeogenesis, as shown via PTT and confirmed by the reduction of key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase, and glucose-6-phosphatase.

NORM3 exerts its hepatoprotective effects, also improving serum lipid profile (triglycerides and cholesterol) and liver enzymes (transaminases and LDH). Indeed, all components of NORM3 had previously shown lipid-lowering activities. For instance, HT suppressed the de novo synthesis and amplified the catabolism of fatty acids(Pirozzi et al., 2016). Accordingly, HT administration showed several anti-atherosclerotic effects, including the regulation of blood lipid profiles, the decrease of inflammatory mediators (Tejada et al., 2017), and the improvement of hepatic mitochondrial dysfunction induced by HFD (Ortiz et al., 2020). Our previous data (already mentioned above) demonstrated that in the same experimental model, PEA (30mg/kg/die *per o*s) increased liver AMPK phosphorylation,

reduced lipid synthesis, and induced PPAR-α expression (Annunziata et al., 2020). Rutin, as well as other phytochemicals, has been shown to improve diabetes in clinical trials not only by lowering blood glucose and reducing IR but also by ameliorating lipid profile and inhibiting inflammation and oxidative stress (Bazyar et al., 2023; Luo et al., 2023).

Here, we show that NORM3 increased the phosphorylation of AMPK, a crucial sensor of cellular metabolism, and restored the expression of CPT1, the first rate-limiting step of β-oxidation of long-chain fatty acids. AMPK has been identified as a therapeutic target for metabolic disease treatment (Day et al., 2017), leading to the phosphorylation of key metabolic mediators and transcriptional regulators, including PPARs. Indeed, AMPK activation redirects metabolism toward inhibited anabolism and increased catabolism, limiting glucose and lipid synthesis and promoting fatty acid oxidation as an energy source (Herzig and Shaw, 2018). Consistently, NORM3 markedly increased the expression of different genes involved in hepatic fatty acid oxidation (PPAR-α and CPT1) and decreased lipid transport and de novo synthesis of fatty acid (CD36 and FAS, respectively), thus improving hepatic lipid metabolism.

The modulation of genes and proteins involved in hepatic glucose and lipid metabolism and steatosis by NORM3 contributes to the improvement of IR and the anti-inflammatory effects. The role of cytokine- and enzyme-induced inflammation and their link with IR and NAFLD has been defined (Duan et al., 2022). The increasing understanding of molecules and signalling pathways involved in diabesity-driven inflammation allows the identification or

development of novel preventive and therapeutic approaches to NAFLD or MAFLD (Song et al., 2023). Here, we show that NORM3 markedly reduced the expression of inflammatory cytokines (TNF-α and IL-6), COX-2, and NFkB activation in the liver, confirming its anti-inflammatory activity, likely associated with its ability to control metabolic alterations through PPAR-α recovery.

Metabolic disarrangements, mitochondrial impairment, and oxidative stress have a crucial role in inducing hepatocyte damage and NAFLD progression (Sanches et al., 2023; Zheng et al., 2023).

Lipid peroxidation products associated with inflammatory cytokines (i.e., TNF-α) contribute to mitochondrial dysfunction, causing the enhancement of ROS production, which in turn self-sustains organelle damage. Oxidative stress is commonly associated with an imbalance between intracellular ROS levels and endogenous enzymatic and nonenzymatic antioxidants (Ezhilarasan and Lakshmi, 2022). NRF2 is a transcriptional factor that orchestrates the cellular defence mechanisms against oxidative stress induced by obesity and other redox insults (Vasileva et al., 2020). Excessive oxidative stress in the cell activates NRF2, which upregulates genes that encode major cytoprotective enzymes such as NQO1 and HO1. Recent studies showed that the activation of the NRF2/HO-1 signalling pathway could attenuate NASH via ameliorating oxidative stress (Xu et al., 2018). Moreover, NRF2 knockout mice showed a more rapid NASH development than wild-type ones on HFD (Okada et al., 2013). Several plant-derived compounds are considered NRF2 activators and hypothetically achieved as cytoprotective compounds in metabolic stress status like diabesity (Vasileva et al., 2020).

Interestingly, NORM3 significantly induced the hepatic transcription of NRF2 and its downstream target genes HO-1 and NQO1, indicating NRF2/HO-1 detoxifying system activation. Notably, the activated AKT promotes NRF2 translocation to the nucleus and then the transactivation of the downstream target genes, inhibiting oxidative stress in HFD-induced obesity (Li et al., 2020). Our results suggest the crucial role of AKT in NORM3 effects, consistently with the involvement of AKT activation in improving insulin sensitivity, regulation of gluconeogenesis, and recovery of hepatic oxidative redox balance in diabetes and obesity (Huang et al., 2018).

Finally, to investigate the synergistic effect of the components of NORM 3, we employed an *in vitro* model of H_2O_2-induced oxidative stress using the hepatocyte cell line HepG2. These cells are sensitive to oxidative stress because NADPH oxidase (NOX)- generated ROS and H_2O_2 challenge increased hepatocyte and DNA damage, causing cytotoxicity (Tu et al., 2022). Interestingly, we show that combining the compounds constituting NORM3 rather than the single components alone reduces the oxidative stress and inflammation induced by the H_2O_2 challenge in hepatocytes, which mimics *in vitro* one of the main detrimental features characterizing hepatic damage and IR.

7.2 TARGETING LIVER AND ADIPOSE TISSUE IN OBESE MICE: EFFECTS OF A N-ACYLETHANOLAMINE MIXTURE ON INSULIN RESISTANCE AND ADIPOCYTE REPROGRAMMING

Obesity results from a chronic energy imbalance, where caloric intake surpasses energy expenditure, leading to weight gain. Lipid accumulation and a positive energy balance contribute to the redistribution of fat within metabolic organs, with the interaction between the liver and adipose tissue playing a key role in maintaining lipid and glucose homeostasis (Zhao et al., 2020).

There is growing evidence supporting the protective role of each NAEs in counteracting metabolic disorders.

NAEs are bioactive lipids that play a crucial role in regulating energy balance, glucose metabolism, and fat storage. In this study, for the first time, we prove the beneficial effects of an olive oil-derived ethanolamide mixture containing more non-canonical cannabinoid NAEs, named OLALIAMID®, on body weight gain, hepatic glucose and lipid metabolism, and the reprogramming of WAT and BAT in HFD-fed obese mice. This mixture has a higher percentage of OEA and lesser of LEA, PEA, and SEA chemically derived from olive oil, keeping the ratio of the respective fatty acids (oleic, linoleic, palmitic, and stearic acids) naturally contained in olive oil. About the nutritional profile of olive oil, the major components are fatty acids (98–99%), mainly MUFA (55.3–86.5%; oleic acid and palmitoleic acid), followed by PUFA (3.5–21%; linoleic and linolenic acids), and saturated fatty acids (8–25.1%; myristic, palmitic and stearic acids) (Isaakidis et al., 2023). The antioxidant and anti-inflammatory properties of olive oil, a key component of the Mediterranean diet, are primarily attributed to

its high content of polyphenols and vitamin E, which contribute to its notable cardiovascular and metabolic benefits (Lama et al., 2017; Pirozzi et al., 2016) (Chrysant and Chrysant, 2024). Evidence suggests that obesity and its associated conditions, such as NAFLD, T2D, are linked to a chronic low-grade inflammation, the extent of which positively correlates with the severity of insulin resistance and diabetes (Fruhbeck et al., 2017; Lumeng and Saltiel, 2011). Here, obesity-driven metainflammation, characterized by the increased pro-inflammatory serum markers, such as TNF-α, IL-1β, IFN-γ, as well as LPS and MCP-1, was markedly reduced in OLA-treated animals. LPS is a key factor contributing to the meta-inflammation associated with HFD feeding, as it triggers the induction of inflammatory cytokines by immune cells and adipocytes (Rohm et al., 2022). We demonstrated that OLA reduced body weight gain and fat mass in obese mice, decreased hyperglycemia and insulin resistance (as indicated by HOMA-IR), and improved the ability of obese mice to regulate blood glucose levels. The key implication of insulin action in hormone systemic effects and AT remodelling was recently reviewed (Santoro et al., 2021), connecting adipocyte metabolism to the regulation of other tissues important for metabolic homeostasis, including liver. The systemic reduction in IR observed in OLA-treated mice is supported by the hepatic activation of AKT and AMPK, key kinases involved in regulating insulin sensitivity and energy homeostasis. Consistent with these findings, OLA reduced the expression of CD36 and limited hepatic de novo lipogenesis by decreasing the transcription of FAS and PPAR-γ, thereby improving liver lipid metabolism (Zhou et al., 2008). Consistently, previous studies showed that in liver adipogenesis-

related genes, PPAR-γ and its targeted gene CD36, are implicated in the HFD-induced steatosis rather than fatty acid oxidation and synthesis (Wilson et al., 2016). Liver-specific disruption of PPAR-γ in leptin-deficient (*ob/ob*) obese mice was associated with a marked improvement of fatty liver as compared to wild-type *ob/ob* mice through a decrease of hepatic TG levels (Matsusue et al., 2003). Here, serum lipid profile (triglycerides and cholesterol) and liver enzymes (transaminases and LDH), significantly increased by HFD, were markedly improved by OLA treatment; similarly, PAI-1, a marker for the progression of metabolic diseases (Wang et al., 2018), was normalized. Long-term obesity leads to a dysfunctional AT and hence the alteration of adipokines, which play a pivotal role not only in metabolic functions (i.e., the regulation of satiety, fat distribution, insulin sensitivity and secretion, energy expenditure), but also in meta-inflammation. OLA restored the levels of serum leptin and adiponectin, two adipokines that inversely regulate glucose and lipid homeostasis through AMPK signaling and fatty acid metabolism in the liver (Jeon, 2016). In both clinical (Fruhbeck et al., 2018) and experimental conditions (Annunziata et al., 2022; Lama et al., 2022) of mild and severe obesity, circulating leptin lost its adipostatic effects, suggestive of leptin resistance (Konner and Bruning, 2012; Meli et al., 2004). Leptin positively correlates with adiposity, providing a mechanistic link between inflammation and obesity-associated metabolic dysfunction (Kiernan and MacIver, 2020). In contrast, adiponectin expression in AT and serum decreases in obese individuals, contributing to the development of dysfunctional AT due to unresolved inflammation (Berezin et al., 2020). Indeed, adiponectin protects

against IR and excessive hepatic lipid accumulation, while also exerting anti-inflammatory effects and demonstrating cardioprotective properties (Stern et al., 2016). Therefore, the leptin/adiponectin ratio, as a marker of AT dysfunction, has been proposed as a surrogate measure of IR, alongside HOMA (Fruhbeck et al., 2017). Considering the interplay between liver and AT, we demonstrated that OLA also targets BAT and WAT functions. Brown adipocytes use FFAs and glucose as fuels, combusting macronutrients to generate heat by thermogenesis, which is aimed mainly by UCP1 (Srivastava and Veech, 2019). This thermogenic activity is impaired in the context of lipid overnutrition or obesity (Cheng et al., 2021). Currently, research focused on identifying the key genes that regulate the differentiation, development, and reprogramming of AT is gaining significant interest due to the potential therapeutic benefits of inducing BAT thermogenesis and/or promoting the beigeing of WAT in the treatment of obesity and related metabolic disorders (Srivastava and Veech, 2019) (Morigny et al., 2021). In this field of research, PRDM16, PPAR-γ, and PGC-1α have been identified as the key nodes in the regulation of adipocyte remodeling. PRDM16 has been identified as a transcriptional activator of the brown fat gene program and a repressor of the WAT-specific gene program (Inagaki et al., 2016). PPAR-γ, mainly expressed in AT, regulates the development and function of fat cells (i.e. adipogenesis and lipogenesis) and their capacity to store lipids (Ma et al., 2018). PGC-1α, a coactivator of PPAR-γ, induces the expression of genes that regulate energy metabolism in AT, promote the differentiation of preadipocytes to brown adipocytes, and the recruitment/trans-differentiation of beige adipocytes (Bostrom et al.,

2012). UCP-1 and CIDEA, apart from their involvement in the thermogenic responses of BAT, can also be used as markers of beigeing in mouse WAT (Garcia et al., 2016). Our data showed that OLA treatment promoted the iBAT thermogenic adaptation to the excessive energy gained from the HFD, inducing the expression of thermogenic genes (*Prdm16, Ucp1, Cidea and Pgc1a, Cox8b*). The beneficial effect of OLA on AT was confirmed by the white-to-beige reprogramming in scWAT, since the beigeing of this tissue is frequently associated with an improvement of metabolic function and insulin sensitivity. Furthermore, OLA significantly reduced the gene expression of the batokine IL-1β in iBAT, as well as the inflammation-associated hormone/cytokine GDF15. Although IL-1β is predominantly produced by innate immune cells, with minimal production in adipocytes, it plays a crucial role in AT inflammation, which subsequently drives systemic inflammation and IR in obese individuals (Chen et al., 2023). Notably, for the first time, we showed the detrimental implication of IL-1β in iBAT following chronic metabolic stress due to prolonged HFD feeding in obese mice. GDF15 is a cytokine belonging to the transforming growth factor beta superfamily with anorectic activity (Wang et al., 2021). Interestingly, serum concentrations of GDF15 increase during obesity in both patients (Vila et al., 2011) and mice (Patel et al., 2019). Our results indicate that GDF15 expression increases following prolonged high-fat feeding, not only in the liver and WAT, but also, more notably, in BAT (Patel et al., 2019). All types of adipose depots typically contain immune cells that surveil and maintain the function and hormonal sensitivity of adipocytes. In obesity, the percentage of resident

macrophages in the AT is increased, with polarization toward the M1 phenotype in AT of both rodents and humans (Yao et al., 2022). M1 phenotype is a hallmark of AT inflammation and is associated with the development of IR and metabolic diseases (Haase et al., 2014). The AT is the main cytokine source in severe obesity rather than the liver, which is the main target for AT-derived inflammatory mediators leading to hepatic damage. Conversely to the resolution of liver inflammation upon weight loss, AT seems to have an obesogenic memory and retains its inflammatory state despite weight loss in response to extreme conditions such as bariatric surgery (Schmitz et al., 2016). Several studies have highlighted that M2 polarization occurs during WAT browning (Reilly and Saltiel, 2017). Our data suggest that OLA capability in decreasing inflammatory cytokines and increasing anti-inflammatory mediators can be predictive of the M1/M2 macrophage shift. Finally, the anti-inflammatory effect of OLA and the increased expression of glucose transporter GLUT-4 in scWAT confirmed the restored insulin sensitivity of this tissue. GLUT-4 is the most abundant glucose transporter in adipose cells, and studies using knockout or overexpressing glucose transporters have shown the key role of AT in glucose homeostasis and organ crosstalk (Chadt and Al-Hasani, 2020). Accordingly, IR, as well as increased inflammation, causes a diminution in GLUT-4 expression in adipocytes of obese mice and patients (Favaretto et al., 2014; Kouidhi et al., 2013), while its overexpression improves insulin sensitivity (Chadt and Al-Hasani, 2020).

7.3 THE IMPACT OF NAES ON CELLULAR PATHWAYS ALTERED BY METABOLIC STRESS

Another key aspect to consider in the context of obesity, at the cellular level, is that the excessive and prolonged lipid exposure triggers the chronic activation of adaptive stress mechanisms (Wellen and Thompson, 2010). As result, cells trigger senescence and oxidative stress, associated with an alteration of beneficial pathways including autophagic pathways (Faraonio, 2022; Kaushik and Cuervo, 2018).

Typically, cells use these mechanisms to cope with stress, but when persistently activated, they can become maladaptive, leading to dysfunction or disease (Wellen and Thompson, 2010).

Indeed, nutrient excess can push cells into a "chronic stress" state, where these adaptive mechanisms become dysregulated, contributing to aging or the development of diseases such as metabolic disorders, cancer, and neurodegeneration(Wellen and Thompson, 2010). Notably, targeting stress-related cellular responses such as senescence or autophagy has recently garnered significant attention from researchers and clinicians as a strategy to prevent or treat various age-related diseases, including metabolic disorders. This approach focuses on restoring cellular homeostasis as basis for therapeutic interventions.

Our *in vitro* results in HepG2 cell line showed that NAEs can induce these protective mechanisms, such as autophagy and mitophagy. Specifically, all NAEs induced the activation of autophagy, increasing the expression of LC3-II, a standard marker for autophagosomes in presence of bafilomycin, and reducing the proteasomal degradation of ubiquitinated proteins, as shown by decreased p62/SQSTM1 (sequestosome 1) protein expression.

In addition, we mimicked the pathological context linked to metabolic stress *in vitro*, exposing HepG2 cells to the saturated fatty acid PA. We demonstrated that 100 uM PA reduced cell viability and induced the compensatory activation of autophagic pathway.

Notably, NAE treatment was able to further increase LC3-II expression suggesting the potential protective effect of these compounds in enhancing the degradation and recycling processes involved in the maintenance of cellular homeostasis. This effect requires further investigation to better understand the beneficial role of NAEs in enhancing cellular homeostasis and improving metabolic alterations.

Very few research studies described *in vitro* models of senescence induced by metabolic damage. Therefore, we first established a new model of senescence induced by stimulation with PA for 7 days, demonstrating the ability of this saturated fatty acid to increase β-galactosidase activity.

Then, we started focusing our attention on PEA (the most studied NAE) possible effect in PA-induced senescence and demonstrated that can reduce the expression of p21 and p16 as well as Il-6 one of the main cytokines involved in SASP senescence related production. Considering and targeting these pathways in the context of metabolic damage associated with obesity could offer an alternative and expanded therapeutic application for NAEs. However, further research is required to elucidate these mechanisms in other cell populations within metabolically active tissues, such as the adipose organ, where NAEs can exert their beneficial effects.

7.4 CHANGES IN NAE LEVELS IN LIVER OF HFD FED MICE

Finally, we examined the expression of NAEs in the livers of mice with obesity, and our findings reveal distinct patterns of modulation for OEA, PEA, and AEA in this experimental model of diabesity. These findings suggest that the liver activates distinct compensatory synthetic pathways in response to lipid overnutrition, highlighting its adaptive mechanisms for managing excessive fat intake. Interestingly, we observed a significant reduction in OEA levels in the livers of mice chronically fed HFD. This decrease suggests that the liver may become less efficient in synthesizing the bioactive lipid OEA under conditions of chronic obesity. Given the known beneficial effects of OEA in regulating lipid metabolism, inflammation, and energy homeostasis, these findings support our idea that supplementation with OEA could help to restore its physiological balance and potentially ameliorate obesity-related metabolic dysfunctions.

These preliminary findings support our idea that targeting OEA as a therapeutic intervention could represent a promising strategy to prevent or mitigate the progression of metabolic alterations and diseases.

8. CONCLUSIONS

Based on our results, NAEs can be considered a therapeutic tool to improve metabolic flexibility impaired by diabesity. The involvement of AMPK in metabolic effects of these endogenous lipids identifies these molecules as lipid and glucose metabolic modulators and regulators, relevant in the improvement of liver insulin sensitivity. The beneficial effects of NAEs alone or in combination may be the result of multiple converging metabolic mechanisms, behind their well-known anti-inflammatory effect, an activity shared with other canonical endocannabinoids (i.e AEA and 2AG). Moreover, our data identifies NAE mixture as a strategy to promote the conversion of energy-storing white adipocytes into energy-consuming brown-like adipocytes. The metabolic, thermogenic, and anti-inflammatory effects of NAEs on adipose tissue reprogramming are the results of several converging mechanisms which may contribute to the weight and fat mass loss, the overcoming of leptin resistance and the recovery of adipose tissue function and plasticity.

Indeed, NAEs association can synergically contribute to the restoration of metabolic homeostasis targeting liver-adipose tissue axis and to ameliorate the altered lipid and glucose dysmetabolism induced by obesity and related diseases.

9. REFERENCES

Abdalla, M. M. I., 2023. Serum resistin and the risk for hepatocellular carcinoma in diabetic patients. World J Gastroenterol. 29**,** 4271-4288. 10.3748/wjg.v29.i27.4271.

Abdou, H. M., Hamaad, F. A., Ali, E. Y., Ghoneum, M. H., 2022. Antidiabetic efficacy of Trifolium alexandrinum extracts hesperetin and quercetin in ameliorating carbohydrate metabolism and activating IR and AMPK signaling in the pancreatic tissues of diabetic rats. Biomed Pharmacother. 149**,** 112838. 10.1016/j.biopha.2022.112838.

Affinati, A. H., Myers, M. G., Jr., Neuroendocrine Control of Body Energy Homeostasis. In: K. R. Feingold, et al., Eds.), Endotext, South Dartmouth (MA), 2000.

Ahern, G. P., 2003. Activation of TRPV1 by the satiety factor oleoylethanolamide. J Biol Chem. 278**,** 30429-34. 10.1074/jbc.M305051200.

Ahn, K., Johnson, D. S., Cravatt, B. F., 2009. Fatty acid amide hydrolase as a potential therapeutic target for the treatment of pain and CNS disorders. Expert Opin Drug Discov. 4**,** 763-784. 10.1517/17460440903018857.

Al-Goblan, A. S., Al-Alfi, M. A., Khan, M. Z., 2014. Mechanism linking diabetes mellitus and obesity. Diabetes Metab Syndr Obes. 7**,** 587-91. 10.2147/DMSO.S67400.

Alam, S., Fahim, S. M., 2021. Transition of an acronym from nonalcoholic fatty liver disease to metabolic dysfunction-associated fatty liver disease. World J Hepatol. 13**,** 1203-1207. 10.4254/wjh.v13.i10.1203.

Alfadda, N. A., Aljuraiban, G. S., Awwad, H. M., Khaleel, M. S., Almaghamsi, A. M., Sherbeeni, S. M., Alqutub, A. N., Aldosary, A. S., Alfadda, A. A., 2022. Higher carbohydrate intake in relation to non-alcoholic fatty liver disease in patients with type 2 diabetes. Front Nutr. 9**,** 996004. 10.3389/fnut.2022.996004.

Alhouayek, M., Muccioli, G. G., 2014. Harnessing the anti-inflammatory potential of palmitoylethanolamide. Drug Discov Today. 19**,** 1632-9. 10.1016/j.drudis.2014.06.007.

Aloe, L., Leon, A., Levi-Montalcini, R., 1993. A proposed autacoid mechanism controlling mastocyte behaviour. Agents Actions. 39 Spec No**,** C145-7. 10.1007/BF01972748.

American Diabetes, A., 2010. Diagnosis and classification of diabetes mellitus. Diabetes Care. 33 Suppl 1**,** S62-9. 10.2337/dc10-S062.

American Diabetes Association Professional Practice, C., 2024. 8. Obesity and Weight Management for the Prevention and Treatment of Type 2 Diabetes: Standards of Care in Diabetes-2024. Diabetes Care. 47**,** S145-S157. 10.2337/dc24-S008.

Annunziata, C., Lama, A., Pirozzi, C., Cavaliere, G., Trinchese, G., Di Guida, F., Nitrato Izzo, A., Cimmino, F., Paciello, O., De Biase, D., Murru, E., Banni, S., Calignano, A., Mollica, M. P., Mattace Raso, G., Meli, R., 2020. Palmitoylethanolamide counteracts hepatic metabolic inflexibility modulating mitochondrial function and efficiency in diet-induced obese mice. FASEB J. 34**,** 350-364. 10.1096/fj.201901510RR.

Annunziata, C., Pirozzi, C., Lama, A., Senzacqua, M., Comella, F., Bordin, A., Monnolo, A., Pelagalli, A., Ferrante, M. C., Mollica, M. P., Iossa, A., De Falco, E., Mattace Raso, G., Cinti, S., Giordano, A., Meli, R., 2022. Palmitoylethanolamide Promotes White-to-Beige Conversion and Metabolic Reprogramming of Adipocytes: Contribution of PPAR-alpha. Pharmaceutics. 14. 10.3390/pharmaceutics14020338.

Arner, P., Ryden, M., 2022. Human white adipose tissue: A highly dynamic metabolic organ. J Intern Med. 291**,** 611-621. 10.1111/joim.13435.

Au-Yong, I. T., Thorn, N., Ganatra, R., Perkins, A. C., Symonds, M. E., 2009. Brown adipose tissue and seasonal variation in humans. Diabetes. 58**,** 2583-7. 10.2337/db09-0833.

Balistreri, C. R., Caruso, C., Candore, G., 2010. The role of adipose tissue and adipokines in obesity-related inflammatory diseases. Mediators Inflamm. 2010**,** 802078. 10.1155/2010/802078.

Battaglia, G. M., Zheng, D., Hickner, R. C., Houmard, J. A., 2012. Effect of exercise training on metabolic flexibility in response to a high-fat diet in obese individuals. Am J Physiol Endocrinol Metab. 303**,** E1440-5. 10.1152/ajpendo.00355.2012.

Bays, H. E., Gonzalez-Campoy, J. M., Bray, G. A., Kitabchi, A. E., Bergman, D. A., Schorr, A. B., Rodbard, H. W., Henry, R. R., 2008. Pathogenic potential of adipose tissue and metabolic consequences of adipocyte hypertrophy and increased visceral adiposity. Expert Rev Cardiovasc Ther. 6**,** 343-68. 10.1586/14779072.6.3.343.

Bazyar, H., Moradi, L., Zaman, F., Zare Javid, A., 2023. The effects of rutin flavonoid supplement on glycemic status, lipid profile, atherogenic index of plasma, brain-derived neurotrophic factor (BDNF), some serum inflammatory, and oxidative stress factors in patients with type 2 diabetes mellitus: A double-blind, placebo-controlled trial. Phytother Res. 37**,** 271-284. 10.1002/ptr.7611.

Bean, M. K., Stewart, K., Olbrisch, M. E., 2008. Obesity in America: implications for clinical and health psychologists. J Clin Psychol Med Settings. 15**,** 214-24. 10.1007/s10880-008-9124-9.

Bellentani, S., Scaglioni, F., Marino, M., Bedogni, G., 2010. Epidemiology of non-alcoholic fatty liver disease. Dig Dis. 28**,** 155-61. 10.1159/000282080.

Berezin, A. E., Berezin, A. A., Lichtenauer, M., 2020. Emerging Role of Adipocyte Dysfunction in Inducing Heart Failure Among Obese Patients With Prediabetes and Known Diabetes Mellitus. Front Cardiovasc Med. 7**,** 583175. 10.3389/fcvm.2020.583175.

Bernal-Chico, A., Tepavcevic, V., Manterola, A., Utrilla, C., Matute, C., Mato, S., 2023. Endocannabinoid signaling in brain diseases: Emerging relevance of glial cells. Glia. 71**,** 103-126. 10.1002/glia.24172.

Bertelli, M., Kiani, A. K., Paolacci, S., Manara, E., Kurti, D., Dhuli, K., Bushati, V., Miertus, J., Pangallo, D., Baglivo, M., Beccari, T., Michelini, S., 2020. Hydroxytyrosol: A natural compound with promising pharmacological activities. J Biotechnol. 309**,** 29-33. 10.1016/j.jbiotec.2019.12.016.

Biddinger, S. B., Kahn, C. R., 2006. From mice to men: insights into the insulin resistance syndromes. Annu Rev Physiol. 68**,** 123-58. 10.1146/annurev.physiol.68.040104.124723.

Bienboire-Frosini, C., Wang, D., Marcet-Rius, M., Villanueva-Garcia, D., Gazzano, A., Dominguez-Oliva, A., Olmos-Hernandez, A., Hernandez-Avalos, I., Lezama-Garcia, K., Verduzco-Mendoza, A., Gomez-Prado, J., Mota-Rojas, D., 2023. The Role of Brown Adipose Tissue and Energy Metabolism in Mammalian Thermoregulation during the Perinatal Period. Animals (Basel). 13. 10.3390/ani13132173.

Bilal, R. M., Liu, C., Zhao, H., Wang, Y., Farag, M. R., Alagawany, M., Hassan, F. U., Elnesr, S. S., Elwan, H. A. M., Qiu, H., Lin, Q., 2021. Olive Oil: Nutritional Applications, Beneficial

Health Aspects and its Prospective Application in Poultry Production. Front Pharmacol. 12**,** 723040. 10.3389/fphar.2021.723040.

Bjorndal, B., Burri, L., Staalesen, V., Skorve, J., Berge, R. K., 2011. Different adipose depots: their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents. J Obes. 2011**,** 490650. 10.1155/2011/490650.

Blondin, D. P., Nielsen, S., Kuipers, E. N., Severinsen, M. C., Jensen, V. H., Miard, S., Jespersen, N. Z., Kooijman, S., Boon, M. R., Fortin, M., Phoenix, S., Frisch, F., Guerin, B., Turcotte, E. E., Haman, F., Richard, D., Picard, F., Rensen, P. C. N., Scheele, C., Carpentier, A. C., 2020. Human Brown Adipocyte Thermogenesis Is Driven by beta2-AR Stimulation. Cell Metab. 32**,** 287-300 e7. 10.1016/j.cmet.2020.07.005.

Borrelli, F., Izzo, A. A., 2009. Role of acylethanolamides in the gastrointestinal tract with special reference to food intake and energy balance. Best Pract Res Clin Endocrinol Metab. 23**,** 33-49. 10.1016/j.beem.2008.10.003.

Bostrom, P., Wu, J., Jedrychowski, M. P., Korde, A., Ye, L., Lo, J. C., Rasbach, K. A., Bostrom, E. A., Choi, J. H., Long, J. Z., Kajimura, S., Zingaretti, M. C., Vind, B. F., Tu, H., Cinti, S., Hojlund, K., Gygi, S. P., Spiegelman, B. M., 2012. A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature. 481**,** 463-8. 10.1038/nature10777.

Brown, J. D., Karimian Azari, E., Ayala, J. E., 2017. Oleoylethanolamide: A fat ally in the fight against obesity. Physiol Behav. 176**,** 50-58. 10.1016/j.physbeh.2017.02.034.

Burton, D. G. A., Faragher, R. G. A., 2018. Obesity and type-2 diabetes as inducers of premature cellular senescence and ageing. Biogerontology. 19**,** 447-459. 10.1007/s10522-018-9763-7.

Buzzetti, E., Pinzani, M., Tsochatzis, E. A., 2016. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism. 65**,** 1038-48. 10.1016/j.metabol.2015.12.012.

Calignano, A., La Rana, G., Giuffrida, A., Piomelli, D., 1998. Control of pain initiation by endogenous cannabinoids. Nature. 394**,** 277-81. 10.1038/28393.

Capasso, R., Izzo, A. A., Fezza, F., Pinto, A., Capasso, F., Mascolo, N., Di Marzo, V., 2001. Inhibitory effect of

palmitoylethanolamide on gastrointestinal motility in mice. Br J Pharmacol. 134**,** 945-50. 10.1038/sj.bjp.0704339.

Chadt, A., Al-Hasani, H., 2020. Glucose transporters in adipose tissue, liver, and skeletal muscle in metabolic health and disease. Pflugers Arch. 472**,** 1273-1298. 10.1007/s00424-020-02417-x.

Chen, J. L., Feng, Z. L., Zhou, F., Lou, R. H., Peng, C., Ye, Y., Lin, L. G., 2023. 14-Deoxygarcinol improves insulin sensitivity in high-fat diet-induced obese mice via mitigating NF-kappaB/Sirtuin 2-NLRP3-mediated adipose tissue remodeling. Acta Pharmacol Sin. 44**,** 434-445. 10.1038/s41401-022-00958-8.

Chen, S. X., Zhang, L. J., Gallo, R. L., 2019. Dermal White Adipose Tissue: A Newly Recognized Layer of Skin Innate Defense. J Invest Dermatol. 139**,** 1002-1009. 10.1016/j.jid.2018.12.031.

Chen, Z., Tian, R., She, Z., Cai, J., Li, H., 2021. Corrigendum to "Role of oxidative stress in the pathogenesis of nonalcoholic fatty liver disease" [Free Radic. Biol. Med. 152 (2020) 116-141]. Free Radic Biol Med. 162**,** 174. 10.1016/j.freeradbiomed.2020.06.011.

Cheng, L., Wang, J., Dai, H., Duan, Y., An, Y., Shi, L., Lv, Y., Li, H., Wang, C., Ma, Q., Li, Y., Li, P., Du, H., Zhao, B., 2021. Brown and beige adipose tissue: a novel therapeutic strategy for obesity and type 2 diabetes mellitus. Adipocyte. 10**,** 48-65. 10.1080/21623945.2020.1870060.

Cheng, Z., White, M. F., 2011. Targeting Forkhead box O1 from the concept to metabolic diseases: lessons from mouse models. Antioxid Redox Signal. 14**,** 649-61. 10.1089/ars.2010.3370.

Choi, A. M., Ryter, S. W., Levine, B., 2013. Autophagy in human health and disease. N Engl J Med. 368**,** 1845-6. 10.1056/NEJMc1303158.

Chrysant, S. G., Chrysant, G. S., 2024. Olive Oil Consumption and Cardiovascular Protection: Mechanism of Action. Cardiol Rev. 32**,** 57-61. 10.1097/CRD.0000000000000449.

Cinti, S., 2009. Transdifferentiation properties of adipocytes in the adipose organ. Am J Physiol Endocrinol Metab. 297**,** E977-86. 10.1152/ajpendo.00183.2009.

Cinti, S., 2012. The adipose organ at a glance. Dis Model Mech. 5**,** 588-94. 10.1242/dmm.009662.

Ciumarnean, L., Milaciu, M. V., Runcan, O., Vesa, S. C., Rachisan, A. L., Negrean, V., Perne, M. G., Donca, V. I., Alexescu, T. G.,

Para, I., Dogaru, G., 2020. The Effects of Flavonoids in Cardiovascular Diseases. Molecules. 25. 10.3390/molecules25184320.

Colditz, G. A., Willett, W. C., Rotnitzky, A., Manson, J. E., 1995. Weight gain as a risk factor for clinical diabetes mellitus in women. Ann Intern Med. 122**,** 481-6. 10.7326/0003-4819-122-7-199504010-00001.

Collaborators, G. U. O. F., 2024. National-level and state-level prevalence of overweight and obesity among children, adolescents, and adults in the USA, 1990-2021, and forecasts up to 2050. Lancet. 404**,** 2278-2298. 10.1016/S0140-6736(24)01548-4.

Comella, F., Lama, A., Pirozzi, C., Annunziata, C., Piegari, G., Sodano, F., Melini, S., Paciello, O., Lago Paz, F., Meli, R., Mattace Raso, G., 2024. Oleoylethanolamide attenuates acute-to-chronic kidney injury: in vivo and in vitro evidence of PPAR-alpha involvement. Biomed Pharmacother. 171**,** 116094. 10.1016/j.biopha.2023.116094.

Cory, H., Passarelli, S., Szeto, J., Tamez, M., Mattei, J., 2018. The Role of Polyphenols in Human Health and Food Systems: A Mini-Review. Front Nutr. 5**,** 87. 10.3389/fnut.2018.00087.

Cypess, A. M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A. B., Kuo, F. C., Palmer, E. L., Tseng, Y. H., Doria, A., Kolodny, G. M., Kahn, C. R., 2009. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 360**,** 1509-17. 10.1056/NEJMoa0810780.

Dalle Carbonare, M., Del Giudice, E., Stecca, A., Colavito, D., Fabris, M., D'Arrigo, A., Bernardini, D., Dam, M., Leon, A., 2008. A saturated N-acylethanolamine other than N-palmitoyl ethanolamine with anti-inflammatory properties: a neglected story. J Neuroendocrinol. 20 Suppl 1**,** 26-34. 10.1111/j.1365-2826.2008.01689.x.

Day, E. A., Ford, R. J., Steinberg, G. R., 2017. AMPK as a Therapeutic Target for Treating Metabolic Diseases. Trends Endocrinol Metab. 28**,** 545-560. 10.1016/j.tem.2017.05.004.

de Pablos, R. M., Espinosa-Oliva, A. M., Hornedo-Ortega, R., Cano, M., Arguelles, S., 2019. Hydroxytyrosol protects from aging process via AMPK and autophagy; a review of its effects on cancer, metabolic syndrome, osteoporosis, immune-mediated

and neurodegenerative diseases. Pharmacol Res. 143**,** 58-72. 10.1016/j.phrs.2019.03.005.

De Petrocellis, L., Davis, J. B., Di Marzo, V., 2001. Palmitoylethanolamide enhances anandamide stimulation of human vanilloid VR1 receptors. FEBS Lett. 506**,** 253-6. 10.1016/s0014-5793(01)02934-9.

De Santis, S., Cariello, M., Piccinin, E., Sabba, C., Moschetta, A., 2019. Extra Virgin Olive Oil: Lesson from Nutrigenomics. Nutrients. 11. 10.3390/nu11092085.

Debnath, J., Gammoh, N., Ryan, K. M., 2023. Autophagy and autophagy-related pathways in cancer. Nat Rev Mol Cell Biol. 24**,** 560-575. 10.1038/s41580-023-00585-z.

Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., Mechoulam, R., 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science. 258**,** 1946-9. 10.1126/science.1470919.

Devarshi, P. P., Jangale, N. M., Ghule, A. E., Bodhankar, S. L., Harsulkar, A. M., 2013. Beneficial effects of flaxseed oil and fish oil diet are through modulation of different hepatic genes involved in lipid metabolism in streptozotocin-nicotinamide induced diabetic rats. Genes Nutr. 8**,** 329-42. 10.1007/s12263-012-0326-2.

Di Cesare Mannelli, L., D'Agostino, G., Pacini, A., Russo, R., Zanardelli, M., Ghelardini, C., Calignano, A., 2013. Palmitoylethanolamide is a disease-modifying agent in peripheral neuropathy: pain relief and neuroprotection share a PPAR-alpha-mediated mechanism. Mediators Inflamm. 2013**,** 328797. 10.1155/2013/328797.

Di Lorenzo, C., Colombo, F., Biella, S., Stockley, C., Restani, P., 2021. Polyphenols and Human Health: The Role of Bioavailability. Nutrients. 13. 10.3390/nu13010273.

Di Marzo, V., Goparaju, S. K., Wang, L., Liu, J., Batkai, S., Jarai, Z., Fezza, F., Miura, G. I., Palmiter, R. D., Sugiura, T., Kunos, G., 2001. Leptin-regulated endocannabinoids are involved in maintaining food intake. Nature. 410**,** 822-5. 10.1038/35071088.

Diep, T. A., Madsen, A. N., Holst, B., Kristiansen, M. M., Wellner, N., Hansen, S. H., Hansen, H. S., 2011. Dietary fat decreases

intestinal levels of the anorectic lipids through a fat sensor. FASEB J. 25**,** 765-74. 10.1096/fj.10-166595.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., et al., 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A. 92**,** 9363-7. 10.1073/pnas.92.20.9363.

DiPatrizio, N. V., Astarita, G., Schwartz, G., Li, X., Piomelli, D., 2011. Endocannabinoid signal in the gut controls dietary fat intake. Proc Natl Acad Sci U S A. 108**,** 12904-8. 10.1073/pnas.1104675108.

Dobrzyn, A., Ntambi, J. M., 2005. Stearoyl-CoA desaturase as a new drug target for obesity treatment. Obes Rev. 6**,** 169-74. 10.1111/j.1467-789X.2005.00177.x.

Donaldson, P. T., 2004. Genetics of liver disease: immunogenetics and disease pathogenesis. Gut. 53**,** 599-608. 10.1136/gut.2003.031732.

Duan, Y., Pan, X., Luo, J., Xiao, X., Li, J., Bestman, P. L., Luo, M., 2022. Association of Inflammatory Cytokines With Non-Alcoholic Fatty Liver Disease. Front Immunol. 13**,** 880298. 10.3389/fimmu.2022.880298.

Dugani, C. B., Klip, A., 2005. Glucose transporter 4: cycling, compartments and controversies. EMBO Rep. 6**,** 1137-42. 10.1038/sj.embor.7400584.

Duwaerts, C. C., Maher, J. J., 2019. Macronutrients and the Adipose-Liver Axis in Obesity and Fatty Liver. Cell Mol Gastroenterol Hepatol. 7**,** 749-761. 10.1016/j.jcmgh.2019.02.001.

Erichsen, J. M., Fadel, J. R., Reagan, L. P., 2022. Peripheral versus central insulin and leptin resistance: Role in metabolic disorders, cognition, and neuropsychiatric diseases. Neuropharmacology. 203**,** 108877. 10.1016/j.neuropharm.2021.108877.

Eslam, M., Newsome, P. N., Sarin, S. K., Anstee, Q. M., Targher, G., Romero-Gomez, M., Zelber-Sagi, S., Wai-Sun Wong, V., Dufour, J. F., Schattenberg, J. M., Kawaguchi, T., Arrese, M., Valenti, L., Shiha, G., Tiribelli, C., Yki-Jarvinen, H., Fan, J. G., Gronbaek, H., Yilmaz, Y., Cortez-Pinto, H., Oliveira, C. P., Bedossa, P., Adams, L. A., Zheng, M. H., Fouad, Y., Chan, W. K., Mendez-Sanchez, N., Ahn, S. H., Castera, L., Bugianesi, E., Ratziu, V., George, J., 2020. A new definition for metabolic

dysfunction-associated fatty liver disease: An international expert consensus statement. J Hepatol. 73**,** 202-209. 10.1016/j.jhep.2020.03.039.

Ezhilarasan, D., Lakshmi, T., 2022. A Molecular Insight into the Role of Antioxidants in Nonalcoholic Fatty Liver Diseases. Oxid Med Cell Longev. 2022**,** 9233650. 10.1155/2022/9233650.

Faraonio, R., 2022. Oxidative Stress and Cell Senescence Process. Antioxidants (Basel). 11. 10.3390/antiox11091718.

Favaretto, F., Milan, G., Collin, G. B., Marshall, J. D., Stasi, F., Maffei, P., Vettor, R., Naggert, J. K., 2014. GLUT4 defects in adipose tissue are early signs of metabolic alterations in Alms1GT/GT, a mouse model for obesity and insulin resistance. PLoS One. 9**,** e109540. 10.1371/journal.pone.0109540.

Feng, Y., He, D., Yao, Z., Klionsky, D. J., 2014. The machinery of macroautophagy. Cell Res. 24**,** 24-41. 10.1038/cr.2013.168.

Feng, Y., Sun, W., Sun, F., Yin, G., Liang, P., Chen, S., Liu, X., Jiang, T., Zhang, F., 2022. Biological Mechanisms and Related Natural Inhibitors of CD36 in Nonalcoholic Fatty Liver. Drug Des Devel Ther. 16**,** 3829-3845. 10.2147/DDDT.S386982.

Fernandes, A. A., Novelli, E. L., Okoshi, K., Okoshi, M. P., Di Muzio, B. P., Guimaraes, J. F., Fernandes Junior, A., 2010. Influence of rutin treatment on biochemical alterations in experimental diabetes. Biomed Pharmacother. 64**,** 214-9. 10.1016/j.biopha.2009.08.007.

Finicelli, M., Squillaro, T., Galderisi, U., Peluso, G., 2021. Polyphenols, the Healthy Brand of Olive Oil: Insights and Perspectives. Nutrients. 13. 10.3390/nu13113831.

Frayn, K. N., 2010. Fat as a fuel: emerging understanding of the adipose tissue-skeletal muscle axis. Acta Physiol (Oxf). 199**,** 509-18. 10.1111/j.1748-1716.2010.02128.x.

Fromenty, B., Roden, M., 2023. Mitochondrial alterations in fatty liver diseases. J Hepatol. 78**,** 415-429. 10.1016/j.jhep.2022.09.020.

Fruhbeck, G., Catalan, V., Rodriguez, A., Gomez-Ambrosi, J., 2018. Adiponectin-leptin ratio: A promising index to estimate adipose tissue dysfunction. Relation with obesity-associated cardiometabolic risk. Adipocyte. 7**,** 57-62. 10.1080/21623945.2017.1402151.

Fruhbeck, G., Catalan, V., Rodriguez, A., Ramirez, B., Becerril, S., Salvador, J., Portincasa, P., Colina, I., Gomez-Ambrosi, J., 2017. Involvement of the leptin-adiponectin axis in

inflammation and oxidative stress in the metabolic syndrome. Sci Rep. 7**,** 6619. 10.1038/s41598-017-06997-0.

Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodriguez De Fonseca, F., Rosengarth, A., Luecke, H., Di Giacomo, B., Tarzia, G., Piomelli, D., 2003. Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. Nature. 425**,** 90-3. 10.1038/nature01921.

Fu, J., Oveisi, F., Gaetani, S., Lin, E., Piomelli, D., 2005. Oleoylethanolamide, an endogenous PPAR-alpha agonist, lowers body weight and hyperlipidemia in obese rats. Neuropharmacology. 48**,** 1147-53. 10.1016/j.neuropharm.2005.02.013.

Fu, Z., Gilbert, E. R., Liu, D., 2013. Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. Curr Diabetes Rev. 9**,** 25-53.

Gaetani, S., Fu, J., Cassano, T., Dipasquale, P., Romano, A., Righetti, L., Cianci, S., Laconca, L., Giannini, E., Scaccianoce, S., Mairesse, J., Cuomo, V., Piomelli, D., 2010. The fat-induced satiety factor oleoylethanolamide suppresses feeding through central release of oxytocin. J Neurosci. 30**,** 8096-101. 10.1523/JNEUROSCI.0036-10.2010.

Gaforio, J. J., Visioli, F., Alarcon-de-la-Lastra, C., Castaner, O., Delgado-Rodriguez, M., Fito, M., Hernandez, A. F., Huertas, J. R., Martinez-Gonzalez, M. A., Menendez, J. A., Osada, J., Papadaki, A., Parron, T., Pereira, J. E., Rosillo, M. A., Sanchez-Quesada, C., Schwingshackl, L., Toledo, E., Tsatsakis, A. M., 2019. Virgin Olive Oil and Health: Summary of the III International Conference on Virgin Olive Oil and Health Consensus Report, JAEN (Spain) 2018. Nutrients. 11. 10.3390/nu11092039.

Galluzzi, L., Pietrocola, F., Levine, B., Kroemer, G., 2014. Metabolic control of autophagy. Cell. 159**,** 1263-76. 10.1016/j.cell.2014.11.006.

Gancheva, S., Jelenik, T., Alvarez-Hernandez, E., Roden, M., 2018. Interorgan Metabolic Crosstalk in Human Insulin Resistance. Physiol Rev. 98**,** 1371-1415. 10.1152/physrev.00015.2017.

Ganeshpurkar, A., Saluja, A. K., 2017. The Pharmacological Potential of Rutin. Saudi Pharm J. 25**,** 149-164. 10.1016/j.jsps.2016.04.025.

Gao, Y., Zhang, M., Zhang, R., You, L., Li, T., Liu, R. H., 2019. Whole Grain Brown Rice Extrudate Ameliorates the Symptoms of Diabetes by Activating the IRS1/PI3K/AKT Insulin Pathway in db/db Mice. J Agric Food Chem. 67**,** 11657-11664. 10.1021/acs.jafc.9b04684.

Garcia, R. A., Roemmich, J. N., Claycombe, K. J., 2016. Evaluation of markers of beige adipocytes in white adipose tissue of the mouse. Nutr Metab (Lond). 13**,** 24. 10.1186/s12986-016-0081-2.

Ghorbani, A., 2017. Mechanisms of antidiabetic effects of flavonoid rutin. Biomed Pharmacother. 96**,** 305-312. 10.1016/j.biopha.2017.10.001.

Giordano, A., Smorlesi, A., Frontini, A., Barbatelli, G., Cinti, S., 2014. White, brown and pink adipocytes: the extraordinary plasticity of the adipose organ. Eur J Endocrinol. 170**,** R159-71. 10.1530/EJE-13-0945.

Giralt, M., Villarroya, F., 2013. White, brown, beige/brite: different adipose cells for different functions? Endocrinology. 154**,** 2992-3000. 10.1210/en.2013-1403.

Giudetti, A. M., Vergara, D., Longo, S., Friuli, M., Eramo, B., Tacconi, S., Fidaleo, M., Dini, L., Romano, A., Gaetani, S., 2021. Oleoylethanolamide Reduces Hepatic Oxidative Stress and Endoplasmic Reticulum Stress in High-Fat Diet-Fed Rats. Antioxidants (Basel). 10. 10.3390/antiox10081289.

Godlewski, G., Offertaler, L., Wagner, J. A., Kunos, G., 2009. Receptors for acylethanolamides-GPR55 and GPR119. Prostaglandins Other Lipid Mediat. 89**,** 105-11. 10.1016/j.prostaglandins.2009.07.001.

Gomez-Virgilio, L., Silva-Lucero, M. D., Flores-Morelos, D. S., Gallardo-Nieto, J., Lopez-Toledo, G., Abarca-Fernandez, A. M., Zacapala-Gomez, A. E., Luna-Munoz, J., Montiel-Sosa, F., Soto-Rojas, L. O., Pacheco-Herrero, M., Cardenas-Aguayo, M. D., 2022. Autophagy: A Key Regulator of Homeostasis and Disease: An Overview of Molecular Mechanisms and Modulators. Cells. 11. 10.3390/cells11152262.

Gonzalez-Aparicio, R., Moratalla, R., 2014. Oleoylethanolamide reduces L-DOPA-induced dyskinesia via TRPV1 receptor in a mouse model of Parkinson s disease. Neurobiol Dis. 62**,** 416-25. 10.1016/j.nbd.2013.10.008.

Grabska-Kobylecka, I., Szpakowski, P., Krol, A., Ksiazek-Winiarek, D., Kobylecki, A., Glabinski, A., Nowak, D., 2023. Polyphenols and Their Impact on the Prevention of Neurodegenerative Diseases and Development. Nutrients. 15. 10.3390/nu15153454.

Grant, R. W., Dixit, V. D., 2015. Adipose tissue as an immunological organ. Obesity (Silver Spring). 23**,** 512-8. 10.1002/oby.21003.

Guerreiro, V. A., Carvalho, D., Freitas, P., 2022. Obesity, Adipose Tissue, and Inflammation Answered in Questions. J Obes. 2022**,** 2252516. 10.1155/2022/2252516.

Guillemot-Legris, O., Muccioli, G. G., 2017. Obesity-Induced Neuroinflammation: Beyond the Hypothalamus. Trends Neurosci. 40**,** 237-253. 10.1016/j.tins.2017.02.005.

Gupta, S., Sharma, N., Arora, S., Verma, S., 2024. Diabetes: a review of its pathophysiology, and advanced methods of mitigation. Curr Med Res Opin. 40**,** 773-780. 10.1080/03007995.2024.2333440.

Guzman, M., Lo Verme, J., Fu, J., Oveisi, F., Blazquez, C., Piomelli, D., 2004. Oleoylethanolamide stimulates lipolysis by activating the nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR-alpha). J Biol Chem. 279**,** 27849-54. 10.1074/jbc.M404087200.

Haase, J., Weyer, U., Immig, K., Kloting, N., Bluher, M., Eilers, J., Bechmann, I., Gericke, M., 2014. Local proliferation of macrophages in adipose tissue during obesity-induced inflammation. Diabetologia. 57**,** 562-71. 10.1007/s00125-013-3139-y.

Habegger, K. M., 2022. Cross Talk Between Insulin and Glucagon Receptor Signaling in the Hepatocyte. Diabetes. 71**,** 1842-1851. 10.2337/dbi22-0002.

Habibullah, M., Jemmieh, K., Ouda, A., Haider, M. Z., Malki, M. I., Elzouki, A. N., 2024. Metabolic-associated fatty liver disease: a selective review of pathogenesis, diagnostic approaches, and therapeutic strategies. Front Med (Lausanne). 11**,** 1291501. 10.3389/fmed.2024.1291501.

Hagiwara, A., Cornu, M., Cybulski, N., Polak, P., Betz, C., Trapani, F., Terracciano, L., Heim, M. H., Ruegg, M. A., Hall, M. N., 2012. Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. Cell Metab. 15**,** 725-38. 10.1016/j.cmet.2012.03.015.

Hamilton, J. A., Johnson, R. A., Corkey, B., Kamp, F., 2001. Fatty acid transport: the diffusion mechanism in model and biological membranes. J Mol Neurosci. 16**,** 99-108; discussion 151-7. 10.1385/JMN:16:2-3:99.

Hansen, H. S., 2014. Role of anorectic N-acylethanolamines in intestinal physiology and satiety control with respect to dietary fat. Pharmacol Res. 86**,** 18-25. 10.1016/j.phrs.2014.03.006.

Harms, M., Seale, P., 2013. Brown and beige fat: development, function and therapeutic potential. Nat Med. 19**,** 1252-63. 10.1038/nm.3361.

Hasnat, H., Shompa, S. A., Islam, M. M., Alam, S., Richi, F. T., Emon, N. U., Ashrafi, S., Ahmed, N. U., Chowdhury, M. N. R., Fatema, N., Hossain, M. S., Ghosh, A., Ahmed, F., 2024. Flavonoids: A treasure house of prospective pharmacological potentials. Heliyon. 10**,** e27533. 10.1016/j.heliyon.2024.e27533.

Hernaez, A., Remaley, A. T., Farras, M., Fernandez-Castillejo, S., Subirana, I., Schroder, H., Fernandez-Mampel, M., Munoz-Aguayo, D., Sampson, M., Sola, R., Farre, M., de la Torre, R., Lopez-Sabater, M. C., Nyyssonen, K., Zunft, H. J., Covas, M. I., Fito, M., 2015. Olive Oil Polyphenols Decrease LDL Concentrations and LDL Atherogenicity in Men in a Randomized Controlled Trial. J Nutr. 145**,** 1692-7. 10.3945/jn.115.211557.

Herzig, S., Shaw, R. J., 2018. AMPK: guardian of metabolism and mitochondrial homeostasis. Nat Rev Mol Cell Biol. 19**,** 121-135. 10.1038/nrm.2017.95.

Hill, J. O., Wyatt, H. R., Peters, J. C., 2012. Energy balance and obesity. Circulation. 126**,** 126-32. 10.1161/CIRCULATIONAHA.111.087213.

Honzawa, N., Fujimoto, K., Kitamura, T., 2019. Cell Autonomous Dysfunction and Insulin Resistance in Pancreatic alpha Cells. Int J Mol Sci. 20. 10.3390/ijms20153699.

Huang, X., Liu, G., Guo, J., Su, Z., 2018. The PI3K/AKT pathway in obesity and type 2 diabetes. Int J Biol Sci. 14**,** 1483-1496. 10.7150/ijbs.27173.

Huang, Y., Lin, X., Lin, S., 2021. Neuropeptide Y and Metabolism Syndrome: An Update on Perspectives of Clinical Therapeutic Intervention Strategies. Front Cell Dev Biol. 9**,** 695623. 10.3389/fcell.2021.695623.

Hur, W., Kim, S. W., Lee, Y. K., Choi, J. E., Hong, S. W., Song, M. J., Bae, S. H., Park, T., Um, S. J., Yoon, S. K., 2012. Oleuropein reduces free fatty acid-induced lipogenesis via lowered extracellular signal-regulated kinase activation in hepatocytes. Nutr Res. 32**,** 778-86. 10.1016/j.nutres.2012.06.017.

Impellizzeri, D., Bruschetta, G., Cordaro, M., Crupi, R., Siracusa, R., Esposito, E., Cuzzocrea, S., 2014. Micronized/ultramicronized palmitoylethanolamide displays superior oral efficacy compared to nonmicronized palmitoylethanolamide in a rat model of inflammatory pain. J Neuroinflammation. 11**,** 136. 10.1186/s12974-014-0136-0.

Inagaki, T., Sakai, J., Kajimura, S., 2016. Transcriptional and epigenetic control of brown and beige adipose cell fate and function. Nat Rev Mol Cell Biol. 17**,** 480-95. 10.1038/nrm.2016.62.

Isaakidis, A., Maghariki, J. E., Carvalho-Barros, S., Gomes, A. M., Correia, M., 2023. Is There More to Olive Oil than Healthy Lipids? Nutrients. 15. 10.3390/nu15163625.

Ishida, T., Nishiumi, S., Tanahashi, T., Yamasaki, A., Yamazaki, A., Akashi, T., Miki, I., Kondo, Y., Inoue, J., Kawauchi, S., Azuma, T., Yoshida, M., Mizuno, S., 2013. Linoleoyl ethanolamide reduces lipopolysaccharide-induced inflammation in macrophages and ameliorates 2,4-dinitrofluorobenzene-induced contact dermatitis in mice. Eur J Pharmacol. 699**,** 6-13. 10.1016/j.ejphar.2012.11.030.

James, D. E., Stockli, J., Birnbaum, M. J., 2021. The aetiology and molecular landscape of insulin resistance. Nat Rev Mol Cell Biol. 22**,** 751-771. 10.1038/s41580-021-00390-6.

Javed, H., Khan, M. M., Ahmad, A., Vaibhav, K., Ahmad, M. E., Khan, A., Ashafaq, M., Islam, F., Siddiqui, M. S., Safhi, M. M., Islam, F., 2012. Rutin prevents cognitive impairments by ameliorating oxidative stress and neuroinflammation in rat model of sporadic dementia of Alzheimer type. Neuroscience. 210**,** 340-52. 10.1016/j.neuroscience.2012.02.046.

Jeon, S. M., 2016. Regulation and function of AMPK in physiology and diseases. Exp Mol Med. 48**,** e245. 10.1038/emm.2016.81.

Jhaveri, M. D., Richardson, D., Robinson, I., Garle, M. J., Patel, A., Sun, Y., Sagar, D. R., Bennett, A. J., Alexander, S. P., Kendall, D. A., Barrett, D. A., Chapman, V., 2008. Inhibition of fatty acid amide hydrolase and cyclooxygenase-2 increases levels of

endocannabinoid related molecules and produces analgesia via peroxisome proliferator-activated receptor-alpha in a model of inflammatory pain. Neuropharmacology. 55**,** 85-93. 10.1016/j.neuropharm.2008.04.018.

Jin, X., Qiu, T., Li, L., Yu, R., Chen, X., Li, C., Proud, C. G., Jiang, T., 2023. Pathophysiology of obesity and its associated diseases. Acta Pharm Sin B. 13**,** 2403-2424. 10.1016/j.apsb.2023.01.012.

Jomova, K., Raptova, R., Alomar, S. Y., Alwasel, S. H., Nepovimova, E., Kuca, K., Valko, M., 2023. Reactive oxygen species, toxicity, oxidative stress, and antioxidants: chronic diseases and aging. Arch Toxicol. 97**,** 2499-2574. 10.1007/s00204-023-03562-9.

Jonsson, K. O., Vandevoorde, S., Lambert, D. M., Tiger, G., Fowler, C. J., 2001. Effects of homologues and analogues of palmitoylethanolamide upon the inactivation of the endocannabinoid anandamide. Br J Pharmacol. 133**,** 1263-75. 10.1038/sj.bjp.0704199.

Juarez-Rojas, J. G., Reyes-Soffer, G., Conlon, D., Ginsberg, H. N., 2014. Autophagy and cardiometabolic risk factors. Rev Endocr Metab Disord. 15**,** 307-15. 10.1007/s11154-014-9295-7.

Jurk, D., Wilson, C., Passos, J. F., Oakley, F., Correia-Melo, C., Greaves, L., Saretzki, G., Fox, C., Lawless, C., Anderson, R., Hewitt, G., Pender, S. L., Fullard, N., Nelson, G., Mann, J., van de Sluis, B., Mann, D. A., von Zglinicki, T., 2014. Chronic inflammation induces telomere dysfunction and accelerates ageing in mice. Nat Commun. 2**,** 4172. 10.1038/ncomms5172.

Kanmani, P., Suganya, K., Kim, H., 2020. The Gut Microbiota: How Does It Influence the Development and Progression of Liver Diseases. Biomedicines. 8. 10.3390/biomedicines8110501.

Karkovic Markovic, A., Toric, J., Barbaric, M., Jakobusic Brala, C., 2019. Hydroxytyrosol, Tyrosol and Derivatives and Their Potential Effects on Human Health. Molecules. 24. 10.3390/molecules24102001.

Karkucinska-Wieckowska, A., Simoes, I. C. M., Kalinowski, P., Lebiedzinska-Arciszewska, M., Zieniewicz, K., Milkiewicz, P., Gorska-Ponikowska, M., Pinton, P., Malik, A. N., Krawczyk, M., Oliveira, P. J., Wieckowski, M. R., 2022. Mitochondria, oxidative stress and nonalcoholic fatty liver

disease: A complex relationship. Eur J Clin Invest. 52**,** e13622. 10.1111/eci.13622.

Kasatkina, L. A., Heinemann, A., Hudz, Y. A., Thomas, D., Sturm, E. M., 2020. Stearoylethanolamide interferes with retrograde endocannabinoid signalling and supports the blood-brain barrier integrity under acute systemic inflammation. Biochem Pharmacol. 174**,** 113783. 10.1016/j.bcp.2019.113783.

Kaushik, S., Cuervo, A. M., 2018. The coming of age of chaperone-mediated autophagy. Nat Rev Mol Cell Biol. 19**,** 365-381. 10.1038/s41580-018-0001-6.

Keppel Hesselink, J. M., de Boer, T., Witkamp, R. F., 2013. Palmitoylethanolamide: A Natural Body-Own Anti-Inflammatory Agent, Effective and Safe against Influenza and Common Cold. Int J Inflam. 2013**,** 151028. 10.1155/2013/151028.

Kersten, S., 2014. Integrated physiology and systems biology of PPARalpha. Mol Metab. 3**,** 354-71. 10.1016/j.molmet.2014.02.002.

Kiernan, K., MacIver, N. J., 2020. The Role of the Adipokine Leptin in Immune Cell Function in Health and Disease. Front Immunol. 11**,** 622468. 10.3389/fimmu.2020.622468.

Kim, H., Lee, D. S., An, T. H., Park, H. J., Kim, W. K., Bae, K. H., Oh, K. J., 2021. Metabolic Spectrum of Liver Failure in Type 2 Diabetes and Obesity: From NAFLD to NASH to HCC. Int J Mol Sci. 22. 10.3390/ijms22094495.

Kim, K. H., Lee, M. S., 2014. Autophagy--a key player in cellular and body metabolism. Nat Rev Endocrinol. 10**,** 322-37. 10.1038/nrendo.2014.35.

Klein, S., Gastaldelli, A., Yki-Jarvinen, H., Scherer, P. E., 2022. Why does obesity cause diabetes? Cell Metab. 34**,** 11-20. 10.1016/j.cmet.2021.12.012.

Koga, H., Kaushik, S., Cuervo, A. M., 2010. Altered lipid content inhibits autophagic vesicular fusion. FASEB J. 24**,** 3052-65. 10.1096/fj.09-144519.

Konner, A. C., Bruning, J. C., 2012. Selective insulin and leptin resistance in metabolic disorders. Cell Metab. 16**,** 144-52. 10.1016/j.cmet.2012.07.004.

Kotzbeck, P., Giordano, A., Mondini, E., Murano, I., Severi, I., Venema, W., Cecchini, M. P., Kershaw, E. E., Barbatelli, G., Haemmerle, G., Zechner, R., Cinti, S., 2018. Brown adipose

tissue whitening leads to brown adipocyte death and adipose tissue inflammation. J Lipid Res. 59**,** 784-794. 10.1194/jlr.M079665.

Kouidhi, S., Berrhouma, R., Rouissi, K., Jarboui, S., Clerget-Froidevaux, M. S., Seugnet, I., Bchir, F., Demeneix, B., Guissouma, H., Elgaaied, A. B., 2013. Human subcutaneous adipose tissue Glut 4 mRNA expression in obesity and type 2 diabetes. Acta Diabetol. 50**,** 227-32. 10.1007/s00592-011-0295-8.

Kovsan, J., Bluher, M., Tarnovscki, T., Kloting, N., Kirshtein, B., Madar, L., Shai, I., Golan, R., Harman-Boehm, I., Schon, M. R., Greenberg, A. S., Elazar, Z., Bashan, N., Rudich, A., 2011. Altered autophagy in human adipose tissues in obesity. J Clin Endocrinol Metab. 96**,** E268-77. 10.1210/jc.2010-1681.

Kozlowska, A., Szostak-Wegierek, D., 2022. Targeting Cardiovascular Diseases by Flavonols: An Update. Nutrients. 14. 10.3390/nu14071439.

Kubota, N., Yano, W., Kubota, T., Yamauchi, T., Itoh, S., Kumagai, H., Kozono, H., Takamoto, I., Okamoto, S., Shiuchi, T., Suzuki, R., Satoh, H., Tsuchida, A., Moroi, M., Sugi, K., Noda, T., Ebinuma, H., Ueta, Y., Kondo, T., Araki, E., Ezaki, O., Nagai, R., Tobe, K., Terauchi, Y., Ueki, K., Minokoshi, Y., Kadowaki, T., 2007. Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab. 6**,** 55-68. 10.1016/j.cmet.2007.06.003.

Kuchitsu, Y., Taguchi, T., 2024. Lysosomal microautophagy: an emerging dimension in mammalian autophagy. Trends Cell Biol. 34**,** 606-616. 10.1016/j.tcb.2023.11.005.

Kuipers, E. N., Kantae, V., Maarse, B. C. E., van den Berg, S. M., van Eenige, R., Nahon, K. J., Reifel-Miller, A., Coskun, T., de Winther, M. P. J., Lutgens, E., Kooijman, S., Harms, A. C., Hankemeier, T., van der Stelt, M., Rensen, P. C. N., Boon, M. R., 2018. High Fat Diet Increases Circulating Endocannabinoids Accompanied by Increased Synthesis Enzymes in Adipose Tissue. Front Physiol. 9**,** 1913. 10.3389/fphys.2018.01913.

Kumari, R., Jat, P., 2021. Mechanisms of Cellular Senescence: Cell Cycle Arrest and Senescence Associated Secretory Phenotype. Front Cell Dev Biol. 9**,** 645593. 10.3389/fcell.2021.645593.

Kurtov, M., Rubinic, I., Likic, R., 2024. The endocannabinoid system in appetite regulation and treatment of obesity. Pharmacol Res Perspect. 12**,** e70009. 10.1002/prp2.70009.

Laferrere, B., Panda, S., 2022. Calorie and Time Restriction in Weight Loss. N Engl J Med. 386**,** 1572-1573. 10.1056/NEJMe2202821.

Lakey-Beitia, J., Burillo, A. M., La Penna, G., Hegde, M. L., Rao, K. S., 2021. Polyphenols as Potential Metal Chelation Compounds Against Alzheimer's Disease. J Alzheimers Dis. 82**,** S335-S357. 10.3233/JAD-200185.

Lama, A., Pirozzi, C., Annunziata, C., Morgese, M. G., Senzacqua, M., Severi, I., Calignano, A., Trabace, L., Giordano, A., Meli, R., Mattace Raso, G., 2021. Palmitoylethanolamide counteracts brain fog improving depressive-like behaviour in obese mice: Possible role of synaptic plasticity and neurogenesis. Br J Pharmacol. 178**,** 845-859. 10.1111/bph.15071.

Lama, A., Pirozzi, C., Mollica, M. P., Trinchese, G., Di Guida, F., Cavaliere, G., Calignano, A., Mattace Raso, G., Berni Canani, R., Meli, R., 2017. Polyphenol-rich virgin olive oil reduces insulin resistance and liver inflammation and improves mitochondrial dysfunction in high-fat diet fed rats. Mol Nutr Food Res. 61. 10.1002/mnfr.201600418.

Lama, A., Pirozzi, C., Severi, I., Morgese, M. G., Senzacqua, M., Annunziata, C., Comella, F., Del Piano, F., Schiavone, S., Petrosino, S., Mollica, M. P., Diano, S., Trabace, L., Calignano, A., Giordano, A., Mattace Raso, G., Meli, R., 2022. Palmitoylethanolamide dampens neuroinflammation and anxiety-like behavior in obese mice. Brain Behav Immun. 102**,** 110-123. 10.1016/j.bbi.2022.02.008.

Lambert, D. M., Vandevoorde, S., Diependaele, G., Govaerts, S. J., Robert, A. R., 2001. Anticonvulsant activity of N-palmitoylethanolamide, a putative endocannabinoid, in mice. Epilepsia. 42**,** 321-7. 10.1046/j.1528-1157.2001.41499.x.

Lennicke, C., Cocheme, H. M., 2021. Redox regulation of the insulin signalling pathway. Redox Biol. 42**,** 101964. 10.1016/j.redox.2021.101964.

Li, S., Eguchi, N., Lau, H., Ichii, H., 2020. The Role of the Nrf2 Signaling in Obesity and Insulin Resistance. Int J Mol Sci. 21. 10.3390/ijms21186973.

Li, Y., Yao, J., Han, C., Yang, J., Chaudhry, M. T., Wang, S., Liu, H., Yin, Y., 2016. Quercetin, Inflammation and Immunity. Nutrients. 8**,** 167. 10.3390/nu8030167.

Lin, Y., Wang, Y., Li, P. F., 2022. PPARalpha: An emerging target of metabolic syndrome, neurodegenerative and cardiovascular diseases. Front Endocrinol (Lausanne). 13**,** 1074911. 10.3389/fendo.2022.1074911.

Liu, B. N., Liu, X. T., Liang, Z. H., Wang, J. H., 2021. Gut microbiota in obesity. World J Gastroenterol. 27**,** 3837-3850. 10.3748/wjg.v27.i25.3837.

Liu, Q., Pan, R., Ding, L., Zhang, F., Hu, L., Ding, B., Zhu, L., Xia, Y., Dou, X., 2017. Rutin exhibits hepatoprotective effects in a mouse model of non-alcoholic fatty liver disease by reducing hepatic lipid levels and mitigating lipid-induced oxidative injuries. Int Immunopharmacol. 49**,** 132-141. 10.1016/j.intimp.2017.05.026.

Lo Verme, J., Fu, J., Astarita, G., La Rana, G., Russo, R., Calignano, A., Piomelli, D., 2005. The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide. Mol Pharmacol. 67**,** 15-9. 10.1124/mol.104.006353.

Loomba, R., Morgan, E., Watts, L., Xia, S., Hannan, L. A., Geary, R. S., Baker, B. F., Bhanot, S., 2020. Novel antisense inhibition of diacylglycerol O-acyltransferase 2 for treatment of non-alcoholic fatty liver disease: a multicentre, double-blind, randomised, placebo-controlled phase 2 trial. Lancet Gastroenterol Hepatol. 5**,** 829-838. 10.1016/S2468-1253(20)30186-2.

Lu, B., Bridges, D., Yang, Y., Fisher, K., Cheng, A., Chang, L., Meng, Z. X., Lin, J. D., Downes, M., Yu, R. T., Liddle, C., Evans, R. M., Saltiel, A. R., 2014. Metabolic crosstalk: molecular links between glycogen and lipid metabolism in obesity. Diabetes. 63**,** 2935-48. 10.2337/db13-1531.

Lu, Y., Li, Z., Zhang, S., Zhang, T., Liu, Y., Zhang, L., 2023. Cellular mitophagy: Mechanism, roles in diseases and small molecule pharmacological regulation. Theranostics. 13**,** 736-766. 10.7150/thno.79876.

Lumeng, C. N., Saltiel, A. R., 2011. Inflammatory links between obesity and metabolic disease. J Clin Invest. 121**,** 2111-7. 10.1172/JCI57132.

Luo, L., Liu, M., 2016. Adipose tissue in control of metabolism. J Endocrinol. 231**,** R77-R99. 10.1530/JOE-16-0211.

Luo, Y., Zeng, Y., Peng, J., Zhang, K., Wang, L., Feng, T., Nhamdriel, T., Fan, G., 2023. Phytochemicals for the treatment of metabolic diseases: Evidence from clinical studies. Biomed Pharmacother. 165**,** 115274. 10.1016/j.biopha.2023.115274.

Luppino, F. S., de Wit, L. M., Bouvy, P. F., Stijnen, T., Cuijpers, P., Penninx, B. W., Zitman, F. G., 2010. Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies. Arch Gen Psychiatry. 67**,** 220-9. 10.1001/archgenpsychiatry.2010.2.

Ma, X., Wang, D., Zhao, W., Xu, L., 2018. Deciphering the Roles of PPARgamma in Adipocytes via Dynamic Change of Transcription Complex. Front Endocrinol (Lausanne). 9**,** 473. 10.3389/fendo.2018.00473.

Makarewicz, M., Drozdz, I., Tarko, T., Duda-Chodak, A., 2021. The Interactions between Polyphenols and Microorganisms, Especially Gut Microbiota. Antioxidants (Basel). 10. 10.3390/antiox10020188.

Mancina, R. M., Dongiovanni, P., Petta, S., Pingitore, P., Meroni, M., Rametta, R., Boren, J., Montalcini, T., Pujia, A., Wiklund, O., Hindy, G., Spagnuolo, R., Motta, B. M., Pipitone, R. M., Craxi, A., Fargion, S., Nobili, V., Kakela, P., Karja, V., Mannisto, V., Pihlajamaki, J., Reilly, D. F., Castro-Perez, J., Kozlitina, J., Valenti, L., Romeo, S., 2016. The MBOAT7-TMC4 Variant rs641738 Increases Risk of Nonalcoholic Fatty Liver Disease in Individuals of European Descent. Gastroenterology. 150**,** 1219-1230 e6. 10.1053/j.gastro.2016.01.032.

Mann, V., Sundaresan, A., Shishodia, S., 2024. Overnutrition and Lipotoxicity: Impaired Efferocytosis and Chronic Inflammation as Precursors to Multifaceted Disease Pathogenesis. Biology (Basel). 13. 10.3390/biology13040241.

Manning, B. D., Cantley, L. C., 2007. AKT/PKB signaling: navigating downstream. Cell. 129**,** 1261-74. 10.1016/j.cell.2007.06.009.

Martyn, J. A., Kaneki, M., Yasuhara, S., 2008. Obesity-induced insulin resistance and hyperglycemia: etiologic factors and molecular mechanisms. Anesthesiology. 109**,** 137-48. 10.1097/ALN.0b013e3181799d45.

Matsusue, K., Haluzik, M., Lambert, G., Yim, S. H., Gavrilova, O., Ward, J. M., Brewer, B., Jr., Reitman, M. L., Gonzalez, F. J.,

2003. Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. J Clin Invest. 111**,** 737-47. 10.1172/JCI17223.

Mattace Raso, G., Russo, R., Calignano, A., Meli, R., 2014a. Palmitoylethanolamide in CNS health and disease. Pharmacol Res. 86**,** 32-41. 10.1016/j.phrs.2014.05.006.

Mattace Raso, G., Santoro, A., Russo, R., Simeoli, R., Paciello, O., Di Carlo, C., Diano, S., Calignano, A., Meli, R., 2014b. Palmitoylethanolamide prevents metabolic alterations and restores leptin sensitivity in ovariectomized rats. Endocrinology. 155**,** 1291-301. 10.1210/en.2013-1823.

Maurer, S., Harms, M., Boucher, J., 2021. The colorful versatility of adipocytes: white-to-brown transdifferentiation and its therapeutic potential in humans. FEBS J. 288**,** 3628-3646. 10.1111/febs.15470.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., Gopher, A., Almog, S., Martin, B. R., Compton, D. R., et al., 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem Pharmacol. 50**,** 83-90. 10.1016/0006-2952(95)00109-d.

Meli, R., Pacilio, M., Raso, G. M., Esposito, E., Coppola, A., Nasti, A., Di Carlo, C., Nappi, C., Di Carlo, R., 2004. Estrogen and raloxifene modulate leptin and its receptor in hypothalamus and adipose tissue from ovariectomized rats. Endocrinology. 145**,** 3115-21. 10.1210/en.2004-0129.

Melini, S., Lama, A., Comella, F., Opallo, N., Del Piano, F., Annunziata, C., Mollica, M. P., Ferrante, M. C., Pirozzi, C., Mattace Raso, G., Meli, R., 2024. Targeting liver and adipose tissue in obese mice: Effects of a N-acylethanolamine mixture on insulin resistance and adipocyte reprogramming. Biomed Pharmacother. 174**,** 116531. 10.1016/j.biopha.2024.116531.

Miao, M. Q., Han, Y. B., Liu, L., 2023. Mitophagy in metabolic syndrome. J Clin Hypertens (Greenwich). 25**,** 397-403. 10.1111/jch.14650.

Michaelidou, M., Pappachan, J. M., Jeeyavudeen, M. S., 2023. Management of diabesity: Current concepts. World J Diabetes. 14**,** 396-411. 10.4239/wjd.v14.i4.396.

Miller, A. A., Spencer, S. J., 2014. Obesity and neuroinflammation: a pathway to cognitive impairment. Brain Behav Immun. 42**,** 10-21. 10.1016/j.bbi.2014.04.001.

Mittal, B., 2019. Subcutaneous adipose tissue & visceral adipose tissue. Indian J Med Res. 149**,** 571-573. 10.4103/ijmr.IJMR_1910_18.

Mock, E. D., Gagestein, B., van der Stelt, M., 2023. Anandamide and other N-acylethanolamines: A class of signaling lipids with therapeutic opportunities. Prog Lipid Res. 89**,** 101194. 10.1016/j.plipres.2022.101194.

Mollica, M. P., Mattace Raso, G., Cavaliere, G., Trinchese, G., De Filippo, C., Aceto, S., Prisco, M., Pirozzi, C., Di Guida, F., Lama, A., Crispino, M., Tronino, D., Di Vaio, P., Berni Canani, R., Calignano, A., Meli, R., 2017. Butyrate Regulates Liver Mitochondrial Function, Efficiency, and Dynamics in Insulin-Resistant Obese Mice. Diabetes. 66**,** 1405-1418. 10.2337/db16-0924.

Moore, T. M., Cheng, L., Wolf, D. M., Ngo, J., Segawa, M., Zhu, X., Strumwasser, A. R., Cao, Y., Clifford, B. L., Ma, A., Scumpia, P., Shirihai, O. S., Vallim, T. Q. A., Laakso, M., Lusis, A. J., Hevener, A. L., Zhou, Z., 2022. Parkin regulates adiposity by coordinating mitophagy with mitochondrial biogenesis in white adipocytes. Nat Commun. 13**,** 6661. 10.1038/s41467-022-34468-2.

Morigny, P., Boucher, J., Arner, P., Langin, D., 2021. Lipid and glucose metabolism in white adipocytes: pathways, dysfunction and therapeutics. Nat Rev Endocrinol. 17**,** 276-295. 10.1038/s41574-021-00471-8.

Morino, K., Petersen, K. F., Shulman, G. I., 2006. Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. Diabetes. 55 Suppl 2**,** S9-S15. 10.2337/db06-S002.

Mosca, A., Crudele, A., Smeriglio, A., Braghini, M. R., Panera, N., Comparcola, D., Alterio, A., Sartorelli, M. R., Tozzi, G., Raponi, M., Trombetta, D., Alisi, A., 2021. Antioxidant activity of Hydroxytyrosol and Vitamin E reduces systemic inflammation in children with paediatric NAFLD. Dig Liver Dis. 53**,** 1154-1158. 10.1016/j.dld.2020.09.021.

Murano, I., Barbatelli, G., Parisani, V., Latini, C., Muzzonigro, G., Castellucci, M., Cinti, S., 2008. Dead adipocytes, detected as

crown-like structures, are prevalent in visceral fat depots of genetically obese mice. J Lipid Res. 49**,** 1562-8. 10.1194/jlr.M800019-JLR200.

Nakatogawa, H., 2013. Two ubiquitin-like conjugation systems that mediate membrane formation during autophagy. Essays Biochem. 55**,** 39-50. 10.1042/bse0550039.

Narasimhan, A., Flores, R. R., Camell, C. D., Bernlohr, D. A., Robbins, P. D., Niedernhofer, L. J., 2022. Cellular Senescence in Obesity and Associated Complications: a New Therapeutic Target. Curr Diab Rep. 22**,** 537-548. 10.1007/s11892-022-01493-w.

Narita, M., 2007. Cellular senescence and chromatin organisation. Br J Cancer. 96**,** 686-91. 10.1038/sj.bjc.6603636.

Nedergaard, J., Cannon, B., 2014. The browning of white adipose tissue: some burning issues. Cell Metab. 20**,** 396-407. 10.1016/j.cmet.2014.07.005.

Nedergaard, J., Golozoubova, V., Matthias, A., Asadi, A., Jacobsson, A., Cannon, B., 2001. UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. Biochim Biophys Acta. 1504**,** 82-106. 10.1016/s0005-2728(00)00247-4.

Negroiu, C. E., Tudorascu, I., Bezna, C. M., Godeanu, S., Diaconu, M., Danoiu, R., Danoiu, S., 2024. Beyond the Cold: Activating Brown Adipose Tissue as an Approach to Combat Obesity. J Clin Med. 13. 10.3390/jcm13071973.

Noguera-Navarro, C., Montoro-Garcia, S., Orenes-Pinero, E., 2023. Hydroxytyrosol: Its role in the prevention of cardiovascular diseases. Heliyon. 9**,** e12963. 10.1016/j.heliyon.2023.e12963.

Norton, L., Shannon, C., Gastaldelli, A., DeFronzo, R. A., 2022. Insulin: The master regulator of glucose metabolism. Metabolism. 129**,** 155142. 10.1016/j.metabol.2022.155142.

Obradovic, M., Sudar-Milovanovic, E., Soskic, S., Essack, M., Arya, S., Stewart, A. J., Gojobori, T., Isenovic, E. R., 2021. Leptin and Obesity: Role and Clinical Implication. Front Endocrinol (Lausanne). 12**,** 585887. 10.3389/fendo.2021.585887.

Ogrodnik, M., Zhu, Y., Langhi, L. G. P., Tchkonia, T., Kruger, P., Fielder, E., Victorelli, S., Ruswhandi, R. A., Giorgadze, N., Pirtskhalava, T., Podgorni, O., Enikolopov, G., Johnson, K. O., Xu, M., Inman, C., Palmer, A. K., Schafer, M., Weigl, M., Ikeno, Y., Burns, T. C., Passos, J. F., von Zglinicki, T., Kirkland, J. L., Jurk, D., 2019. Obesity-Induced Cellular

Senescence Drives Anxiety and Impairs Neurogenesis. Cell Metab. 29**,** 1061-1077 e8. 10.1016/j.cmet.2018.12.008.

Oguntibeju, O. O., 2019. Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links. Int J Physiol Pathophysiol Pharmacol. 11**,** 45-63.

Ohara, M., Ohnishi, S., Hosono, H., Yamamoto, K., Fu, Q., Maehara, O., Suda, G., Sakamoto, N., 2018. Palmitoylethanolamide Ameliorates Carbon Tetrachloride-Induced Liver Fibrosis in Rats. Front Pharmacol. 9**,** 709. 10.3389/fphar.2018.00709.

Okada, K., Warabi, E., Sugimoto, H., Horie, M., Gotoh, N., Tokushige, K., Hashimoto, E., Utsunomiya, H., Takahashi, H., Ishii, T., Yamamoto, M., Shoda, J., 2013. Deletion of Nrf2 leads to rapid progression of steatohepatitis in mice fed atherogenic plus high-fat diet. J Gastroenterol. 48**,** 620-32. 10.1007/s00535-012-0659-z.

Omran, F., Christian, M., 2020. Inflammatory Signaling and Brown Fat Activity. Front Endocrinol (Lausanne). 11**,** 156. 10.3389/fendo.2020.00156.

Onopchenko, O. V., Kosiakova, G. V., Oz, M., Klimashevsky, V. M., Gula, N. M., 2015. N-stearoylethanolamine restores pancreas lipid composition in obesity-induced insulin resistant rats. Lipids. 50**,** 13-21. 10.1007/s11745-014-3960-1.

Ortega, M. A., Fraile-Martinez, O., Naya, I., Garcia-Honduvilla, N., Alvarez-Mon, M., Bujan, J., Asunsolo, A., de la Torre, B., 2020. Type 2 Diabetes Mellitus Associated with Obesity (Diabesity). The Central Role of Gut Microbiota and Its Translational Applications. Nutrients. 12. 10.3390/nu12092749.

Ortiz, M., Soto-Alarcon, S. A., Orellana, P., Espinosa, A., Campos, C., Lopez-Arana, S., Rincon, M. A., Illesca, P., Valenzuela, R., Videla, L. A., 2020. Suppression of high-fat diet-induced obesity-associated liver mitochondrial dysfunction by docosahexaenoic acid and hydroxytyrosol co-administration. Dig Liver Dis. 52**,** 895-904. 10.1016/j.dld.2020.04.019.

Osei-Hyiaman, D., DePetrillo, M., Pacher, P., Liu, J., Radaeva, S., Batkai, S., Harvey-White, J., Mackie, K., Offertaler, L., Wang, L., Kunos, G., 2005. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. J Clin Invest. 115**,** 1298-305. 10.1172/JCI23057.

Pal, S. C., Mendez-Sanchez, N., 2023. Insulin resistance and adipose tissue interactions as the cornerstone of metabolic (dysfunction)-associated fatty liver disease pathogenesis. World J Gastroenterol. 29**,** 3999-4008. 10.3748/wjg.v29.i25.3999.

Panche, A. N., Diwan, A. D., Chandra, S. R., 2016. Flavonoids: an overview. J Nutr Sci. 5**,** e47. 10.1017/jns.2016.41.

Park, H. J., Choi, J., Kim, H., Yang, D. Y., An, T. H., Lee, E. W., Han, B. S., Lee, S. C., Kim, W. K., Bae, K. H., Oh, K. J., 2023. Cellular heterogeneity and plasticity during NAFLD progression. Front Mol Biosci. 10**,** 1221669. 10.3389/fmolb.2023.1221669.

Paschos, P., Paletas, K., 2009. Non alcoholic fatty liver disease and metabolic syndrome. Hippokratia. 13**,** 9-19.

Patel, S., Alvarez-Guaita, A., Melvin, A., Rimmington, D., Dattilo, A., Miedzybrodzka, E. L., Cimino, I., Maurin, A. C., Roberts, G. P., Meek, C. L., Virtue, S., Sparks, L. M., Parsons, S. A., Redman, L. M., Bray, G. A., Liou, A. P., Woods, R. M., Parry, S. A., Jeppesen, P. B., Kolnes, A. J., Harding, H. P., Ron, D., Vidal-Puig, A., Reimann, F., Gribble, F. M., Hulston, C. J., Farooqi, I. S., Fafournoux, P., Smith, S. R., Jensen, J., Breen, D., Wu, Z., Zhang, B. B., Coll, A. P., Savage, D. B., O'Rahilly, S., 2019. GDF15 Provides an Endocrine Signal of Nutritional Stress in Mice and Humans. Cell Metab. 29**,** 707-718 e8. 10.1016/j.cmet.2018.12.016.

Piomelli, D., 2013. A fatty gut feeling. Trends Endocrinol Metab. 24**,** 332-41. 10.1016/j.tem.2013.03.001.

Pirazzi, C., Adiels, M., Burza, M. A., Mancina, R. M., Levin, M., Stahlman, M., Taskinen, M. R., Orho-Melander, M., Perman, J., Pujia, A., Andersson, L., Maglio, C., Montalcini, T., Wiklund, O., Boren, J., Romeo, S., 2012. Patatin-like phospholipase domain-containing 3 (PNPLA3) I148M (rs738409) affects hepatic VLDL secretion in humans and in vitro. J Hepatol. 57**,** 1276-82. 10.1016/j.jhep.2012.07.030.

Pirozzi, C., Coretti, L., Opallo, N., Bove, M., Annunziata, C., Comella, F., Turco, L., Lama, A., Trabace, L., Meli, R., Lembo, F., Mattace Raso, G., 2023. Palmitoylethanolamide counteracts high-fat diet-induced gut dysfunction by reprogramming microbiota composition and affecting tryptophan metabolism. Front Nutr. 10**,** 1143004. 10.3389/fnut.2023.1143004.

Pirozzi, C., Lama, A., Annunziata, C., Cavaliere, G., De Caro, C., Citraro, R., Russo, E., Tallarico, M., Iannone, M., Ferrante, M. C., Mollica, M. P., Mattace Raso, G., De Sarro, G., Calignano, A., Meli, R., 2020. Butyrate prevents valproate-induced liver injury: In vitro and in vivo evidence. FASEB J. 34**,** 676-690. 10.1096/fj.201900927RR.

Pirozzi, C., Lama, A., Simeoli, R., Paciello, O., Pagano, T. B., Mollica, M. P., Di Guida, F., Russo, R., Magliocca, S., Canani, R. B., Raso, G. M., Calignano, A., Meli, R., 2016. Hydroxytyrosol prevents metabolic impairment reducing hepatic inflammation and restoring duodenal integrity in a rat model of NAFLD. J Nutr Biochem. 30**,** 108-15. 10.1016/j.jnutbio.2015.12.004.

Poitout, V., Robertson, R. P., 2008. Glucolipotoxicity: fuel excess and beta-cell dysfunction. Endocr Rev. 29**,** 351-66. 10.1210/er.2007-0023.

Popoviciu, M. S., Paduraru, L., Yahya, G., Metwally, K., Cavalu, S., 2023. Emerging Role of GLP-1 Agonists in Obesity: A Comprehensive Review of Randomised Controlled Trials. Int J Mol Sci. 24. 10.3390/ijms241310449.

Pouwels, S., Sakran, N., Graham, Y., Leal, A., Pintar, T., Yang, W., Kassir, R., Singhal, R., Mahawar, K., Ramnarain, D., 2022. Non-alcoholic fatty liver disease (NAFLD): a review of pathophysiology, clinical management and effects of weight loss. BMC Endocr Disord. 22**,** 63. 10.1186/s12902-022-00980-1.

Prasun, P., Ginevic, I., Oishi, K., 2021. Mitochondrial dysfunction in nonalcoholic fatty liver disease and alcohol related liver disease. Transl Gastroenterol Hepatol. 6**,** 4. 10.21037/tgh-20-125.

Priore, P., Siculella, L., Gnoni, G. V., 2014. Extra virgin olive oil phenols down-regulate lipid synthesis in primary-cultured rat-hepatocytes. J Nutr Biochem. 25**,** 683-91. 10.1016/j.jnutbio.2014.01.009.

Provensi, G., Coccurello, R., Umehara, H., Munari, L., Giacovazzo, G., Galeotti, N., Nosi, D., Gaetani, S., Romano, A., Moles, A., Blandina, P., Passani, M. B., 2014. Satiety factor oleoylethanolamide recruits the brain histaminergic system to inhibit food intake. Proc Natl Acad Sci U S A. 111**,** 11527-32. 10.1073/pnas.1322016111.

Purnell, J. Q., Definitions, Classification, and Epidemiology of Obesity. In: K. R. Feingold, et al., Eds.), Endotext, South Dartmouth (MA), 2000.

Rada, P., Gonzalez-Rodriguez, A., Garcia-Monzon, C., Valverde, A. M., 2020. Understanding lipotoxicity in NAFLD pathogenesis: is CD36 a key driver? Cell Death Dis. 11**,** 802. 10.1038/s41419-020-03003-w.

Rana, A., Samtiya, M., Dhewa, T., Mishra, V., Aluko, R. E., 2022. Health benefits of polyphenols: A concise review. J Food Biochem. 46**,** e14264. 10.1111/jfbc.14264.

Reggio, P. H., 2010. Endocannabinoid binding to the cannabinoid receptors: what is known and what remains unknown. Curr Med Chem. 17**,** 1468-86. 10.2174/092986710790980005.

Reilly, S. M., Saltiel, A. R., 2017. Adapting to obesity with adipose tissue inflammation. Nat Rev Endocrinol. 13**,** 633-643. 10.1038/nrendo.2017.90.

Reyes, J. J., Villanueva, B., Lopez-Villodres, J. A., De La Cruz, J. P., Romero, L., Rodriguez-Perez, M. D., Rodriguez-Gutierrez, G., Fernandez-Bolanos, J., Gonzalez-Correa, J. A., 2017. Neuroprotective Effect of Hydroxytyrosol in Experimental Diabetes Mellitus. J Agric Food Chem. 65**,** 4378-4383. 10.1021/acs.jafc.6b02945.

Robertson, R. P., Harmon, J., Tran, P. O., Tanaka, Y., Takahashi, H., 2003. Glucose toxicity in beta-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. Diabetes. 52**,** 581-7. 10.2337/diabetes.52.3.581.

Rodriguez de Fonseca, F., Navarro, M., Gomez, R., Escuredo, L., Nava, F., Fu, J., Murillo-Rodriguez, E., Giuffrida, A., LoVerme, J., Gaetani, S., Kathuria, S., Gall, C., Piomelli, D., 2001. An anorexic lipid mediator regulated by feeding. Nature. 414**,** 209-12. 10.1038/35102582.

Rohm, T. V., Meier, D. T., Olefsky, J. M., Donath, M. Y., 2022. Inflammation in obesity, diabetes, and related disorders. Immunity. 55**,** 31-55. 10.1016/j.immuni.2021.12.013.

Rosen, E. D., Spiegelman, B. M., 2014. What we talk about when we talk about fat. Cell. 156**,** 20-44. 10.1016/j.cell.2013.12.012.

Ross, F. C., Mayer, D. E., Horn, J., Cryan, J. F., Del Rio, D., Randolph, E., Gill, C. I. R., Gupta, A., Ross, R. P., Stanton, C., Mayer, E. A., 2024. Potential of dietary polyphenols for protection from age-related decline and neurodegeneration: a role for gut

microbiota? Nutr Neurosci. 27, 1058-1076. 10.1080/1028415X.2023.2298098.
Rutter, K., Hennoste, L., Ward, L. C., Cornish, B. H., Thomas, B. J., 1998. Bioelectrical impedance analysis for the estimation of body composition in rats. Lab Anim. 32, 65-71. 10.1258/002367798780559356.
Ruze, R., Liu, T., Zou, X., Song, J., Chen, Y., Xu, R., Yin, X., Xu, Q., 2023. Obesity and type 2 diabetes mellitus: connections in epidemiology, pathogenesis, and treatments. Front Endocrinol (Lausanne). 14, 1161521. 10.3389/fendo.2023.1161521.
Ryberg, E., Larsson, N., Sjogren, S., Hjorth, S., Hermansson, N. O., Leonova, J., Elebring, T., Nilsson, K., Drmota, T., Greasley, P. J., 2007. The orphan receptor GPR55 is a novel cannabinoid receptor. Br J Pharmacol. 152, 1092-101. 10.1038/sj.bjp.0707460.
Safar Zadeh, E., Lungu, A. O., Cochran, E. K., Brown, R. J., Ghany, M. G., Heller, T., Kleiner, D. E., Gorden, P., 2013. The liver diseases of lipodystrophy: the long-term effect of leptin treatment. J Hepatol. 59, 131-7. 10.1016/j.jhep.2013.02.007.
Sakers, A., De Siqueira, M. K., Seale, P., Villanueva, C. J., 2022. Adipose-tissue plasticity in health and disease. Cell. 185, 419-446. 10.1016/j.cell.2021.12.016.
Saltiel, A. R., Pessin, J. E., 2002. Insulin signaling pathways in time and space. Trends Cell Biol. 12, 65-71. 10.1016/s0962-8924(01)02207-3.
Samovski, D., Abumrad, N. A., 2019. Regulation of lipophagy in NAFLD by cellular metabolism and CD36. J Lipid Res. 60, 755-757. 10.1194/jlr.C093674.
Sanches, J. M., Zhao, L. N., Salehi, A., Wollheim, C. B., Kaldis, P., 2023. Pathophysiology of type 2 diabetes and the impact of altered metabolic interorgan crosstalk. FEBS J. 290, 620-648. 10.1111/febs.16306.
Santoro, A., McGraw, T. E., Kahn, B. B., 2021. Insulin action in adipocytes, adipose remodeling, and systemic effects. Cell Metab. 33, 748-757. 10.1016/j.cmet.2021.03.019.
Santos, M. M., Piccirillo, C., Castro, P. M., Kalogerakis, N., Pintado, M. E., 2012. Bioconversion of oleuropein to hydroxytyrosol by lactic acid bacteria. World J Microbiol Biotechnol. 28, 2435-40. 10.1007/s11274-012-1036-z.

Schafer, M. J., White, T. A., Evans, G., Tonne, J. M., Verzosa, G. C., Stout, M. B., Mazula, D. L., Palmer, A. K., Baker, D. J., Jensen, M. D., Torbenson, M. S., Miller, J. D., Ikeda, Y., Tchkonia, T., van Deursen, J. M., Kirkland, J. L., LeBrasseur, N. K., 2016. Exercise Prevents Diet-Induced Cellular Senescence in Adipose Tissue. Diabetes. 65**,** 1606-15. 10.2337/db15-0291.

Schirinzi, V., Poli, C., Berteotti, C., Leone, A., 2023. Browning of Adipocytes: A Potential Therapeutic Approach to Obesity. Nutrients. 15. 10.3390/nu15092229.

Schmitz, J., Evers, N., Awazawa, M., Nicholls, H. T., Bronneke, H. S., Dietrich, A., Mauer, J., Bluher, M., Bruning, J. C., 2016. Obesogenic memory can confer long-term increases in adipose tissue but not liver inflammation and insulin resistance after weight loss. Mol Metab. 5**,** 328-339. 10.1016/j.molmet.2015.12.001.

Schofield, J. H., Schafer, Z. T., 2021. Mitochondrial Reactive Oxygen Species and Mitophagy: A Complex and Nuanced Relationship. Antioxid Redox Signal. 34**,** 517-530. 10.1089/ars.2020.8058.

Schwartz, G. J., Fu, J., Astarita, G., Li, X., Gaetani, S., Campolongo, P., Cuomo, V., Piomelli, D., 2008. The lipid messenger OEA links dietary fat intake to satiety. Cell Metab. 8**,** 281-288. 10.1016/j.cmet.2008.08.005.

Schwingshackl, L., Hoffmann, G., 2014. Monounsaturated fatty acids, olive oil and health status: a systematic review and meta-analysis of cohort studies. Lipids Health Dis. 13**,** 154. 10.1186/1476-511X-13-154.

Scuteri, D., Guida, F., Boccella, S., Palazzo, E., Maione, S., Rodriguez-Landa, J. F., Martinez-Mota, L., Tonin, P., Bagetta, G., Corasaniti, M. T., 2022. Effects of Palmitoylethanolamide (PEA) on Nociceptive, Musculoskeletal and Neuropathic Pain: Systematic Review and Meta-Analysis of Clinical Evidence. Pharmaceutics. 14. 10.3390/pharmaceutics14081672.

Seale, P., Bjork, B., Yang, W., Kajimura, S., Chin, S., Kuang, S., Scime, A., Devarakonda, S., Conroe, H. M., Erdjument-Bromage, H., Tempst, P., Rudnicki, M. A., Beier, D. R., Spiegelman, B. M., 2008. PRDM16 controls a brown fat/skeletal muscle switch. Nature. 454**,** 961-7. 10.1038/nature07182.

Semova, I., Biddinger, S. B., 2021. Triglycerides in Nonalcoholic Fatty Liver Disease: Guilty Until Proven Innocent. Trends Pharmacol Sci. 42**,** 183-190. 10.1016/j.tips.2020.12.001.

Serreli, G., Le Sayec, M., Diotallevi, C., Teissier, A., Deiana, M., Corona, G., 2021. Conjugated Metabolites of Hydroxytyrosol and Tyrosol Contribute to the Maintenance of Nitric Oxide Balance in Human Aortic Endothelial Cells at Physiologically Relevant Concentrations. Molecules. 26. 10.3390/molecules26247480.

Sheikh, A. B., Nasrullah, A., Haq, S., Akhtar, A., Ghazanfar, H., Nasir, A., Afzal, R. M., Bukhari, M. M., Chaudhary, A. Y., Naqvi, S. W., 2017. The Interplay of Genetics and Environmental Factors in the Development of Obesity. Cureus. 9**,** e1435. 10.7759/cureus.1435.

Shreeya, T., Ansari, M. S., Kumar, P., Saifi, M., Shati, A. A., Alfaifi, M. Y., Elbehairi, S. E. I., 2023. Senescence: A DNA damage response and its role in aging and Neurodegenerative Diseases. Front Aging. 4**,** 1292053. 10.3389/fragi.2023.1292053.

Simon, T. G., Roelstraete, B., Hagstrom, H., Loomba, R., Ludvigsson, J. F., 2023. Progression of non-alcoholic fatty liver disease and long-term outcomes: A nationwide paired liver biopsy cohort study. J Hepatol. 79**,** 1366-1373. 10.1016/j.jhep.2023.08.008.

Singh, A., Hardin, B. I., Keyes, D., Epidemiologic and Etiologic Considerations of Obesity. StatPearls, Treasure Island (FL), 2024.

Snel, M., Jonker, J. T., Schoones, J., Lamb, H., de Roos, A., Pijl, H., Smit, J. W., Meinders, A. E., Jazet, I. M., 2012. Ectopic fat and insulin resistance: pathophysiology and effect of diet and lifestyle interventions. Int J Endocrinol. 2012**,** 983814. 10.1155/2012/983814.

Solano-Urrusquieta, A., Morales-Gonzalez, J. A., Castro-Narro, G. E., Cerda-Reyes, E., Flores-Rangel, P. D., Fierros-Oceguera, R., 2020. NRF-2 and nonalcoholic fatty liver disease. Ann Hepatol. 19**,** 458-465. 10.1016/j.aohep.2019.11.010.

Son, J., Accili, D., 2023. Reversing pancreatic beta-cell dedifferentiation in the treatment of type 2 diabetes. Exp Mol Med. 55**,** 1652-1658. 10.1038/s12276-023-01043-8.

Song, C., Long, X., He, J., Huang, Y., 2023. Recent evaluation about inflammatory mechanisms in nonalcoholic fatty liver disease. Front Pharmacol. 14**,** 1081334. 10.3389/fphar.2023.1081334.

Spiegelman, B. M., Flier, J. S., 2001. Obesity and the regulation of energy balance. Cell. 104**,** 531-43. 10.1016/s0092-8674(01)00240-9.

Srivastava, S., Veech, R. L., 2019. Brown and Brite: The Fat Soldiers in the Anti-obesity Fight. Front Physiol. 10**,** 38. 10.3389/fphys.2019.00038.

Stern, J. H., Rutkowski, J. M., Scherer, P. E., 2016. Adiponectin, Leptin, and Fatty Acids in the Maintenance of Metabolic Homeostasis through Adipose Tissue Crosstalk. Cell Metab. 23**,** 770-84. 10.1016/j.cmet.2016.04.011.

Su, Z., Guo, Y., Huang, X., Feng, B., Tang, L., Zheng, G., Zhu, Y., 2021. Phytochemicals: Targeting Mitophagy to Treat Metabolic Disorders. Front Cell Dev Biol. 9**,** 686820. 10.3389/fcell.2021.686820.

Suchacki, K. J., Tavares, A. A. S., Mattiucci, D., Scheller, E. L., Papanastasiou, G., Gray, C., Sinton, M. C., Ramage, L. E., McDougald, W. A., Lovdel, A., Sulston, R. J., Thomas, B. J., Nicholson, B. M., Drake, A. J., Alcaide-Corral, C. J., Said, D., Poloni, A., Cinti, S., Macpherson, G. J., Dweck, M. R., Andrews, J. P. M., Williams, M. C., Wallace, R. J., van Beek, E. J. R., MacDougald, O. A., Morton, N. M., Stimson, R. H., Cawthorn, W. P., 2020. Bone marrow adipose tissue is a unique adipose subtype with distinct roles in glucose homeostasis. Nat Commun. 11**,** 3097. 10.1038/s41467-020-16878-2.

Sugiura, T., Kishimoto, S., Oka, S., Gokoh, M., 2006. Biochemistry, pharmacology and physiology of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand. Prog Lipid Res. 45**,** 405-46. 10.1016/j.plipres.2006.03.003.

Sun, K., Tordjman, J., Clement, K., Scherer, P. E., 2013. Fibrosis and adipose tissue dysfunction. Cell Metab. 18**,** 470-7. 10.1016/j.cmet.2013.06.016.

Sun, L., Luo, C., Liu, J., 2014. Hydroxytyrosol induces apoptosis in human colon cancer cells through ROS generation. Food Funct. 5**,** 1909-14. 10.1039/c4fo00187g.

Syed, S. K., Bui, H. H., Beavers, L. S., Farb, T. B., Ficorilli, J., Chesterfield, A. K., Kuo, M. S., Bokvist, K., Barrett, D. G., Efanov, A. M., 2012. Regulation of GPR119 receptor activity with endocannabinoid-like lipids. Am J Physiol Endocrinol Metab. 303**,** E1469-78. 10.1152/ajpendo.00269.2012.

Tabernero, M., Sarria, B., Largo, C., Martinez-Lopez, S., Madrona, A., Espartero, J. L., Bravo, L., Mateos, R., 2014. Comparative evaluation of the metabolic effects of hydroxytyrosol and its lipophilic derivatives (hydroxytyrosyl acetate and ethyl hydroxytyrosyl ether) in hypercholesterolemic rats. Food Funct. 5**,** 1556-63. 10.1039/c3fo60677e.

Tabuchi, C., Sul, H. S., 2021. Signaling Pathways Regulating Thermogenesis. Front Endocrinol (Lausanne). 12**,** 595020. 10.3389/fendo.2021.595020.

Taheri, R., Mokhtari, Y., Yousefi, A. M., Bashash, D., 2024. The PI3K/Akt signaling axis and type 2 diabetes mellitus (T2DM): From mechanistic insights into possible therapeutic targets. Cell Biol Int. 48**,** 1049-1068. 10.1002/cbin.12189.

Tang, S., Geng, Y., Lin, Q., 2024. The role of mitophagy in metabolic diseases and its exercise intervention. Front Physiol. 15**,** 1339128. 10.3389/fphys.2024.1339128.

Tejada, S., Pinya, S., Del Mar Bibiloni, M., Tur, J. A., Pons, A., Sureda, A., 2017. Cardioprotective Effects of the Polyphenol Hydroxytyrosol from Olive Oil. Curr Drug Targets. 18**,** 1477-1486. 10.2174/1389450117666161005150650.

Terrazzino, S., Berto, F., Dalle Carbonare, M., Fabris, M., Guiotto, A., Bernardini, D., Leon, A., 2004. Stearoylethanolamide exerts anorexic effects in mice via down-regulation of liver stearoyl-coenzyme A desaturase-1 mRNA expression. FASEB J. 18**,** 1580-2. 10.1096/fj.03-1080fje.

Thilakarathna, S. H., Rupasinghe, H. P., 2013. Flavonoid bioavailability and attempts for bioavailability enhancement. Nutrients. 5**,** 3367-87. 10.3390/nu5093367.

Todisco, S., Santarsiero, A., Convertini, P., De Stefano, G., Gilio, M., Iacobazzi, V., Infantino, V., 2022. PPAR Alpha as a Metabolic Modulator of the Liver: Role in the Pathogenesis of Nonalcoholic Steatohepatitis (NASH). Biology (Basel). 11. 10.3390/biology11050792.

Tong, X., Li, P., Zhang, D., VanDommelen, K., Gupta, N., Rui, L., Omary, M. B., Yin, L., 2016. E4BP4 is an insulin-induced stabilizer of nuclear SREBP-1c and promotes SREBP-1c-mediated lipogenesis. J Lipid Res. 57**,** 1219-30. 10.1194/jlr.M067181.

Tovar, R., de Ceglia, M., Ubaldi, M., Rodriguez-Pozo, M., Soverchia, L., Cifani, C., Rojo, G., Gavito, A., Hernandez-Folgado, L.,

Jagerovic, N., Ciccocioppo, R., Baixeras, E., Rodriguez de Fonseca, F., Decara, J., 2023. Administration of Linoleoylethanolamide Reduced Weight Gain, Dyslipidemia, and Inflammation Associated with High-Fat-Diet-Induced Obesity. Nutrients. 15. 10.3390/nu15204448.

Tripathi, D., Kant, S., Pandey, S., Ehtesham, N. Z., 2020. Resistin in metabolism, inflammation, and disease. FEBS J. 287**,** 3141-3149. 10.1111/febs.15322.

Tsuboi, K., Ikematsu, N., Uyama, T., Deutsch, D. G., Tokumura, A., Ueda, N., 2013. Biosynthetic pathways of bioactive N-acylethanolamines in brain. CNS Neurol Disord Drug Targets. 12**,** 7-16. 10.2174/1871527311312010005.

Tsuboi, K., Uyama, T., Okamoto, Y., Ueda, N., 2018. Endocannabinoids and related N-acylethanolamines: biological activities and metabolism. Inflamm Regen. 38**,** 28. 10.1186/s41232-018-0086-5.

Tu, M., Fan, X., Shi, J., Jing, S., Xu, X., Wang, Y., 2022. 2-Fluorofucose Attenuates Hydrogen Peroxide-Induced Oxidative Stress in HepG2 Cells via Nrf2/keap1 and NF-kappaB Signaling Pathways. Life (Basel). 12. 10.3390/life12030406.

Tutunchi, H., Ostadrahimi, A., Saghafi-Asl, M., Hosseinzadeh-Attar, M. J., Shakeri, A., Asghari-Jafarabadi, M., Roshanravan, N., Farrin, N., Naemi, M., Hasankhani, M., 2020. Oleoylethanolamide supplementation in obese patients newly diagnosed with non-alcoholic fatty liver disease: Effects on metabolic parameters, anthropometric indices, and expression of PPAR-alpha, UCP1, and UCP2 genes. Pharmacol Res. 156**,** 104770. 10.1016/j.phrs.2020.104770.

Tutunchi, H., Ostadrahimi, A., Saghafi-Asl, M., Maleki, V., 2019. The effects of oleoylethanolamide, an endogenous PPAR-alpha agonist, on risk factors for NAFLD: A systematic review. Obes Rev. 20**,** 1057-1069. 10.1111/obr.12853.

Ueda, N., Tsuboi, K., Uyama, T., 2013. Metabolism of endocannabinoids and related N-acylethanolamines: canonical and alternative pathways. FEBS J. 280**,** 1874-94. 10.1111/febs.12152.

Ulasov, A. V., Rosenkranz, A. A., Georgiev, G. P., Sobolev, A. S., 2022. Nrf2/Keap1/ARE signaling: Towards specific regulation. Life Sci. 291**,** 120111. 10.1016/j.lfs.2021.120111.

Utzschneider, K. M., Kahn, S. E., 2006. Review: The role of insulin resistance in nonalcoholic fatty liver disease. J Clin Endocrinol Metab. 91**,** 4753-61. 10.1210/jc.2006-0587.

Valenza, M., Facchinetti, R., Steardo, L., Scuderi, C., 2022. Palmitoylethanolamide and White Matter Lesions: Evidence for Therapeutic Implications. Biomolecules. 12. 10.3390/biom12091191.

Vasileva, L. V., Savova, M. S., Amirova, K. M., Dinkova-Kostova, A. T., Georgiev, M. I., 2020. Obesity and NRF2-mediated cytoprotection: Where is the missing link? Pharmacol Res. 156**,** 104760. 10.1016/j.phrs.2020.104760.

Vijakumaran, U., Shanmugam, J., Heng, J. W., Azman, S. S., Yazid, M. D., Haizum Abdullah, N. A., Sulaiman, N., 2023. Effects of Hydroxytyrosol in Endothelial Functioning: A Comprehensive Review. Molecules. 28. 10.3390/molecules28041861.

Vila, G., Riedl, M., Anderwald, C., Resl, M., Handisurya, A., Clodi, M., Prager, G., Ludvik, B., Krebs, M., Luger, A., 2011. The relationship between insulin resistance and the cardiovascular biomarker growth differentiation factor-15 in obese patients. Clin Chem. 57**,** 309-16. 10.1373/clinchem.2010.153726.

Viola, A., Munari, F., Sanchez-Rodriguez, R., Scolaro, T., Castegna, A., 2019. The Metabolic Signature of Macrophage Responses. Front Immunol. 10**,** 1462. 10.3389/fimmu.2019.01462.

Vlavcheski, F., Young, M., Tsiani, E., 2019. Antidiabetic Effects of Hydroxytyrosol: In Vitro and In Vivo Evidence. Antioxidants (Basel). 8. 10.3390/antiox8060188.

Wang, C., 2020. A Sensitive and Quantitative mKeima Assay for Mitophagy via FACS. Curr Protoc Cell Biol. 86**,** e99. 10.1002/cpcb.99.

Wang, D., Day, E. A., Townsend, L. K., Djordjevic, D., Jorgensen, S. B., Steinberg, G. R., 2021. GDF15: emerging biology and therapeutic applications for obesity and cardiometabolic disease. Nat Rev Endocrinol. 17**,** 592-607. 10.1038/s41574-021-00529-7.

Wang, G., Bonkovsky, H. L., de Lemos, A., Burczynski, F. J., 2015. Recent insights into the biological functions of liver fatty acid binding protein 1. J Lipid Res. 56**,** 2238-47. 10.1194/jlr.R056705.

Wang, N., Liu, Y., Ma, Y., Wen, D., 2018. Hydroxytyrosol ameliorates insulin resistance by modulating endoplasmic reticulum stress

and prevents hepatic steatosis in diet-induced obesity mice. J Nutr Biochem. 57**,** 180-188. 10.1016/j.jnutbio.2018.03.018.

Wang, Q. A., Tao, C., Gupta, R. K., Scherer, P. E., 2013. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. Nat Med. 19**,** 1338-44. 10.1038/nm.3324.

Wang, S., Moustaid-Moussa, N., Chen, L., Mo, H., Shastri, A., Su, R., Bapat, P., Kwun, I., Shen, C. L., 2014. Novel insights of dietary polyphenols and obesity. J Nutr Biochem. 25**,** 1-18. 10.1016/j.jnutbio.2013.09.001.

Wellen, K. E., Thompson, C. B., 2010. Cellular metabolic stress: considering how cells respond to nutrient excess. Mol Cell. 40**,** 323-32. 10.1016/j.molcel.2010.10.004.

Wen, X., Tang, S., Wan, F., Zhong, R., Chen, L., Zhang, H., 2024. The PI3K/Akt-Nrf2 Signaling Pathway and Mitophagy Synergistically Mediate Hydroxytyrosol to Alleviate Intestinal Oxidative Damage. Int J Biol Sci. 20**,** 4258-4276. 10.7150/ijbs.97263.

Wiley, C. D., Campisi, J., 2021. The metabolic roots of senescence: mechanisms and opportunities for intervention. Nat Metab. 3**,** 1290-1301. 10.1038/s42255-021-00483-8.

Wilson, C. G., Tran, J. L., Erion, D. M., Vera, N. B., Febbraio, M., Weiss, E. J., 2016. Hepatocyte-Specific Disruption of CD36 Attenuates Fatty Liver and Improves Insulin Sensitivity in HFD-Fed Mice. Endocrinology. 157**,** 570-85. 10.1210/en.2015-1866.

Xu, D., Xu, M., Jeong, S., Qian, Y., Wu, H., Xia, Q., Kong, X., 2018. The Role of Nrf2 in Liver Disease: Novel Molecular Mechanisms and Therapeutic Approaches. Front Pharmacol. 9**,** 1428. 10.3389/fphar.2018.01428.

Xu, M., Palmer, A. K., Ding, H., Weivoda, M. M., Pirtskhalava, T., White, T. A., Sepe, A., Johnson, K. O., Stout, M. B., Giorgadze, N., Jensen, M. D., LeBrasseur, N. K., Tchkonia, T., Kirkland, J. L., 2015. Targeting senescent cells enhances adipogenesis and metabolic function in old age. Elife. 4**,** e12997. 10.7554/eLife.12997.

Yang, L., Guo, H., Li, Y., Meng, X., Yan, L., Dan, Z., Wu, S., Zhou, H., Peng, L., Xie, Q., Jin, X., 2016. Oleoylethanolamide exerts anti-inflammatory effects on LPS-induced THP-1 cells by enhancing PPARalpha signaling and inhibiting the NF-kappaB

and ERK1/2/AP-1/STAT3 pathways. Sci Rep. 6, 34611. 10.1038/srep34611.

Yang, L., Li, P., Fu, S., Calay, E. S., Hotamisligil, G. S., 2010. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. Cell Metab. 11, 467-78. 10.1016/j.cmet.2010.04.005.

Yao, J., Wu, D., Qiu, Y., 2022. Adipose tissue macrophage in obesity-associated metabolic diseases. Front Immunol. 13, 977485. 10.3389/fimmu.2022.977485.

Yao, J., Wu, D., Zhang, C., Yan, T., Zhao, Y., Shen, H., Xue, K., Huang, X., Wang, Z., Qiu, Y., 2021. Macrophage IRX3 promotes diet-induced obesity and metabolic inflammation. Nat Immunol. 22, 1268-1279. 10.1038/s41590-021-01023-y.

Young, P., Arch, J. R., Ashwell, M., 1984. Brown adipose tissue in the parametrial fat pad of the mouse. FEBS Lett. 167, 10-4. 10.1016/0014-5793(84)80822-4.

Yuan, X., Wei, G., You, Y., Huang, Y., Lee, H. J., Dong, M., Lin, J., Hu, T., Zhang, H., Zhang, C., Zhou, H., Ye, R., Qi, X., Zhai, B., Huang, W., Liu, S., Xie, W., Liu, Q., Liu, X., Cui, C., Li, D., Zhan, J., Cheng, J., Yuan, Z., Jin, W., 2017. Rutin ameliorates obesity through brown fat activation. FASEB J. 31, 333-345. 10.1096/fj.201600459RR.

Zhang, Y., Sowers, J. R., Ren, J., 2018. Targeting autophagy in obesity: from pathophysiology to management. Nat Rev Endocrinol. 14, 356-376. 10.1038/s41574-018-0009-1.

Zhao, Y., Zhao, M. F., Jiang, S., Wu, J., Liu, J., Yuan, X. W., Shen, D., Zhang, J. Z., Zhou, N., He, J., Fang, L., Sun, X. T., Xue, B., Li, C. J., 2020. Liver governs adipose remodelling via extracellular vesicles in response to lipid overload. Nat Commun. 11, 719. 10.1038/s41467-020-14450-6.

Zheng, Y., Wang, S., Wu, J., Wang, Y., 2023. Mitochondrial metabolic dysfunction and non-alcoholic fatty liver disease: new insights from pathogenic mechanisms to clinically targeted therapy. J Transl Med. 21, 510. 10.1186/s12967-023-04367-1.

Zhou, J., Febbraio, M., Wada, T., Zhai, Y., Kuruba, R., He, J., Lee, J. H., Khadem, S., Ren, S., Li, S., Silverstein, R. L., Xie, W., 2008. Hepatic fatty acid transporter Cd36 is a common target of LXR, PXR, and PPARgamma in promoting steatosis. Gastroenterology. 134, 556-67. 10.1053/j.gastro.2007.11.037.

Zhuang, X. Y., Zhang, Y. H., Xiao, A. F., Zhang, A. H., Fang, B. S., 2022. Corrigendum: Key Enzymes in Fatty Acid Synthesis Pathway for Bioactive Lipids Biosynthesis. Front Nutr. 9**,** 914273. 10.3389/fnut.2022.914273.

Ziolkowska, S., Binienda, A., Jablkowski, M., Szemraj, J., Czarny, P., 2021. The Interplay between Insulin Resistance, Inflammation, Oxidative Stress, Base Excision Repair and Metabolic Syndrome in Nonalcoholic Fatty Liver Disease. Int J Mol Sci. 22. 10.3390/ijms222011128.

Ziqubu, K., Dludla, P. V., Mthembu, S. X. H., Nkambule, B. B., Mabhida, S. E., Jack, B. U., Nyambuya, T. M., Mazibuko-Mbeje, S. E., 2023. An insight into brown/beige adipose tissue whitening, a metabolic complication of obesity with the multifactorial origin. Front Endocrinol (Lausanne). 14**,** 1114767. 10.3389/fendo.2023.1114767.

Zou, S., Kumar, U., 2018. Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. Int J Mol Sci. 19. 10.3390/ijms19030833.

Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D., Hogestatt, E. D., 1999. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature. 400**,** 452-7. 10.1038/22761.