UNIVERSITAT ROVIRA i VIRGILI

Grup de Recerca NUTRIGENÒMICA URV

Grape seed proanthocyanidin extract consumption modulated *Sucnr1* gene expresion rhythm in cafeteria diet-induced obese rats

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Final Degree Project in Biochemistry and Molecular Biology

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Abbreviations

AT → Adipose tissue(s)
CAF diet → Cafeteria diet
CVD → Cardiovascular diseases
DIO → Diet-induced obesity
eWAT → Epididymal white adipose tissue
GSPE → Grape Seed Proanthocyanidin Extract
HFD → High-fat diet
iWAT →Inguinal white adipose tissue
IR → Insulin resistance
LD → Liver dysfunction
MAFLD → Metabolic-associated fatty liver disease
MD → Metabolic dysfunction
NAFLD → Non-alcoholic fatty liver disease
ROS → Reactive oxygen species
SAT →Subcutaneous adipose tissue
SCN → Suprachiasmatic nucleus
STD diet → Standard diet
TCA cycle → Tricarboxylic acid cycle
T2D →Type 2 Diabetes
VAT → Visceral adipose tissue
WAT → White adipose tissue
ZT12 → Dark/nocturnal phase

Abstract

The obesity, a complex and multifactorial pathology, is related to fat accumulation, chronic inflammation, and insulin resistance, affecting energy homeostasis and metabolic health. It is characterized by the triggering of inflammatory processes mediated by succinate and its receptor (SUCNR1). Previous studies have demonstrated the existence of a rhythmic pattern in SUCNR1 and, on the other hand, the ability of proanthocyanidins to regulate the gene expression of various metabolites. This work focuses on investigating the effects of a cafeteria diet (CAF) on the expression of the succinate receptor (*Sucnr1*) in white adipose tissue (WAT) and liver of rats, and to analyze whether the grape seed procyanidin extract (GSPE) can restore the circadian rhythmic patterns of gene expression altered by such a diet. The hypothesis of this study therefore proposes that the cafeteria diet alters the circadian expression of the *Sucnr1* receptor in these tissues, while the consumption of GSPE could restore these rhythmic patterns.

To test this hypothesis, Fischer 344 male rats were fed with a standard diet (STD) or CAF for 9 weeks, and some received GSPE in the last 4 weeks. Samples of epididymal, inguinal adipose tissue, and liver were taken at different times of the day and analyzed by RT-qPCR to evaluate the circadian rhythmicity of *Sucnr1* gene expression. The results showed that *Sucnr1* gene expression follows a rhythmic pattern in adipose tissue, but not in the liver. The CAF diet significantly altered this rhythm in adipose tissues, reducing the amplitude of oscillations and shifting the acrophase, indicating a disruption of the circadian rhythm, while the liver did not show a clear rhythmic pattern. Additionally, GSPE modulated these gene expression rhythmic patterns, which could be relevant for the treatment of metabolic disorders associated with obesity.

In conclusion, the CAF diet deregulates the circadian rhythms of *Sucnr1* receptor expression in adipose tissue, potentially contributing to metabolic complications, while GSPE emerges as a possible intervention to mitigate these effects, regulating circadian rhythmicity and improving metabolic health in the context of obesity.

Keywords: Diet-induced obesity, eWAT, inflammation, iWAT and liver

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1. Introduction

Obesity is a complex and multifactorial pathology that can be associated with altered eating behaviours or be a secondary consequence of genetic, hypothalamic, iatrogenic, or endocrine diseases(1). This condition affects numerous organs in the body, causing metabolic deterioration characterized by inflammatory processes, which are influenced by the interaction between the genome and the environment, resulting in a specific phenotypic expression, mainly an increase in adipose tissue (AT)(2).

The cornerstone of obesity is adiposopathy, also known as "sick fat," defined as the pathological, anatomical and functional alterations of AT, driven by a positive caloric balance in individuals susceptible both environmentally and genetically, leading to immune and endocrine responses that can cause or exacerbate metabolic diseases. Therefore, we can define obesity as a primary disease since adiposopathy causes dysregulation of metabolic pathways(1).

Obesity is closely related to numerous health problems, such as type 2 diabetes (T2D), cardiovascular diseases (CVD), and liver dysfunction (LD), increasing the risk of developing more than 200 chronic diseases. It is considered a severely debilitating condition on its own, as excess body fat can cause structural and functional abnormalities that diminish the quality of life, such as gastroesophageal reflux, osteoarthritis, obesity hypoventilation, psychological and eating disorders, decreased physical performance, anxiety, or depression. Among the most severe consequences, we can include diabetes, hypertension, myocardial infarction, and other major cardiovascular events(1).

The progression from overweight to obesity involves fat accumulation, chronic inflammation, tissue hypoxia, endoplasmic reticulum stress, and insulin resistance (IR)(2). Increased fat triggers cytokine production, leading to IR, inflammation, and CVD. Excessive secretion of proinflammatory adipokines by adipocytes and macrophages in people with obesity creates a low-grade systemic inflammatory state(3). Macrophages play a central role in inflammation and IR as they infiltrate target organs, become activated, and produce abundant proinflammatory cytokines, ultimately negatively affecting insulin signalling(4).

AT plays a central role in maintaining energy homeostasis and body weight. Adipocytes in white, brown, and beige adipose tissue are essential for this role(5). These mature adipocytes are highly specialized cells, fundamental for energy storage and supply, under strict central and peripheral control. The primary function of white adipose tissue (WAT) is energy storage in the form of triglycerides, lipid metabolism and mobilization, along with the secretion of adipokines and lipokines that act as metabolic hormones. Additionally, it produces a wide variety of proteins related to immunity, inflammation, the fibrinolytic system, and the renin-angiotensin system(2,5,6).

In contrast, brown and beige adipocytes promote energy expenditure and heat production through stored energy, thereby regulating body temperature through thermogenesis, using uncoupling proteins

like UCP1 and activating beta-adrenergic receptors. Beige adipocytes arise in response to stress situations(2,5,6).

The hormonal and molecular regulation of energy metabolism in adipocytes is complex. For example, estrogen helps combat obesity by regulating energy intake and expenditure, influencing fat distribution, and acting as a protective barrier against visceral adiposity through its receptors(7). Additionally, AT interacts with the nervous system to maintain energy homeostasis: the sympathetic nervous system regulates thermogenic activation of brown and beige tissues, fatty acid oxidation, lipolysis, lipogenesis, and browning of white tissue(8). On the other hand, the enzyme AMPK acts as a regulator of intake, energy balance, thermogenesis, and glucose homeostasis, as excess glucose can inhibit the hypothalamus's ability to activate AMPK, leading to prolonged hypoglycemia(2).

Hypercaloric diets are characterized by an excess intake of calories, significantly impacting metabolic health. Chronic overeating with these types of diets, which exceed energy expenditure and storage, can cause a metabolic imbalance that will ultimately lead to cellular and tissue dysfunction and disease(9).

It has been shown that hypercaloric diets rich in fats and sucrose can cause diabetes, obesity, fatty liver disease, and chronic inflammation, as shown in *Figure 1*. Additionally, they can exacerbate age-related dysmetabolism, affecting the carotid body function and metabolic parameters, including the deterioration of organ structure and function due to oxidative stress, increased inflammation, genetic instability, and alteration of homeostatic pathways(10).

These diets affect circadian rhythms in various ways: firstly, diet-induced obesity (DIO) alters locomotor activity, decreasing during the dark phase and, in some cases, also disrupting the light phase, creating an arrhythmic pattern. Additionally, eating behaviour is primarily affected, evidenced by overeating and a fragmented feeding pattern. These diets quickly increase fat stores, thus affecting leptin and corticosterone levels within a few days. Furthermore, they significantly alter the expression of so-called “clock genes” in peripheral organs such as AT and the liver(11,12).

DIO also causes leptin resistance, T2D, and alterations in glucose tolerance. In rats, short-term consumption of high-fat diets (HFD) affects the circadian rhythm of the dorsomedial hypothalamus, modifying cellular activity and food intake regulation. Likewise, the diet influences the composition of the gut microbiome, which in turn impacts the circadian rhythms of the liver and hypothalamus(13).

Hypercaloric diets, especially those high in fats or salt, affect circadian rhythms through various mechanisms, as they modify gene expression and hormonal activity, impacting metabolic processes. Dietary factors reprogram the circadian clock through epigenetic processes (such as transcriptional and post-translational modifications), consequently leading to dysregulation of metabolic homeostasis and the development of metabolism-related diseases(12–15).

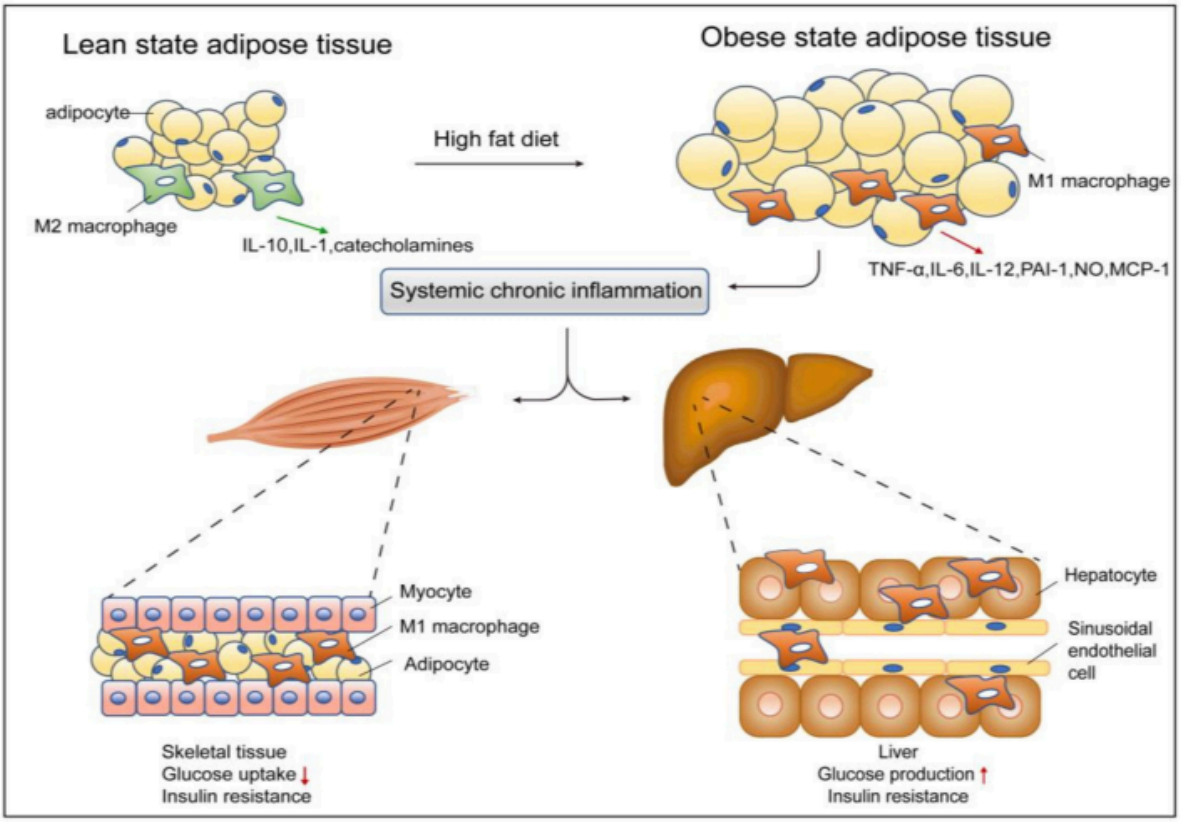

Figure 1. Representation of the effects that obesity produces on adipose tissue, liver and skeletal tissue. *In lean states, macrophages in AT help maintain insulin sensitivity. In obesity, excess nutrients cause inflammation in adipose and other tissues, worsening IR*(4).

1.1. White adipose tissue

1.1.1. Characteristics and functions of WAT

Most WAT is classified as subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). In rodents, SAT is subdivided into anterior and posterior, analogous to upper and lower body subcutaneous fat in humans. The main VAT depots include those surrounding the heart (epicardial/pericardial) and intra-abdominal organs (mesenteric, omental, perigonadal, perirenal, and retroperitoneal) in both humans and rodents (*Figure 2*)(16). The location of WAT is critical, as an increase in visceral adipose mass is associated with a higher risk of metabolic disorders like IR, while SAT can have a protective effect and improve insulin sensitivity(8).

WAT is a crucial metabolic organ involved in energy storage, metabolic regulation, and adipocytokine secretion. Its main function is to store excess energy in triglycerides, contributing to overall energy balance. During times of positive energy balance, such as a HFD, WAT accumulates energy in the form of lipids; however, during times of negative energy balance, such as during exercise or fasting, it mobilizes this energy by releasing fatty acids and glycerol through lipolysis. Thus, WAT plays a fundamental role in energy homeostasis(8).

WAT also produces proteins with various functions related to immunity, proinflammatory cytokines, complement, the fibrinolytic system, the renin-angiotensin system, lipid mobilization, and steroid enzymes. Additionally, it produces large amounts of adipokines and lipokines, which act as regulatory metabolic hormones, but under conditions of excess energy, adipocyte functionality decreases, causing an imbalance in adipokine production. However, when there is an excess of energy, WAT can expand through hyperplasia and hypertrophy, eventually leading to dysfunctions, increasing cardiometabolic risk due to greater lipid deposition and decreased utilization(2).

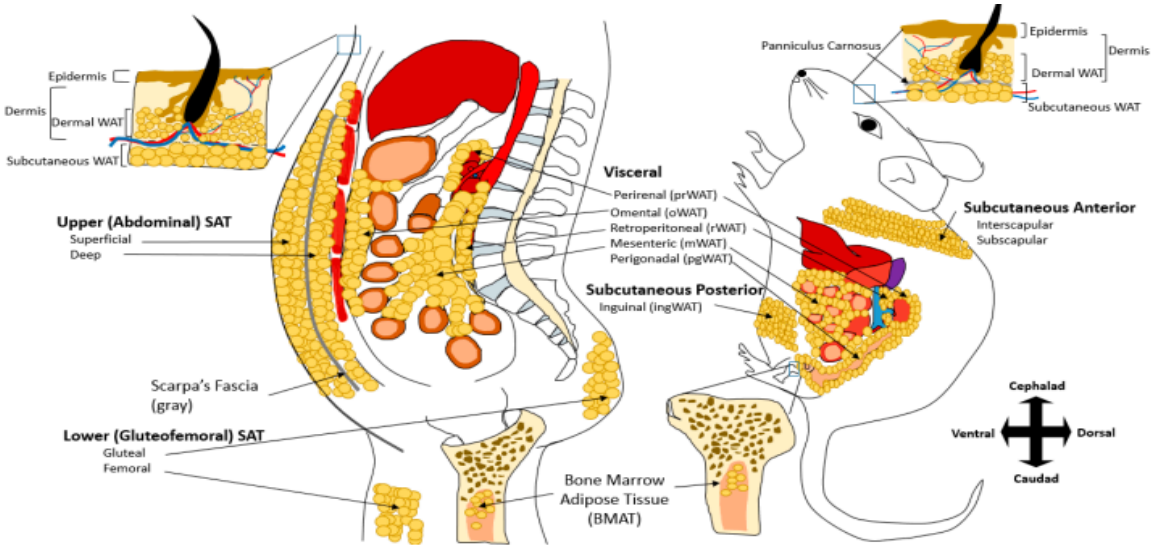

Figure 2. Comparative analysis of human and rodent adipose tissues. *In the figure, the different types of WAT and their locations in both humans and rodents can be observed, highlighting which are found in both models and which are specific to each species*(16).

1.1.2. Epididymal white adipose tissue (eWAT)

The epididymal adipose tissue in rats is located around the testes and is an integral part of VAT, situated adjacent to the epididymis, a tubular structure at the back of the testis. Its proximity to the male reproductive system allows it to influence reproductive function and health. This tissue is primarily composed of mature adipocytes, along with immune and endothelial cells, and is known for its role in inflammation and lipid metabolism(17).

Various studies have shown that eWAT responds specifically to hormonal and dietary changes, showing particular sensitivity to leptin and other factors related to obesity and IR(17). This tissue is crucial for studying adipose tissue macrophages and their influence on metabolic disorders. It has been demonstrated that macrophage infiltration in eWAT contributes to obesity-induced inflammation and metabolic abnormalities, making it a key area for understanding the pathophysiology of this condition(18).

On the other hand, excessive hypertrophy of adipocytes in eWAT can lead to fibrosis, inflammation, and metabolic dysfunction (MD), contributing to the development of obesity-related complications. Furthermore, alteration in the remodelling of the extracellular matrix in this tissue can affect

adipogenesis, the process of forming new fat cells, further exacerbating the metabolic complications associated with obesity(19).

In this pathology, the accumulation of ectopic adipose tissue, especially around visceral areas like eWAT, can cause IR, chronic inflammation, and metabolic alterations. This is why this tissue plays an essential role in regulating metabolism by secreting cytokines that affect various organs and tissues, especially in DIO(20).

Additionally, eWAT participates in regulating the body's energy homeostasis and acts as an immunological effector site, containing immune cells that produce factors with immunoregulatory properties, indicating its role beyond energy balance regulation in host defense against pathogens. It also involves collecting, storing, and releasing energy in the form of lipids, contributing to systemic energy homeostasis(21).

1.1.3. Inguinal white adipose tissue (iWAT)

The inguinal adipose tissue in rats is located subcutaneously in the inguinal region, extending towards the lower abdomen. It surrounds the main blood vessels and nerves of the groin and is divided into compartments by thin connective tissue sheets. This tissue stands out for its ability to respond to environmental and metabolic stimuli, such as changes in diet and temperature. Additionally, it can experience variations in its cellular composition and gene expression, influenced by hormonal and nutritional factors(22).

iWAT is fundamental in the research of obesity and MD due to its involvement in the plasticity and dysfunction of AT. Various studies have demonstrated that hypertrophic inguinal adipocytes, or enlarged fat cells, can contribute to systemic IR, a key factor in MD(23). This tissue plays a crucial role in regulating metabolism and energy homeostasis through compensatory mechanisms in response to disturbances in other fat depots. This tissue shows greater insulin signalling, significantly contributing to overall body insulin sensitivity, highlighting its importance in maintaining metabolic homeostasis(24).

Additionally, inguinal fat exhibits remarkable flexibility compared to perigonadal fat, adapting better to changes in nutrient load and various metabolic conditions: this adaptability reflects its capacity to respond to different metabolic demands(24). iWAT also plays a vital role in metabolic regulation by collecting, storing, and releasing energy in the form of lipids, thus contributing to systemic energy homeostasis. Therefore, it functions as an essential anabolic tissue for the body's energy balance. It also contains various innate and adaptive immune cells, making it an important immunological organ with roles in antimicrobial defense, wound healing, and inflammation(21).

1.1.4. Metabolism and dysfunction of WAT

In a positive energy balance, AT stores the excess energy as triglycerides, leading to its expansion, as previously mentioned. Although this expansion is a physiological mechanism for storing energy, unhealthy expansion is associated with MD. During overfeeding, there is a compensatory increase in total energy expenditure, but it is usually not enough to balance energy intake, leading to AT expansion(25).

WAT dysfunction plays a crucial role in the development of obesity and its associated complications. This dysfunctional tissue is characterized by the presence of enlarged fat cells, low-grade inflammation, altered redox homeostasis, and cellular senescence. The inability of AT precursor cells to form new adipocytes is a key factor in this dysregulation, causing ectopic lipid overflow into non-adipose tissues (lipotoxicity) and altered secretion of adipose-derived hormone(26).

The term "unhealthy expansion" of AT refers to its dysfunctionality, characterized by hypersecretion of proinflammatory adipokines, decreased secretion of anti-inflammatory adipokines, loss of energy storage capacity, and lack of coordination between adipocyte expansion and extracellular matrix remodelling. This harmful expansion and the resulting metaflammation impair insulin signalling pathways in the adipocyte, reducing its ability to store energy. As a result, there is a chronic increase in circulating free fatty acids, promoting lipid deposition in ectopic tissues and causing lipotoxicity. Understanding and measuring WAT dysfunction can help identify individuals at risk of developing obesity-related complications, allowing for targeted interventions to effectively prevent or manage these health issues(25).

WAT dysfunction is closely related to the disruption of circadian rhythms, especially under the influence of hypercaloric diets. Consumption of a HFD disrupts the diurnal rhythm of food intake and can have pathogenic consequences, affecting circadian rhythms in peripheral organs such as WAT. In DIO, AT undergoes circadian reprogramming, and HFD feeding disrupts rhythmic pathways related to the circadian rhythm, ribosome biogenesis, and nucleosome organization. In obesogenic contexts, this tissue's dysfunction is associated with changes in the expression of circadian clock genes: the circadian clock in WAT is more susceptible to attenuation by HFD compared to the hepatic circadian clock, highlighting the impact of hypercaloric diets on circadian rhythms in different organs(27).

1.2. Liver

1.2.1. Importance of the liver in metabolism

The liver is the central organ in the metabolism of carbohydrates, lipids, and proteins, processes that are closely interconnected. It plays a crucial role in maintaining blood glucose levels through glycogenesis, glycogenolysis, and gluconeogenesis, storing glucose as glycogen and releasing it as needed to stabilize blood sugar levels(28,29).

In lipid metabolism, it produces cholesterol, phospholipids, and lipoproteins, and converts excess carbohydrates and proteins into fatty acids and triglycerides for storage or energy production. It regulates lipogenesis, converting glucose into fatty acids, and lipolysis, breaking down fats into fatty acids and glycerol for energy(28,29).

In protein metabolism, the liver synthesizes plasma proteins such as albumin, clotting factors, and complement proteins. It also metabolizes amino acids, synthesizes urea, and detoxifies ammonia, a byproduct of protein metabolism. Additionally, it participates in the deamination of amino acids to produce energy or convert them into glucose or fat as needed by the body(28,29).

The liver also regulates various metabolic pathways in response to hormonal signals and nutrient levels, coordinating the balance between energy storage and utilization to ensure a constant supply of nutrients. Besides its role in metabolism, it detoxifies harmful substances such as drugs and toxins, converting them into less harmful compounds for excretion, and removes waste products from the blood, maintaining the body's overall homeostasis(28).

1.2.2. Impact of cafeteria (CAF) diets on liver function

Exposure to a CAF diet has been shown to induce obesity and increase liver fat accumulation in animal models. Various studies indicate that rats fed a CAF diet exhibit significant weight gain, dyslipidemia, and elevated levels of triacylglycerol and cholesterol in the liver, evidencing the development of obesity and hepatic fat accumulation. This type of diet causes a notable increase in fat accumulation in the rats' livers, leading to alterations in liver structure and function, reflected in changes in liver function markers(30).

CAF diets, particularly those rich in fats, have been shown to induce LD function in male rats, leading to conditions such as obesity, glucose intolerance, and high blood pressure. These diets can also trigger oxidative stress in the liver, characterized by an imbalance between the production of reactive oxygen species (ROS) and antioxidant defense mechanisms, resulting in liver damage. Inflammation is another key mechanism associated with LD induced by CAF diets(31).

LD can lead to the development of metabolic syndrome, often associated with risk factors such as obesity and diabetes, further exacerbating the impact on overall health. This dysfunction also affects lipid management and metabolism, which can cause issues with cellular lipid handling, mitochondrial function, insulin sensitivity, and ion pump exchange, all of which are crucial for maintaining metabolic health(32).

1.2.3. Influence of circadian rhythms on hepatic metabolism and energy homeostasis

Circadian rhythms play a crucial role in regulating hepatic metabolism and energy homeostasis. The liver, being a central organ in energy metabolism, has approximately 10% of its transcriptome

expressed rhythmically, suggesting that the circadian clock regulates a significant number of liver genes. This clock controls the expression of key transcription factors in the liver, regulating the expression of metabolic genes in a time-specific manner, contributing to the overall coordination of energy metabolism(33).

Circadian rhythms affect the timing of food intake, hepatic glucose production, lipid metabolism, and bile acid synthesis, thereby contributing to overall energy homeostasis. Disruptions in these rhythms, such as those caused by shift work or irregular eating patterns, can lead to MD in the liver, contributing to conditions like metabolic-associated fatty liver disease (MAFLD). Proper alignment of circadian rhythms with daily light-dark cycles is essential for optimal liver function and metabolic health(34).

The alteration of the hepatic clock, frequently observed in conditions like non-alcoholic fatty liver disease (NAFLD), can cause metabolic disorders and glucose metabolism disruption, ultimately contributing to liver cancer development. Disruption of circadian rhythms can activate oncogenic pathways in the liver, promoting hepatocellular carcinoma development. Therefore, understanding the interaction between circadian rhythms, hepatic metabolism, and energy homeostasis is of particular interest in identifying potential targets for liver cancer prevention and treatment(35).

1.3. Succinate

1.3.1. Function of succinate in cellular metabolism

Succinate is an essential metabolite in the tricarboxylic acid (TCA) cycle, performing multiple roles in metabolism and cellular signaling. During the TCA cycle, succinate is converted into fumarate by the action of succinate dehydrogenase (SDH), a key enzyme that also acts as a tumor suppressor. As an intermediate in the TCA cycle, succinate is crucial for the cell. In this process, succinyl-CoA, along with CO2 and nicotinamide adenine dinucleotide (NADH), is produced by the oxoglutarate dehydrogenase complex (OGDC), also known as the alpha-ketoglutarate dehydrogenase complex (α-KGDH). Subsequently, succinyl-CoA is utilized by succinate-CoA ligase (SUCL), composed of a heterodimer that includes an invariant α subunit (SUCLG1) and a β subunit, which can be SUCLA2 or SUCLG2. Depending on the association with SUCLA2 or SUCLG2, the products will be the substrate-level phosphorylation of ADP or GDP, along with the release of a succinate molecule. Succinate dehydrogenase (SDH) plays a crucial role at the intersection of the TCA cycle and the electron transport chain (ETC), as SDH is complex II of the ETC. The reaction catalyzed by SDH converts succinate into fumarate and FADH2 (flavin adenine dinucleotide). Besides its involvement in energy metabolism, succinate and succinyl-CoA also serve as entry points into the TCA cycle for anaplerotic reactions of branched-chain amino acids or even propionate. This reinforces the role of succinate as a key node in mitochondrial metabolism(36,37).

Succinate, long known as a key intermediary in mitochondrial metabolism, also acts as a crucial oncometabolite influencing cell differentiation and proliferation, especially due to its effect on various

methylases involved in gene expression control. Thus, the metabolic balance of succinate is essential during the development of living organisms. Through succinate-dependent prolyl hydroxylases, it also controls the response to important factors in aerobic organisms and their various responses to hypoxia(38).

1.3.2. Regulation of inflammation by succinate

Succinate, a key immunometabolite, modulates inflammatory responses through various mechanisms in different tissues. Depending on the cellular context, it can act as both a pro-inflammatory and anti-inflammatory mediator. Its interaction with the SUCNR1 receptor regulates immune responses, inflammation, and tissue repair. When succinate levels rise, it inhibits prolyl hydroxylases (PHD) by product inhibition, activating the HIF-1 transcriptional pathway. This activation induces the secretion of interleukin-1β (IL-1β) in inflammatory macrophages, potentially leading to the development of inflammatory diseases. Although reducing PHD inhibition could mitigate disease progression, succinate can induce inflammatory responses through an alternative pathway(39).

In certain activated innate immune cells or cells relying on anaerobic glycolysis under hypoxic conditions, the amount of mitochondrial succinate increases and is then released into the cytosol. Its accumulation in the cytosol is linked to protein succinylation, stabilization of the HIF-1α transcription factor, epigenetic regulation, and the production of ROS(40).

The succinate-SUCNR1 signalling axis plays a complex role in immune responses. Dendritic cells detect immunological danger through SUCNR1, enhancing antigen-presenting functions necessary for optimal immune activation. There is solid evidence that succinate-SUCNR1 signalling serves as a crucial link between metabolic stress and inflammation(40).

1.3.3. SUCNR1 signalling pathway

Succinate interacts with its membrane receptor, SUCNR1, modulating immune responses. This receptor is expressed in various tissues and immune cells, with its expression regulated by extracellular succinate concentration, which is normally low under physiological conditions. The SUCNR1 receptor, also known as GPR91, is a G-protein-coupled receptor composed of a βγ dimer and an α subunit. When succinate binds to SUCNR1, the α and βγ subunits dissociate, inducing signalling through the mitogen-activated protein kinase (MAPK) pathway. This results in the transcription of a set of genes that vary depending on the cell type(39).

Succinate is produced from α-ketoglutarate and rapidly oxidized to fumarate under normal conditions. However, under stress or metabolic alterations, mitochondrial succinate can accumulate and be transported to the cytosol and extracellular space. Extracellular succinate can be reabsorbed by the solute carrier family 13 (SLC13) and monocarboxylate transporters (MCT), as observed in *Figure 3*. Activation of SUCNR1 by high levels of succinate triggers the activation of Gq and Gi proteins, which initiate various signalling pathways. The Gq pathway activates protein kinase C (PKC) and MAPK

cascades, as well as calcium mobilization. The Gi pathway inhibits cyclic adenosine monophosphate (cAMP) production. This succinate-SUCNR1 axis regulates inflammatory processes through these signalling routes(41).

Alterations in succinate levels are associated with inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease, obesity, and atherosclerosis due to exaggerated immune responses. Targeting succinate pathways offers potential therapies for these diseases, highlighting the importance of understanding the role of succinate in inflammation to develop therapeutic interventions(41).

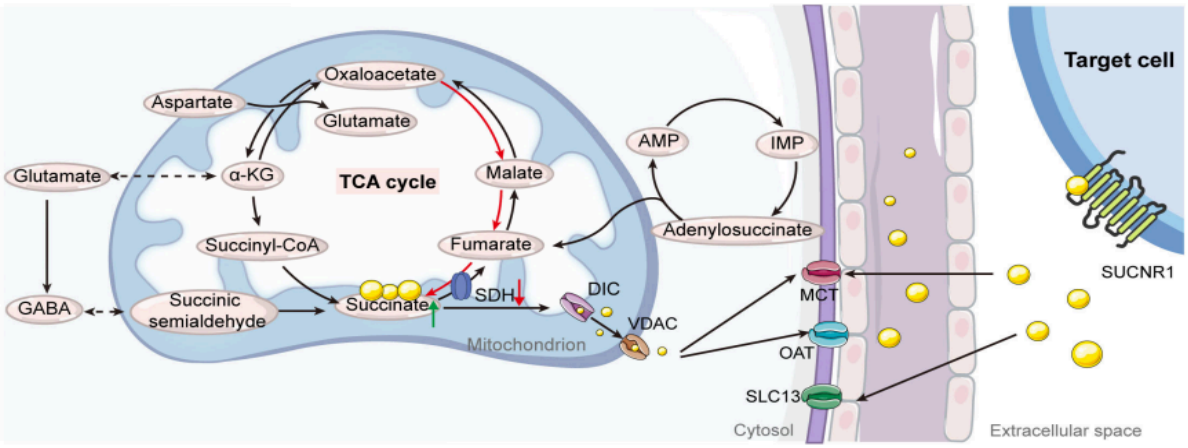

Figure 3. Illustration of the pathways of succinate production and transport. *When mitochondrial succinate levels exceed cellular requirements, it is transported into the cytosol through mitochondrial dicarboxylate carriers (DICs) and voltage-dependent anion channels (VDACs). The efflux of succinate from the cytosol to the extracellular space is mediated by organic anion transporters (OATs) and monocarboxylate transporters (MCTs)*(41).

1.3.4. Evidence of circadian patterning in SUCNR1

The most recent studies have demonstrated the existence of a rhythmic expression pattern of SUCNR1, a succinate receptor. This circadian rhythm is regulated by the central clock component BMAL1, which directly binds to the SUCNR1 promoter to modulate its expression and is crucial for the regulation of energy metabolism and adipocyte function. The circadian regulation of SUCNR1 in adipocytes is interconnected with the molecular clock machinery, as evidenced by altered expression of central clock genes such as Per2, Cry1, and Rev-erbα in SUCNR1 knockout adipocytes. This indicates a bidirectional relationship between SUCNR1 signaling and the circadian clock. Moreover, studies have found that the elimination of SUCNR1 disrupts the circadian expression of leptin, a key adipokine involved in energy balance. This disruption leads to MD, highlighting the importance of SUCNR1 in coordinating circadian rhythms and metabolic processes in adipocytes(42). Additionally, unpublished previous Nutrigenomics studies demonstrate that this metabolite follows a circadian pattern in peripheral tissues and plasma.

The alteration of the circadian expression of SUCNR1 can lead to significant metabolic dysregulation. This is particularly evident in the altered expression of leptin, a key adipokine involved in energy balance. Changes in leptin levels can affect appetite regulation and energy expenditure, potentially leading to obesity or weight gain. Furthermore, the circadian regulation of SUCNR1 is crucial for maintaining energy metabolism. An altered rhythm can disrupt normal metabolic processes, leading to inefficient energy utilization and storage. This can manifest as increased fat accumulation or altered glucose metabolism, contributing to conditions such as IR(43).

SUCNR1 also plays a critical role in adipocyte function. Changes in its expression can affect adipocyte differentiation and lipid accumulation. The relationship between SUCNR1 and central clock genes such as Per2, Cry1, and Rev-Erbα suggests that altered SUCNR1 expression can change the expression of these genes. This alteration can further exacerbate metabolic issues since the circadian clock is essential for regulating various physiological processes, including hormone secretion and metabolism. The interaction between SUCNR1 signaling and circadian rhythms indicates that alterations in SUCNR1 could increase the risk of metabolic disorders such as obesity, diabetes, and CVD(43).

1.4. Importance of circadian rhythms in health and metabolism

The suprachiasmatic nucleus (SCN) receives light information from the retina and regulates peripheral clocks to coordinate circadian rhythms. Additionally, non-light factors such as food, exercise, sleep, and temperature also influence circadian rhythms through central and peripheral clocks (*Figure 4*). Various biological processes, such as sleep-wake cycles, blood pressure, body temperature, hormone secretion, and energy metabolism, exhibit rhythmic fluctuations. Disruption of the circadian system, whether by deactivation of circadian genes, changes in the light/dark cycle, shift work, or jet lag, contributes to obesity and complications like hyperglycemia and IR, imposing a significant burden on health and the economy(44).

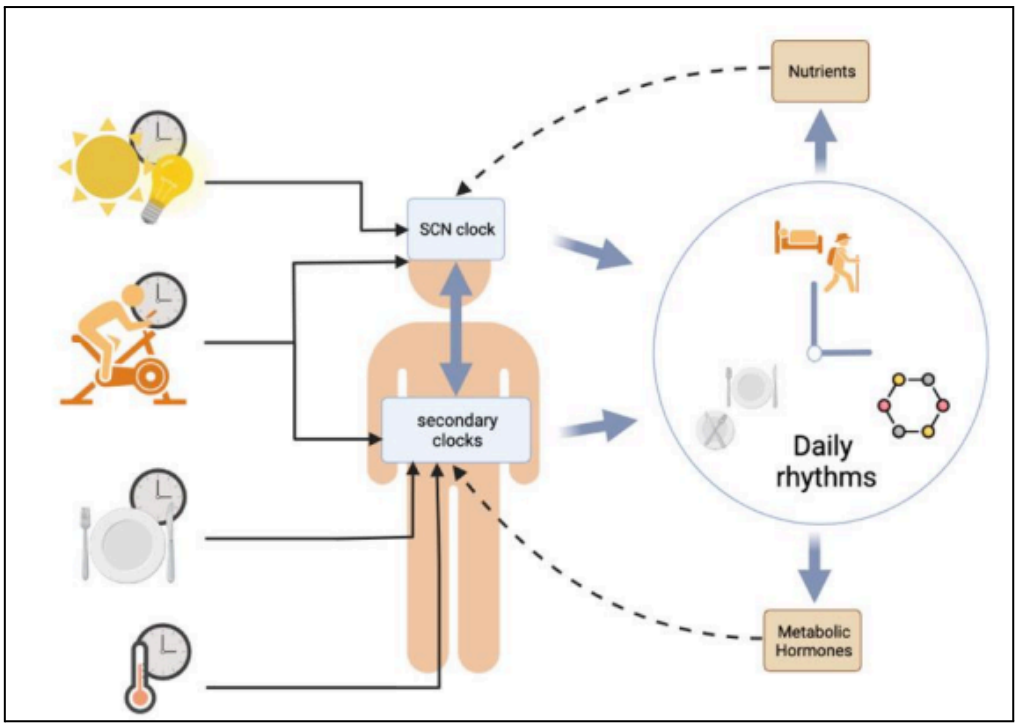

Figure 4. Diagram of the connections between food intake, metabolic energy, and circadian clocks. *The main circadian clock in the hypothalamus and peripheral clocks synchronize with light, physical activity, meals, and temperature, generating daily rhythms such as sleep-wake and hormonal cycles, which in turn regulate these clocks through metabolic hormones and nutritional needs*(15).

Metabolism, food intake, and physical activity can regulate or desynchronize circadian rhythms, creating a vicious cycle between circadian disruption and metabolic disturbances. The deregulation of central and peripheral clocks is linked to IR and associated diseases, highlighting the importance of maintaining synchronized lifestyle habits for health. Better understanding these interactions is crucial and will enable the development of new chronotherapeutic dietary approaches to improve overall health(15).

Circadian rhythms play an essential role in maintaining metabolic homeostasis, encompassing the metabolism of carbohydrates, lipids, and proteins. These rhythms regulate the temporal synchronization of energy metabolism, glucose utilization, insulin sensitivity, and lipid homeostasis, influencing the development of metabolic diseases such as obesity and T2D. Our society's 24/7 lifestyle has led to a significant increase in cardiometabolic diseases worldwide, causing chronic desynchronization between the internal circadian system and external synchronizing signals such as light and food, a phenomenon known as circadian misalignment(45).

1.4.1. Factors that affect circadian rhythms

The synchronization of circadian rhythms is strongly influenced by light and dark cycles, as highlighted in various studies. The circadian timing system resembles a complex clockwork with multiple clocks distributed throughout the body, rather than a single central clock. This robust

rhythmicity is essential for the temporal coordination of organ functions, and its disruption or misalignment is closely associated with various widespread diseases(46).

Due to the Earth's rotation, physiological adjustments also correspond to seasonal changes to enhance survival and reproductive success. These adjustments are based on cues such as temperature, rainfall patterns, food availability, and the length of daily light exposure, also known as photoperiod. The latter is the most influential factor in the properties of circadian rhythms, affecting phase, amplitude, and the degree of synchronization(46).

In humans, light exposure affects melatonin secretion, altering circadian rhythms and sleep-wake cycles; thus, interventions like circadian lighting show potential in psychiatric care centers. The absorption of light by receptors in the eyes and skin influences various aspects of human health, including circadian rhythms, mood, perception, and chemical reactions within the body, such as the synthesis of vitamin D3. Therefore, manipulating light exposure during clinical treatment can yield numerous benefits for patients(47).

The sleep-wake cycle is the most evident example of circadian rhythm in humans, but many other physiological processes and behaviours also show rhythmicity, including body temperature, hormone release, metabolism, and gene expression. The role of light in resetting the phase of the SCN depends on the timing, duration, intensity, and wavelength of exposure. Even a small change in the timing of light exposure can disrupt melatonin production and cause a phase shift in circadian rhythms(47).

Stress, defined as the body's nonspecific physiological response to any negative or aversive stimulus, can alter peripheral organ rhythms, affecting overall well-being and health. Uncontrollable stress, especially when chronic, can cause health problems such as depression, anxiety, hypertension, heart attack, obesity, T2D, and impaired immune function. Numerous studies suggest that chronic, uncontrollable stress can also alter endogenous circadian rhythms(48).

Polyphenols, abundant in fruits and vegetables, have the ability to modulate biological rhythms. The timing of their consumption can influence the functioning of these rhythms, suggesting that consuming polyphenol-rich foods at specific times could enhance their beneficial effects on health. Research has shown that a polyphenol-rich diet can impact circadian rhythms, which in turn affects metabolism. This relationship is key to understanding how dietary patterns can be optimized to align with our biological clocks, thereby improving metabolic health. Polyphenols may play a crucial role in preventing or treating chronic diseases by modulating circadian rhythms. For example, their intake can help regulate physiological responses linked to conditions like obesity and metabolic syndrome, emphasizing the importance of timing in dietary consumption(49).

1.5. Grape Seed Proanthocyanidin Extract (GSPE)

1.5.1. GSPE description

Proanthocyanidins are a class of polyphenolic compounds garnering significant interest in the nutraceutical field due to their potential health benefits. These compounds are ubiquitous and represent the second most abundant type of natural phenol after lignin. Structurally, proanthocyanidins belong to the flavonoid class and are oligomers of (epi)catechin monomeric units, which can be differentiated into type A or type B based on their interflavan bonds. Previous studies have shown that type B GSPE have beneficial effects on both WAT physiology and the regulation of the WAT circadian clock(50). The main constituents of GSPE are monomeric, dimeric, and trimeric flavan-3-ol procyanidins, with relatively low proportions of larger polymers (*Figure 5*)(51).

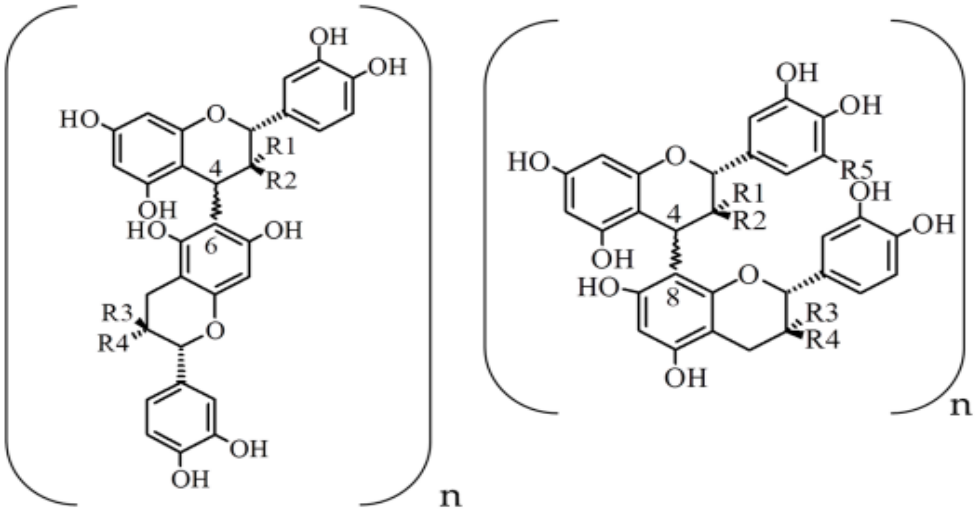

Figure 5. Structural illustration of B-type proanthocyanidin units(52)***.***

These proanthocyanidins possess antioxidant, antimicrobial, anti-inflammatory, anti-allergic, anti-obesity, and vasodilatory properties. Epidemiological evidence has linked the consumption of these compounds to a reduced risk of chronic diseases, including certain types of cancer, CVD, and NAFLD. Additionally, GSPE has demonstrated hepatoprotective effects in DIO animal models, helping to prevent the progression of steatosis and NAFLD. Furthermore, there is increasing evidence suggesting that polyphenols can also affect circadian rhythms by acting on the SCN and the expression of circadian clock genes in peripheral tissues(51).

1.5.2. Antioxidant and anti-inflammatory properties of GSPE

GSPE possesses various pharmacological properties, including anticancer, antiallergic, anti-inflammatory, antihypertensive, and antiviral activities. Recent studies have revealed that GSPE prevents amiodarone-induced pulmonary toxicity, effectively neutralizes ROS, and protects against oxidative stress. Research suggests that GSPE can inhibit apoptosis of alveolar epithelial cells caused by oxidative stress, improve pulmonary fibrosis, and reduce H2O2 levels(53).

Additionally, GSPE has been found to reduce lead-induced oxidative stress by activating the Nrf2 pathway, which is crucial for neutralizing ROS. GSPE also suppresses the levels of miR153 and glycogen synthase kinase 3β (GSK-3β), contributing to reduced oxidative stress and providing protection against lead-induced nephrotoxicity(54). GSPE have demonstrated anti-inflammatory effects by inhibiting the phosphorylated activation of the PI3K/Akt/mTOR pathway molecules in dorsal root ganglion neurons, suggesting that GSPE reduces the production of pro-inflammatory mediators(55).

1.5.3. Potential effects of GSPE in metabolic health

Numerous studies have investigated the impact of GSPE on the regulation of lipid, glucose, and energy metabolism. In a study with healthy F344 rats, GSPE was found to modulate hepatic glucose and lipid metabolism in a season-dependent manner, highlighting the importance of circannual rhythms in the regulation of metabolic homeostasis. It was suggested that consuming this compound is beneficial for modulating photoperiod-dependent changes in glucose and lipid metabolism, potentially influencing metabolic diseases such as diabetes, obesity, and CVD(56).

Research has shown that chronic consumption of GSPE can regulate the daily oscillation of key genes related to lipogenesis and gluconeogenesis, helping to reduce triglyceride accumulation and lipid droplet formation in the liver, thereby alleviating NAFLD induced by a HFD. The administration of this extract has also been found to affect the diurnal rhythm of lipid and glucose metabolism in obese rats, restoring the oscillation of liver mass and improving the rhythmicity of total cholesterol levels in the liver. Additionally, studies have highlighted the importance of the timing of GSPE administration, as it has been shown that administering it at the start of the active phase is particularly effective in modulating hepatic genes and metabolites, emphasizing the time-of-day dependent nature of GSPE's effects on metabolism(51).

Research indicates that GSPE modulates the expression of key metabolic genes in the liver, such as SREBP-1c, Gk, and Acacα, which play crucial roles in glucose and lipid metabolism. It also affects metabolites like glucose-6-phosphate, D-glucose, and D-ribose, thereby improving metabolic function. Additionally, it influences hormone levels of corticosterone and melatonin, which are essential for the regulation of metabolism and circadian rhythms, contributing to metabolic homeostasis. Furthermore, this extract affects the expression of hepatic circadian clock genes, such as Bmal1, Cry1, and Nr1d1, coordinating metabolic processes throughout the day and promoting metabolic health. It also influences genes related to endoplasmic reticulum (ER) stress, such as Atf6, Grp78, and Chop, which are involved in the unfolded protein response and cellular stress, potentially preventing metabolic diseases associated with obesity and dysfunction(56).

Supplementation with this compound increases lipolysis in AT, which can prevent excessive body weight gain in certain photoperiods. It also upregulates mRNA levels of adiponectin in all photoperiods, benefiting metabolic health and interacting with photoperiods to influence the

expression of key genes in metabolic pathways, improving metabolic function and energy balance. Although the CAF diet downregulates the expression of inflammatory genes such as Il6 and Tnfa, GSPE can reverse this decrease, modulating the inflammatory response associated with obesity(57).

1.5.4. Interaction between GSPE and circadian rhythms

GSPE interacts with circadian clocks through various mechanisms (*Figure 6*). One of the primary mechanisms is the restoration of the rhythmic expression of hepatic clock genes. Research has shown that GSPE consumption can correct the altered expression patterns of key clock genes, such as Bmal1, Per2, Cry1, and Rorα, which were disrupted by a CAF diet CAF. This suggests that GSPE may help realign the circadian machinery in the liver. Additionally, GSPE appears to influence mitochondrial dynamics, which are closely linked to circadian rhythms. Some studies have observed an increase in genes associated with mitochondrial fusion and a decrease in fission genes during nocturnal GSPE administration. This shift in mitochondrial dynamics may support the liver's energy needs in a circadian manner, thereby affecting overall metabolic processes. The timing of GSPE administration is crucial for its effectiveness. The bibliography indicated that administering GSPE during the nocturnal phase (ZT12) resulted in a more significant regulation of clock gene rhythms compared to diurnal administration (ZT0). This suggests that GSPE may exert its effects in a time-dependent manner, aligning with the body's natural circadian rhythms. Furthermore, GSPE may influence metabolic pathways regulated by circadian rhythms. It has been noted differential behavior in TCA cycle metabolites between diurnal and nocturnal GSPE administration, indicating that GSPE could help modulate metabolic pathways disrupted by a HFD. This regulation may be linked to the timing of energy metabolism in relation to the circadian clock(58).

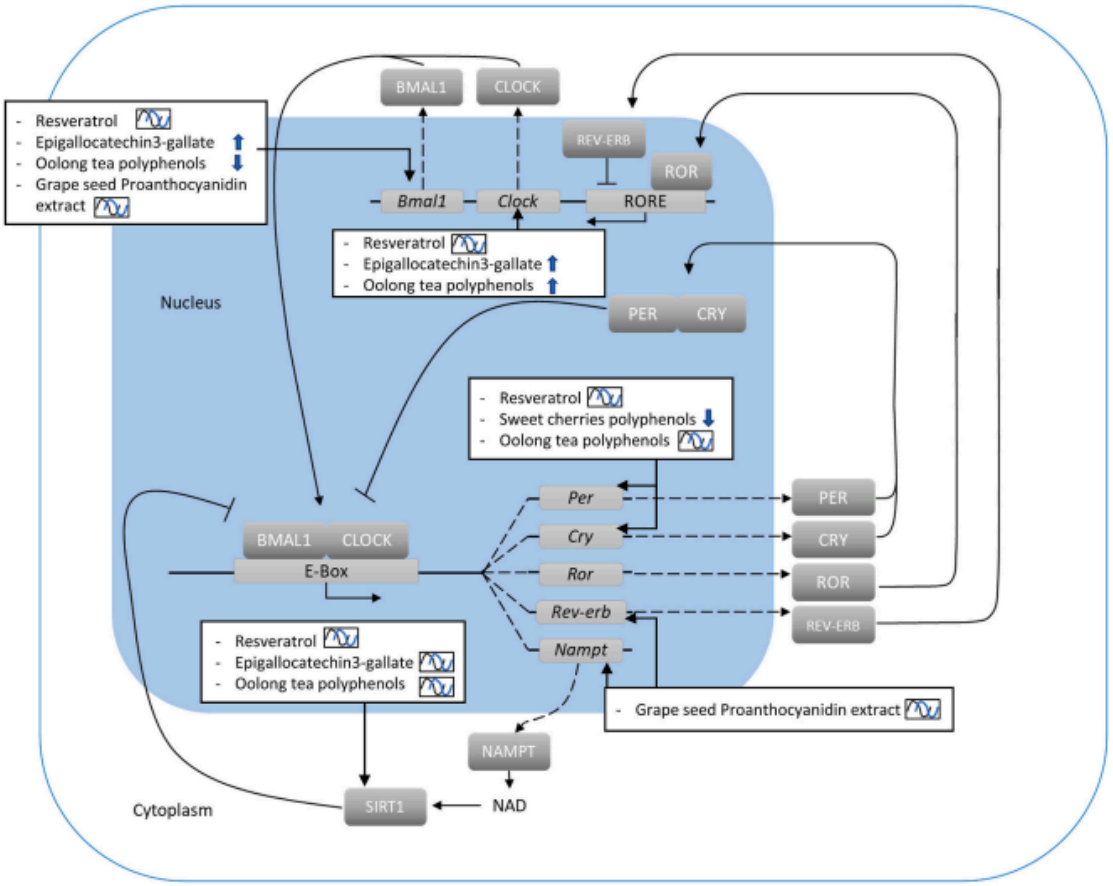

Figure 6. Phenolic regulation of the intracellular circadian machinery. *Phenolic compounds can regulate the intracellular circadian machinery, which operates on a 24-hour cycle. CLOCK and BMAL1 activate circadian genes, while PER and CRY inhibit this activation. These compounds influence key points in this process*(59).

2. Hypothesis and Objectives

The Nutrigenomics research group has previously determined the impact of environmental factors, such as diet, on circadian rhythms and the metabolic homeostasis of peripheral tissues, such as WAT and the liver. Additionally, through metabolomic studies in iWAT and eWAT, the potential existence of a rhythmic pattern in the expression of the succinate has been demonstrated. Likewise, the group's findings suggest that GSPE is a promising candidate for developing a nutritional strategy to reduce inflammation in metabolic diseases, such as obesity. For this reason, it has been decided to study succinate in greater depth, as it is a key immunometabolite in inflammatory processes and could become a clinical target for the development of therapies for inflammation-related pathologies.

Therefore, the hypothesis proposed was that a CAF diet causes a circadian alteration in the gene expression of the succinate receptor in peripheral tissues and that the consumption of GSPE could restore its rhythmic pattern. To demonstrate this hypothesis, the following general and specific objectives were proposed:

1. **To explore the effect of diet and GSPE consumption on the gene expression rhythm of *Sucnr1* in WAT and liver of Fischer 344 male rats**

2. To evaluate whether the gene expression of the succinate receptor *Sucnr1* in WAT and liver of rats shows a circadian rhythmic profile
3. To study the effects of a CAF diet on the gene expression of the succinate receptor *Sucnr1* in WAT and liver of rats
4. To study the effects of GSPE administration on the gene expression of the succinate receptor *Sucnr1* in WAT and liver of rats that were fed with a CAF diet

3. Materials and Methods

3.1. Grape Seed Proanthocyanidin Extract

The GSPE used in this study contained monomers (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%) and proanthocyanidin oligomers of between 5 and 13 units (31.7%), according to the manufacturer (Les Dérivés Résiniques et Terpéniques, Dax, France)(50).

3.2. Experimental Design

The animals used in this experiment were 96 male Fischer 344 rats, 12 weeks old, from Charles River Laboratories (Barcelona, Spain). The animals were housed in pairs under a 12-hour light/dark cycle, at a temperature of 22 °C and 55% humidity, and fed ad libitum with a standard diet (STD) (Panlab A04, Barcelona, Spain) and tap water for one week of adaptation. Subsequently, the animals were randomly divided into two groups according to their diet: 32 rats were fed STD and 64 were fed a CAF diet for 9 weeks. The composition of the STD diet was 76% carbohydrates, 20% proteins, and 4% fats and the CAF diet consisted of cookies with cheese and pâté, bacon, ensaimada, carrot, and milk with 20% sucrose (w/v), with a composition of 51% carbohydrates, 35% fats, and 14% proteins(50).

The treatment period began at week 5 and continued for 4 weeks. The rats fed with STD received 450 µL of the vehicle at the beginning of the light phase (ZT0; n = 16) or the beginning of the dark phase (ZT12; n = 16). The CAF-fed animals were divided into four groups (n = 16 each) that received vehicle or 25 mg of GSPE/kg of body weight at ZT0 or ZT12. The GSPE was dissolved in 450 µL of commercial sweetened skim condensed milk (Nestlé; 100 g: 8.9 g protein, 0.4 g fat, 60.5 g carbohydrates, 1175 kJ). The control groups received the same volume of skim-condensed milk. Two or three days before administration, the rats were trained to lick the milk, thus avoiding oral gavage voluntarily. The treatment was administered orally each day using a syringe(50).

Body weight and food intake were recorded weekly throughout the experiment. At the end of the experiment, each group of 16 animals was randomly divided into four subgroups of four rats to be sacrificed at four different times of the day: ZT1 (9 a.m.), ZT7 (3 p.m.), ZT13 (9 p.m.), and ZT19 (3 a.m.), as shown in *Figure 7*. Before sacrifice, the animals fasted for three hours and were sacrificed by decapitation. Liver and AT samples were stored at -80 °C until further use(50).

Animal experiments were approved by the Animal Ethics Committee of the Universitat Rovira i Virgili (reference number 9495) and were conducted following Council of the European Union Directive 86/609/EEC and the procedures established by the Department of Agriculture, Livestock, and Fisheries of the Generalitat de Catalunya (Barcelona, Spain)(50).

For this work, only the 48 animals corresponding to the ZT12 condition were used, as previous studies by the group have shown that administering the treatment during the dark phase yields better results than during the animals' rest phase.

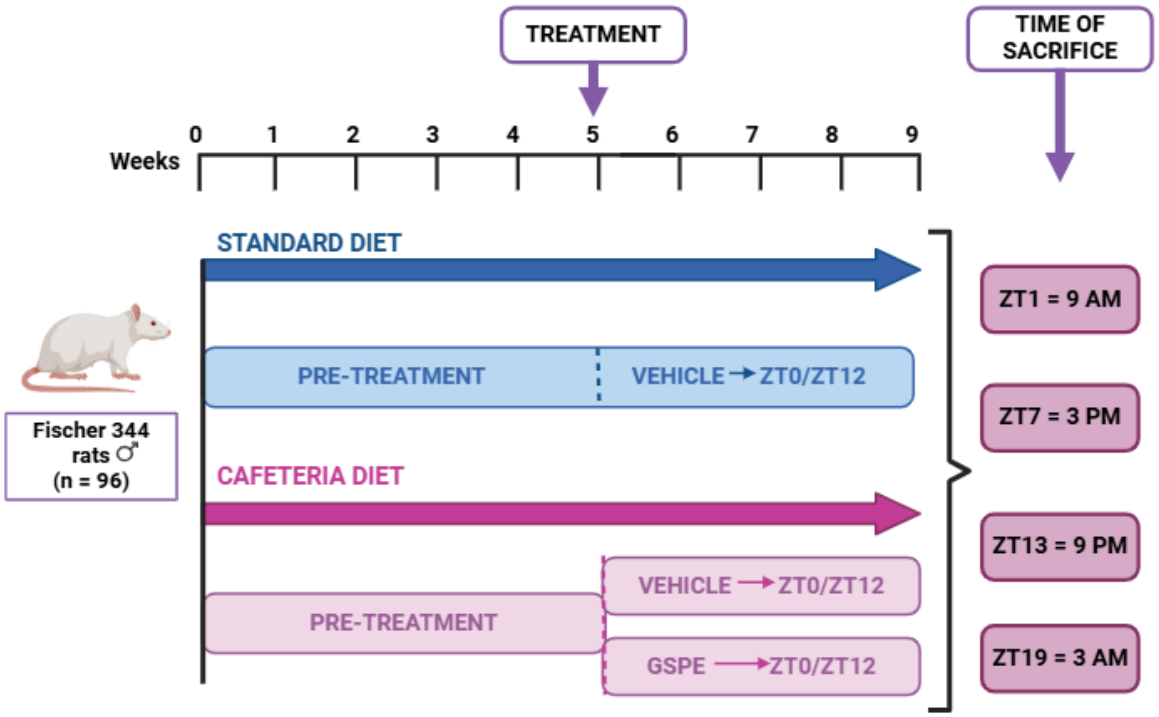

Figure 7. Experimental design for the study of diet-induced obesity in the Fischer 344 rat model. *During the first five weeks of the experiment, one group was fed a STD diet, represented in blue, while the other group was fed a CAF diet, described in pink. From the fifth week onwards, the animals were treated with either a vehicle (VH) or GSPE daily at the beginning of the light phase (ZT0) or the start of the dark phase (ZT12) for four weeks. In the ninth week, the animals were sacrificed at four different times as shown in the figure. Created with BioRender.com*

3.3. Total RNA extraction

The total RNA extraction was performed from eWAT and iWAT, as well as from the liver, previously dissected by the Nutrigenomics group. First, approximately 300 mg of AT and 20-30 mg of liver were weighed in 2 mL Eppendorf using an analytical balance. A metal bead was added to each tube and the samples were kept on ice. Then, 1000 µL of TRIzol® reagent (Invitrogen, Thermo Fisher Scientific, Spain) was added to each sample in the extraction hood, following the manufacturer's protocol. The samples were homogenised at maximum power (50V) for 50 seconds (for liver tissue) and 10 seconds (for adipose tissues), repeating this step until the tissues were completely homogenised in the Fisherbrand™ Bead Mill 24 Homogenizer (Thermo Fisher Scientific, Spain). Subsequently, the tubes were kept on ice for 5 minutes and the samples were centrifuged at 12,000 g in the Hettich® MIKRO 200/200R centrifuge (Andreas Hettich GmbH & Co. KG, Germany) for 10 minutes at 4 °C.(60)

Next, the supernatant was transferred to a new 1.5 mL Eppendorf. A second centrifugation was necessary for the AT to remove fat residues, but this step was not required for the liver. Once the supernatant was obtained from all samples, 250 µL of chloroform (PanReac AppliChem, Spain) was added and immediately vortexed for 15 seconds. The samples were left on ice for 10 minutes until two differentiated phases appeared, after which they were centrifuged at 12,000 g for 15 minutes at 4 °C,

and the upper aqueous phase was transferred to a new 1.5 mL Eppendorf. Then, 500 µL of isopropanol (PanReac AppliChem, Spain) was added, and the samples were manually shaken for 15 seconds, and incubated at room temperature for 10 minutes before being centrifuged again at 12,000 g for 10 minutes at 4 °C. After this centrifugation step, the supernatant was discarded and the pellet was preserved, which was washed with 500 µL of 70% ethanol (Merck KGaA, Darmstadt, Germany). The samples were vortexed to detach the pellet before centrifuging them at 8,000 g for 5 minutes at 4 °C. This process was repeated twice, and after discarding the supernatant the second time, the pellet was allowed to dry completely for approximately 2 hours. Finally, the total RNA was resuspended in 20 µL of RNAse-free water (Invitrogen, Thermo Fisher Scientific, Spain) for AT and in 60 µL of RNAse-free water for liver tissue(60).

Lastly, the quantification and purity measurement of the extracted total RNA was performed using 1 µL of each sample in the NanoDrop® 1000 spectrophotometer (Thermo Fisher Scientific, Spain), using 1 µL of RNAse-free water as a blank(60).

3.3.1. Retrotranscription (RT)

The total RNA extracted from the samples was retrotranscribed to complementary DNA (cDNA) to perform real-time quantitative polymerase chain reactions (RT-qPCR). This protocol consisted of two stages: the first involved eliminating any traces of DNA present in the RNA samples, and the second involved reverse transcribing the RNA in those samples(60).

First, the RNA concentrations of the samples were determined using the NanoDrop. Then, the amount of sample needed to obtain a final RNA concentration of 50 ng/µL in all samples was calculated. These were diluted with the corresponding volume of nuclease-free water to reach a final volume of 30 µL in a 1.5 mL Eppendorf tube (*Annex 1*). Next, 10 µL was taken and added to a 0.5 mL Eppendorf, thus obtaining an RNA concentration of 25 ng/µL in each sample(60).

Second, the reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Spain). The reverse transcription MasterMix included: nuclease-free water, 10x RT Buffer, 10x RT Random Primers, 25x dNTPs Mix, and the MultiScribe Reverse Transcriptase enzyme (50 U/µL). 10 µL of MasterMix was added to each of the samples in the 0.5 mL Eppendorf tubes(60).

Finally, the samples were incubated in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Madrid, Spain) at 25°C for 10 minutes, then at 37°C for 120 minutes, and finally at 85°C for 5 minutes(60). The calculations for each kit component were made according to the number of reactions (samples) with an additional volume to compensate for possible pipetting errors (*Annex 2*).

3.3.2. Quantitative real-time PCR (RT-qPCR)

RT-qPCR is a technique that allows real-time amplification and detection of DNA using specific primers and a fluorescent reporter; in this case, the SYBR Green probe is used. Quantification of the number of amplified DNA molecules is performed by analyzing the intensity of the fluorescent signal. The gene of interest studied in the RT-qPCRs to analyze its expression under obesogenic conditions is *Sucnr1*, the succinate receptor, and *Ppia* (Peptidylprolyl isomerase A) as an endogenous control gene. The cDNA studied came from the 48 samples of WAT and liver, corresponding to the ZT12 condition of the study. For the preparation of the RT-qPCR, a 384-well plate (Applied Biosystems®, ThermoFisher Scientific, USA) was designed with the necessary genes and samples for analysis (*Annex 3*).

First, to determine the optimal concentration of the samples that would allow the primers used to be functional, an efficiency test was performed on a 96-well plate (Azenta Life Sciences, USA) and dilutions with CT values between 20-25 for each tissue were selected (*Annex 4*). To do this, a pool of 8 samples of STD condition was prepared and serial dilutions were performed, while the MasterMix for each gene was prepared in a 0.5 mL Eppendorf, considering the number of reactions and with an additional volume to compensate for possible pipetting errors, as shown in *Annex 5*. Then, 2 µL/well of each cDNA sample (horizontally, against one of the walls) and 8 µL/well of MasterMix (vertically, at the bottom of the well) were added to the PCR plate according to its prior design. The plate was covered with optical adhesive covers (Applied Biosystems®, ThermoFisher Scientific, USA) and the samples were centrifuged. Subsequently, the experimental conditions were established and the quantitative PCR amplification was carried out in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Madrid, Spain)(60).

Secondly, dilutions of all samples were prepared according to the optimal concentration determined previously, and the MasterMix for each gene was prepared in a 1.5 mL Eppendorf, following the same process described above, but considering that in the 384-well plate, the volumes are halved and no RNase-free water is added, as reflected in *Annex 5*. Then, 2 µL/well of each cDNA sample (horizontally, against one of the walls) and 3 µL/well of MasterMix (vertically, at the bottom of the well) were added to the PCR plate according to its prior design. The plate was covered with optical adhesive covers (Applied Biosystems®, ThermoFisher Scientific, USA) and the samples were spun in the Mini Plate Spinner (MPS 1000, Labnet International, USA). Subsequently, the experimental conditions were established and the quantitative PCR amplification was carried out in the QuantStudioTM 5 Real-Time PCR Instrument (Applied Biosystems®, ThermoFisher Scientific, USA) for 2 hours. Once the RT-qPCR was completed, the melting curves for each gene were analysed to verify the formation of non-specific products. Additionally, CT values for each sample were obtained and subsequently analysed using the $2^{-\Delta\Delta Ct}$ method, with the peptidylprolyl isomerase A (*Ppia*) gene serving as the housekeeping control(60). The primers used for the amplification of the target sequence of each gene are described in *Table 1*.

Table 1. Nucleotide sequences of the primers used in RT-qPCR. *The names of the primers, their sequences from 5' to 3', and the supplier are described. For: forward; Rev: reverse.*

Primers	Sequence (5' - 3')	Supplier
Sucnr1-for	ACAGCTGTCGCCCTTTTCTA	(Biomers, Germany)
Sucnr1-rev	TCATGCCAACCTCTACACCA	(Biomers, Germany)
Ppia-for	CTTCGAGCTGAGACAA	(Biomers, Germany)
Ppia-rev	AAGTCACCACCACATG	(Biomers, Germany)

3.4. Statistical analysis

Data were analyzed using the Kruskal-Wallis test to determine significant differences among the three independent groups, as well as the Mann-Whitney U test to explore possible significant differences between two of the groups, employing the statistical software GraphPad Prism 8.0.1 (GraphPad Software, USA). The normal distribution of the data was tested using the Shapiro–Wilk test. Outliers were identified using the ROUT method from GraphPad. Results are presented as the median ± Min to max(60). For the study of rhythmicity, the cosinor method was used through least squares fitting. To achieve this, J.R. S-R developed a script using PyCharm software (v.2018.2.4, JetBrains s.r.o., Prague, Czech Republic) with Python version 3.7.4. The circadian rhythm was estimated using the CosinorPy package (v.1.1). Additionally, this method allowed us to obtain rhythmicity parameters such as the MESOR (average value adjusted for diurnal rhythm), amplitude (the difference between a wave's peak and average), and acrophase (the time when the oscillation peak occurs) of the diurnal oscillations(61). A probability value of $p < 0.05$ was considered statistically significant, and graphs were created with GraphPad Prism 8.0.1(60).

4. Results and Discussion

4.1. The gen expression of *Sucnr1* showed a rhythmic pattern in WAT but not in liver

In *Figure 8A* we observed the data obtained from eWAT, showing the gene expression of the receptor. Despite the lack of significant differences between the different time points, the data suggested the presence of a rhythmic pattern. The variation in expression over the 24-hour cycle, with an increase at ZT19, indicated that *Sucnr1* expression followed a daily cycle. This was consistent with the cosinor analysis results, which demonstrated significant rhythmicity ($p = 0.0359$) and showed a significant amplitude ($p = 0.0021$). The presence of a well-defined acrophase (a clear arrow direction) could be observed.

Figure 8B represents the data obtained from iWAT, showing the gene expression of *Sucnr1*. Although there were no significant differences between the studied time points, a variation in gene expression could be observed throughout the day. This was consistent with the cosinor analysis results, which, despite not significantly demonstrated a rhythmic pattern, showed a trend ($p = 0.0829$). Besides, the amplitude was statistically significant ($p = 0.0132$).

Figure 8C shows the data obtained from the liver, visualizing the gene expression of *Sucnr1*. The analysis showed higher expression at ZT7, with relatively low and constant levels at other time points. Coupled with the lack of significant differences, this did not indicate a clear rhythmic expression pattern. In line with these data, the cosinor method resulted in a low and not significant oscillation ($p = 0.6234$).

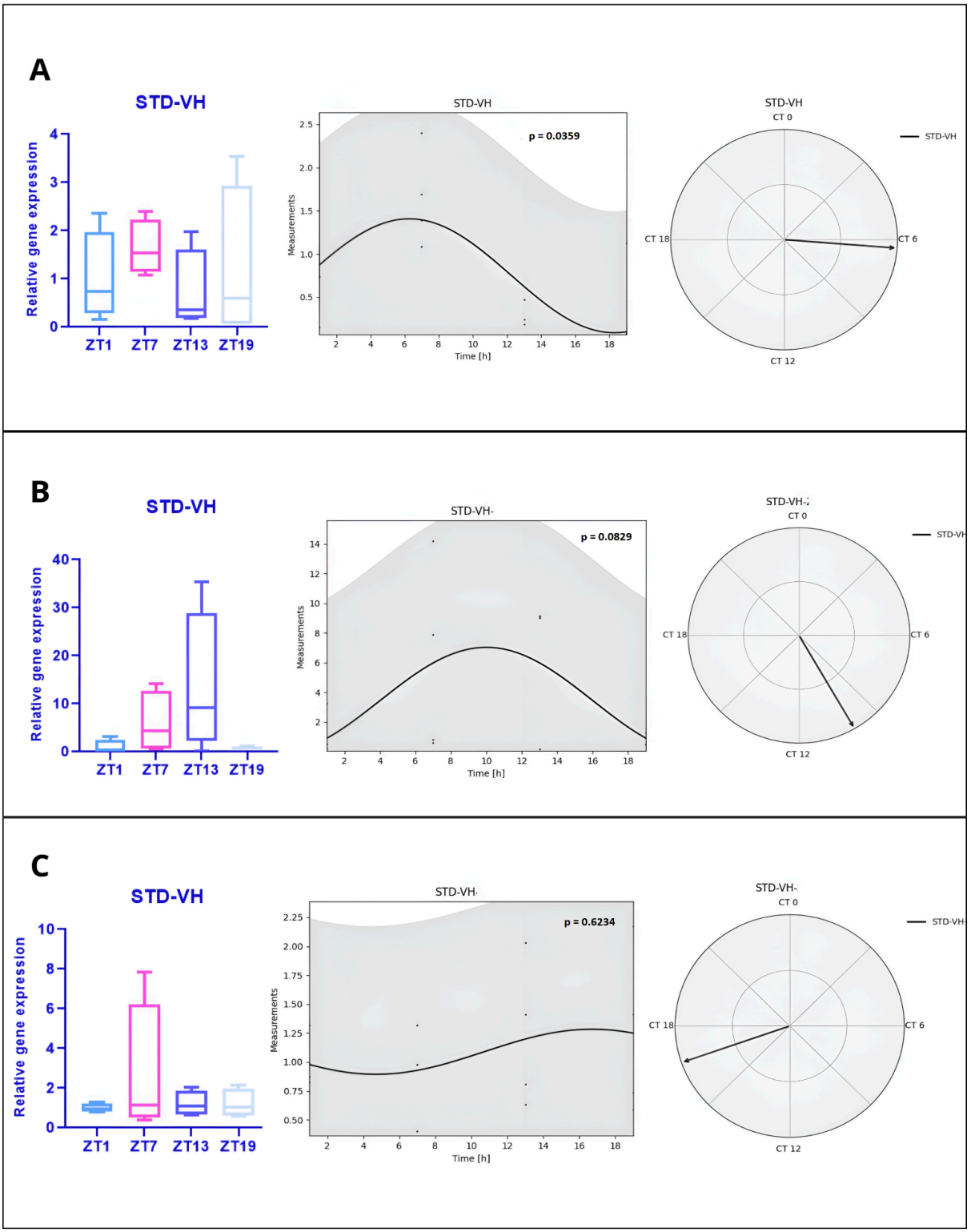

Figure 8. Estimation of the rhythmic pattern of the Sucnr1 gene in eWAT (A), iWAT (B) and liver (C) in Fischer 344 male rats. *Fisher 344 male rats were fed a STD diet for 9 weeks and administrated for the last 4 weeks with a vehicle (VH) at the beginning of ZT12. Relative expression of Sucnr1 in eWAT (A), iWAT (B), and liver (C). DNA levels were normalized to the endogenous Ppia gene and then to the STD-VH control group. Data are shown as the median ± Min to max, its diurnal oscillation, and acrophases with their amplitude. For the analysis of significant differences, the Mann-Whitney U test was used and the cosinor method was used to study rhythmicity.*

According to the results obtained, it is plausible to affirm that there is a rhythmic expression pattern of the *Sucnr1* receptor in WAT. This indicates a common circadian regulation of *Sucnr1*, which could influence the overall physiology of AT and metabolic response, suggesting an important role for *Sucnr1* in regulating energy metabolism and AT homeostasis through circadian mechanisms. The more significant pattern observed in eWAT compared to iWAT may be due to eWAT being the first to expand in the early stages of obesity and being crucial for TAG storage. Additionally, an increase in eWAT is associated with a higher metabolic risk and plays an essential role in regulating metabolism by secreting pro-inflammatory cytokines(8,20). On the other hand, iWAT shows notable flexibility compared to perigonadal fat, better adapting to changes in nutrient load and various metabolic conditions(24).

The expression of *Sucnr1* follows a clear circadian pattern in both AT, suggesting robust circadian regulation of *Sucnr1* in AT physiology. This is supported by studies demonstrating *Sucnr1*'s influence on peripheral clock regulation, causing changes in the expression of central clock genes and leptin levels. Additionally, *Sucnr1* expression in WAT is significantly higher in obese subjects, suggesting a potential link between this receptor and metabolic disorders. It's signalling in adipocytes controls energy metabolism by modulating the circadian clock and leptin expression, highlighting the importance of this receptor in metabolic homeostasis(42,62).

On the other hand, the results of this study show an inconclusive rhythmic expression pattern in the liver, suggesting that *Sucnr1* regulation in the liver could depend on non-circadian factors or that the rhythmic pattern is less pronounced in this tissue. However, various studies have demonstrated the rhythmicity of this gene in the liver, influenced by factors such as dietary interventions and metabolic demands. *Sucnr1* signalling in the liver is associated with controlling extracellular succinate levels and regulating hepatic inflammation through UCP1, highlighting its role in metabolic homeostasis(63). Although our data do not show a clear rhythmic pattern, the literature suggests that *Sucnr1* expression in the liver may be modulated by multiple factors, resulting in a less pronounced rhythmic pattern(62).

In summary, the evidence suggests that *Sucnr1* follows a rhythmic expression pattern in AT, contributing to the circadian regulation of energy metabolism and metabolic homeostasis. However, in the liver, *Sucnr1* expression may be influenced by other factors, resulting in a less pronounced rhythmic pattern. These findings highlight the importance of tissue localization and context in the circadian regulation of *Sucnr1* and its implications for metabolic health.

4.2. The CAF diet produced a disruption in the rhythmic pattern of *Sucnr1* expression in WAT

Figure 9A shows the data obtained in eWAT from the comparison of the two study conditions. We observed higher gene expression in rats following an STD diet, while the CAF diet seemed to significantly reduce *Sucnr1* gene expression at certain times of the day (ZT7; $p = 0.0286$). Moreover, the data showed a more significant oscillation in STD conditions than in obese animals ($p = 0.0309$), indicating a possible loss of rhythmicity. The STD diet showed greater amplitude, indicating a stronger oscillation, while the CAF diet exhibited lesser amplitude ($p = 0.0657$), suggesting a less pronounced oscillation. Adittionally, the shift in acrophase in the CAF diet was significant ($p = 0.0356$).

Figure 9B shows the data obtained in iWAT from the comparison of the two study conditions. We observed a higher gene expression in rats following an STD diet, while the CAF diet seemed to reduce *Sucnr1* gene expression. The STD diet showed a more pronounced oscillation and the CAF diet presented greater variability, suggesting a loss of rhythmicity ($p = 0.3072$). The STD diet showed greater amplitude while the CAF diet presented lesser amplitude, suggesting a weaker oscillation. The acrophase shift in the CAF diet indicated an alteration in the normal circadian rhythm of *Sucnr1* gene expression.

Figure 9C shows the data obtained in the liver from the comparison of the two study conditions. We observed similar gene expression in both groups, with no significant differences so we could not infer a global alteration of the receptor's rhythmic pattern in liver tissue.The STD diet exhibited a less pronounced and more stable oscillation, while the CAF diet showed a more pronounced oscillation and greater variability ($p = 0.2383$). The STD animals presented lesser amplitude than CAF animals, indicating a weaker oscillation of *Sucnr1* gene expression in animals under STD diet. These results differed from the other two tissues studied.

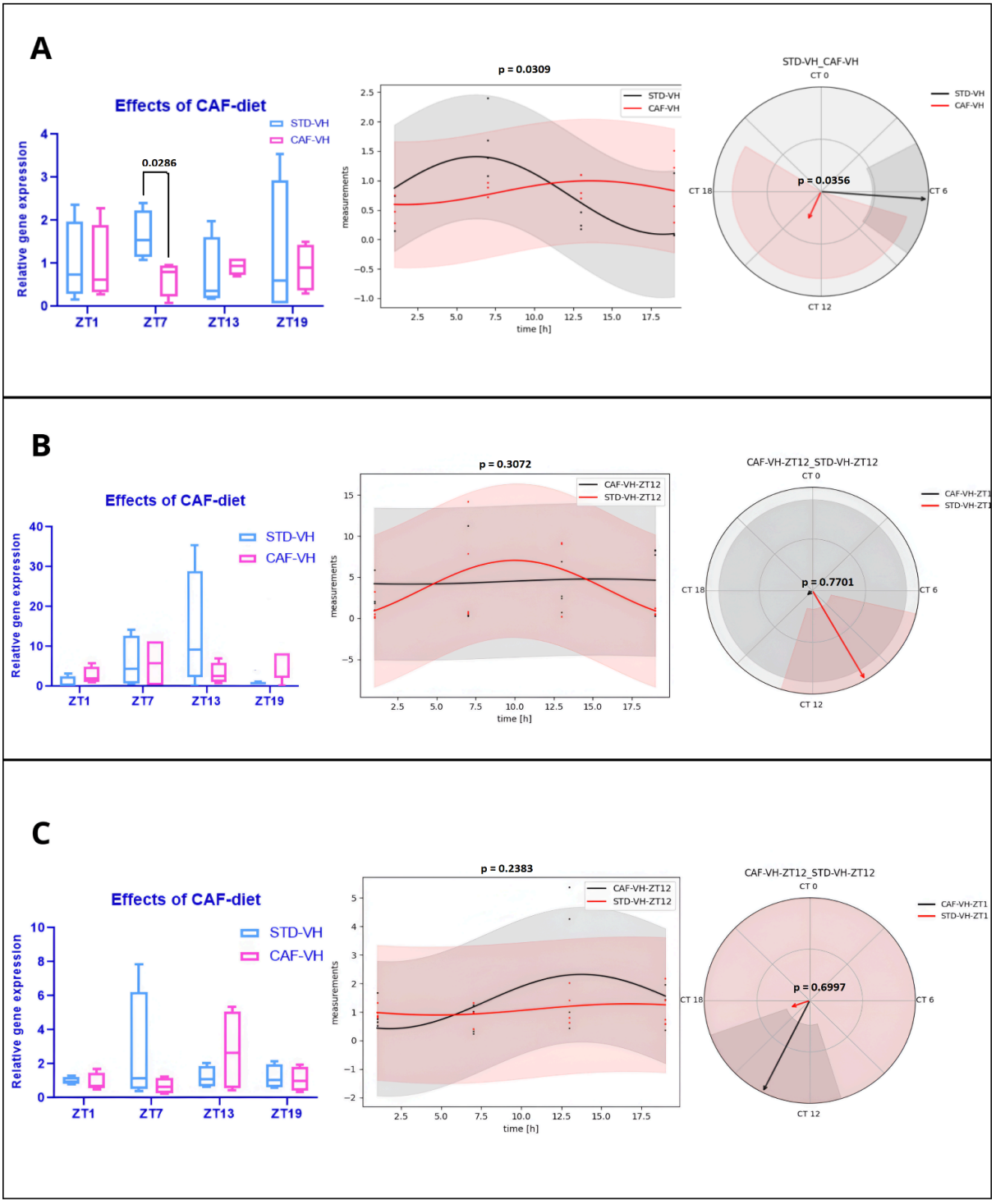

Figure 9. Representation of the effect of the CAF diet on the rhythmic expression pattern of the Sucnr1 gene in eWAT (A), iWAT (B) and liver (C) in Fischer 344 male rats. *Fisher 344 male rats were fed a STD or CAF diet for 9 weeks and administrated for the last 4 weeks with a vehicle (VH) at the beginning of ZT12. Relative expression of Sucnr1 in eWAT (A), iWAT (B), and liver (C). DNA levels were normalized to the endogenous Ppia gene and then to the STD-VH control group. Data are shown as the median ± Min to max, its diurnal oscillation, and acrophases with their amplitude. For the analysis of significant differences, the Mann-Whitney U test was used and the cosinor method was used to study rhythmicity.*

Analyzing the results obtained in animals under CAF diet, we can confirm that *Sucnr1* expression in both AT was significantly altered when rats are fed a CAF diet, thus disrupting the circadian regulation of the receptor and potentially harming energy homeostasis and metabolic regulation. WAT dysfunction is closely related to the disruption of circadian rhythms, especially under the influence of hypercaloric diets. Thus, the consumption of such diets disrupts the diurnal rhythm of food intake and can have pathogenic consequences, affecting circadian rhythms in peripheral organs such as WAT(27).

The CAF diet causes significant alterations in the rhythmic expression of the *Sucnr1* gene in both adipose tissues. This suggests that hypercaloric diets can deregulate the circadian mechanisms controlling *Sucnr1* expression, negatively affecting metabolic homeostasis and energy regulation. Various studies support the data obtained in this study, demonstrating that *Sucnr1* signalling in adipocytes regulates the peripheral clock, affecting the expression of central clock genes and leptin levels. Therefore, a CAF diet, rich in fats and sugars, can deregulate this circadian mechanism(42). Moreover, research indicated that the CAF diet could increase *Sucnr1* expression in obese subjects, linking this increase to metabolic disorders. The alteration observed in the rhythmic patterns of *Sucnr1* in our data suggests a disruption of circadian regulation, exacerbated by the CAF diet(63).

In the liver, we observe a non-clearly rhythmic expression pattern under the STD diet. However, once the CAF diet is administered, some rhythmic fluctuations are seen, leading us to think that *Sucnr1* regulation in the liver might be influenced by additional non-circadian factors or that the rhythmic pattern is less pronounced in this tissue. However, according to the scientific literature, this type of diet causes a notable increase in fat accumulation in the liver of rats, leading to alterations in liver structure and function, reflected in changes in liver function markers(30). Circadian rhythms affect the timing of food intake, hepatic glucose production, lipid metabolism, and bile acid synthesis, thus contributing to overall energy homeostasis. Disruptions in these rhythms, such as those caused by shift work or irregular eating patterns, can lead to metabolic dysfunction in the liver, contributing to conditions like MAFLD(34).

Although a clear rhythmic pattern was not observed in *Sucnr1* expression in the liver under the CAF diet, the literature suggests that *Sucnr1* regulation in this tissue may be influenced by other factors and that a CAF diet could attenuate circadian rhythmicity in the liver, resulting in less pronounced regulation. In summary, the CAF diet has a disruptive impact on the rhythmic expression of *Sucnr1* in AT, but its effect on the liver is inconclusive. These findings underscore the importance of circadian regulation in metabolic function and how a CAF diet can deregulate these mechanisms, contributing to MD and disorders such as obesity and insulin resistance.

4.3. GSPE consumption showed a positive modulatory effect on the rhythmic expression of *Sucnr1* in WAT, although its influence in liver was less evident

In *Figure 10A*, we observed the data obtained in eWAT. First, we analyzed the gene expression of *Sucnr1*, where GSPE appeared to have a stabilizing effect on the gene expression while the cafeteria diet (CAF-VH) showed greater variability and generally lower expression. In addition, the oscillations in the CAF-GSPE diet were less pronounced and more stable ($p = 0.1563$) compared to those produced by the CAF-VH diet. There were significant differences in acrophase ($p = 0.0144$), indicating that GSPE helped to correct the acrophase shift induced by the CAF diet.

In *Figure 10B*, we observed the data obtained in iWAT. First, we examined the gene expression of *Sucnr1*, where the CAF-VH diet, compared to GSPE administration, showed greater variability and generally lower expression of the *Sucnr1* gene expression, while GSPE tended to increase expression and stabilize variability, indicating a more positive regulatory effect. Additionally, the oscillation in CAF-VH was flatter with lower amplitude, whereas the CAF-GSPE condition displayed a more pronounced oscillation with greater amplitude ($p = 0.7764$).

In *Figure 10C*, we observed the data obtained in the liver. First, we analyzed the gene expression of *Sucnr1*, where the CAF-VH diet showed greater variability and generally higher expression while GSPE administration tended to reduce gene expression. Moreover, the CAF-VH condition exhibited more pronounced oscillations with greater amplitude, whereas CAF-GSPE showed less pronounced oscillations with lower amplitude, indicating reduced variability ($p = 0.1892$). As no significant differences in amplitude or acrophase were found, it is possible that *Sucnr1* gene expression does not follow a significant rhythmic pattern in the liver.

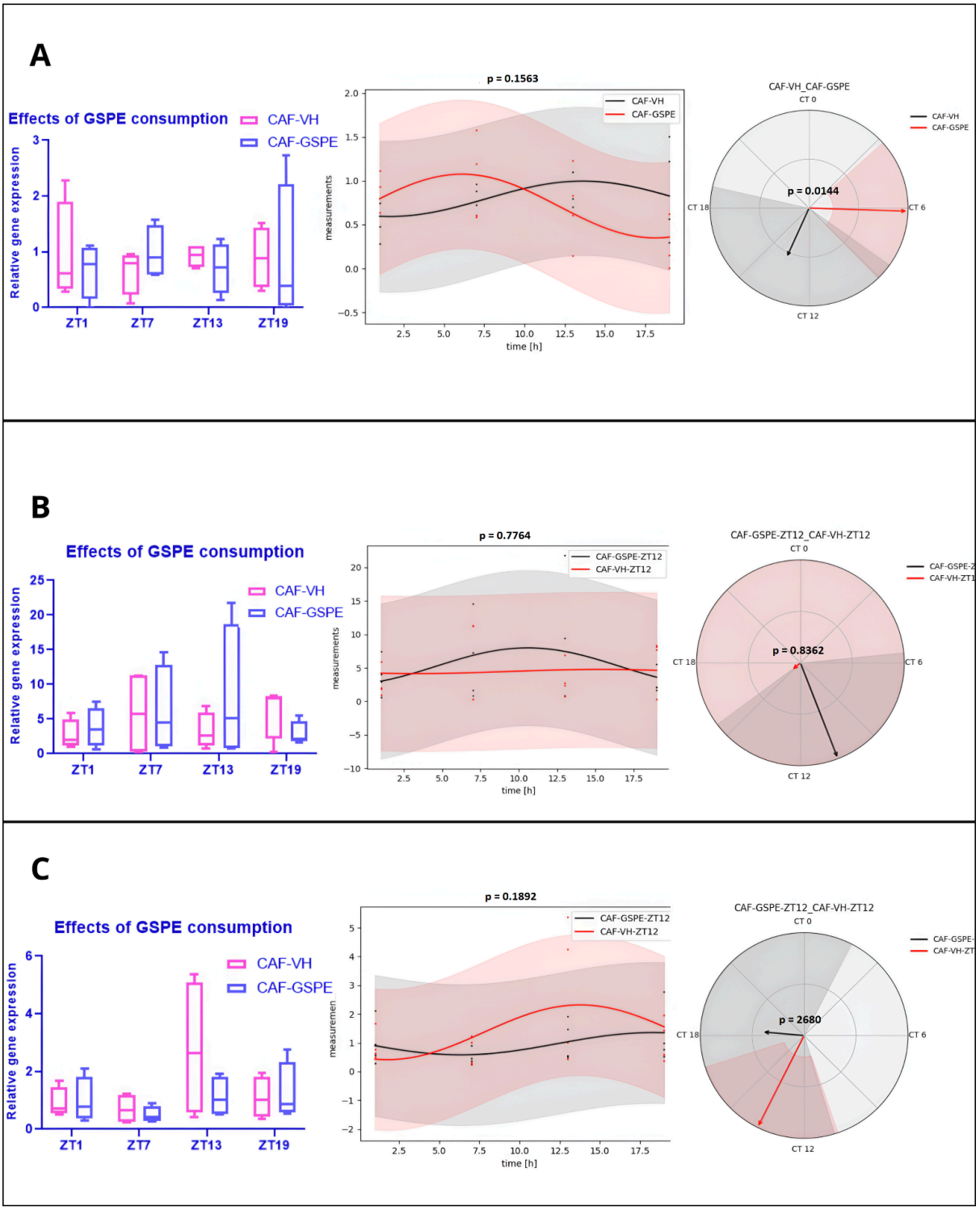

Figure 10. Representation of the effect of GSPE consumption on the recovery of the rhythmic expression pattern of the Sucnr1 gene in eWAT (A), iWAT (B) and liver (C) in Fischer 344 male rats. *Fisher 344 rats were fed a CAF diet for 9 weeks and administrated for the last 4 weeks with vehicle (VH) or GSPE at the beginning of ZT12. Relative expression of Sucnr1 in eWAT (A), iWAT (B), and liver (C). DNA levels were normalized to the endogenous Ppia gene and then to the STD-VH control group. Data are shown as the median ± Min to max, its diurnal oscillation, and acrophases with their amplitude. For the analysis of significant differences, the Mann-Whitney U test was used and the cosinor method was used to study rhythmicity.*

Interpreting the results obtained in this study, we realize that the expression of *Sucnr1* in eWAT, significantly altered by the CAF diet, shows a tendency towards normalization with GSPE administration. This suggests that GSPE mitigates the circadian disruptions induced by the CAF diet, indicating a protective effect on metabolic regulation in eWAT. Meanwhile, the data show that GSPE administration can regulate the circadian expression pattern of the *Sucnr1* gene in iWAT, affected by the CAF diet, attenuating the diet-induced alterations in gene expression and partially restoring circadian regulation in subcutaneous adipose tissue. This confirms the anti-inflammatory properties of proanthocyanidins, which can influence in peripheral circadian clocks, improving the expression of circadian genes and metabolic function(57).

It has been demonstrated that *Sucnr1* expression in WAT exhibits a rhythmic expression pattern, and GSPE administration appears to enhance this regulation in the presence of a CAF diet. This suggests that GSPE can act as a circadian modulator, restoring the rhythmic expression of key genes for energy homeostasis. Studies show the influence of this polyphenolic extract consumption on different AT, indicating that in eWAT, its effect depends on the time of day it is consumed, while in iWAT, its consumption improved AT expansion and decreased adipocyte size(64). Therefore, GSPE administration in rats fed a CAF diet seems to partially restore the circadian rhythmicity of *Sucnr1* expression in eWAT and iWAT, suggesting its capacity to mitigate cafeteria-diet-induced alterations, improving circadian regulation and metabolic homeostasis in these AT.

On the other hand, the data do not show a clear rhythmic pattern in *Sucnr1* expression in the liver under the CAF diet, and GSPE's influence is inconclusive. Thus, GSPE's ability to modulate *Sucnr1* expression in the liver seems limited or dependent on additional factors not captured in this study. However, various studies demonstrate that chronic GSPE consumption can restore the daily oscillation of key genes related to lipogenesis and gluconeogenesis, helping to reduce triglyceride accumulation and lipid droplet formation in the liver, thereby alleviating NAFLD induced by a HFD(51). Additionally, these studies highlight the importance of the timing of GSPE administration, being particularly effective at the beginning of the active phase to modulate hepatic genes and metabolites, emphasizing the time-of-day-dependent nature of GSPE's effects on metabolism(64).

Therefore, although a clear effect of GSPE on the modulation of *Sucnr1* expression in the liver was not observed in our data, the literature suggests that polyphenols can have beneficial effects on overall liver function. GSPE can restore the daily oscillation of key genes, and improve the rhythmicity of total cholesterol levels, and liver mass, highlighting its regulatory potential on hepatic metabolism. In summary, GSPE administration has a positive modulatory effect on the rhythmic expression of *Sucnr1* in AT affected by a CAF diet, but its impact on the liver is less clear. These findings underscore GSPE's potential to improve circadian regulation and metabolic homeostasis in DIO contexts.

Considering all this, more studies similar to this one are needed to verify the results obtained, as the number of rats per group is very small, reproducing the research on a larger scale could provide clearer results. Additionally, it would be interesting to demonstrate the effects observed in AT in the liver. Furthermore, to complete the study, it would have been valuable to validate the gene expression analysis by assessing protein expression through Western Blot or ELISA; to determine whether receptor activation occurs; or to check if this same rhythmic pattern is present in metabolite expression.

5. Conclusions

The conclusions we can draw from this study are as follows:

- The gene expression of *Sucnr1* does show a rhythmic expression pattern, experimentally confirmed in both AT. However, it could not be empirically demonstrated in the liver, although scientific literature suggests that rhythmicity does exist in this tissue.

- The CAF diet causes significant alterations in the rhythmic expression of the *Sucnr1* gene in both AT. This suggests that a CAF diet can deregulate the circadian mechanisms controlling *Sucnr1* expression, negatively affecting metabolic homeostasis and energy regulation, as well as influencing the inflammatory processes characteristic of diet-induced-obesity.

- Although a clear rhythmic pattern in *Sucnr1* expression in the liver under the CAF diet was not observed, the literature suggests that *Sucnr1* regulation in this tissue may be influenced by other factors. The CAF diet could attenuate circadian rhythmicity in the liver, resulting in less pronounced regulation.

- GSPE administration in rats fed a CAF diet seems to partially modulate the circadian rhythmicity of *Sucnr1* expression in eWAT and iWAT. This suggests that GSPE can mitigate the alterations induced by a CAF diet, improving circadian regulation and metabolic homeostasis in these AT.

- Although a clear effect of GSPE on the modulation of *Sucnr1* expression in the liver was not observed in our data, the literature suggests that polyphenols can have beneficial effects on overall liver function. GSPE can restore the daily oscillation of key genes related to lipogenesis and gluconeogenesis, helping to reduce triglyceride accumulation and lipid droplet formation in the liver.

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7. Annexes

Annex 1: Obtaining the concentration necessary for retrotranscription

Table A1. Example of the calculation of the required amount of RNA and RNAse-free water to achieve a concentration of 50 ng/µL of RNA. *An example sample is detailed, including the RNA concentration, the µL of RNA needed to reach 50 ng/µL, and the amount of RNAse-free water needed for a total volume of 30 µL.*

Rat sample (eWAT)	RNA (ng/µL)	µL sample for 50 ng/µL of RNA	µL nuclease-free water (V_T = 30 µL)
49	362.15	4.1	25.9

Annex 2: Reagents used in the retrotranscription *Mastermix*

Table A2. Example of the calculation of the reagents needed for reverse transcription. *The reagents used to form the MasterMix, the amount of each reagent per sample, and the total amount of MasterMix required are detailed.*

Reagents (MasterMix)	Volume (µL/sample)	Total Volume (µL) Nº Samples = 50
Nuclease-free water	4.2	210
10x RT Buffer	2	100
25x dNTPs Mix	0.8	40
10x RT *Random Primers*	2	100
MultiScribe Reverse Transcriptase	1	50
Total Volume	10	500

Annex 3: Plate 384 layout for performing RT-qPCR

Table A3. Example of RT-qPCR distribution in a 384-well plate. *Samples were added vertically in triplicate. Primers were added horizontally: Ppia is represented in purple and Sucnr1 in pink.*

49	49	49	50	50	50	51	51	51	52	52	52	53	53	53	54	54	54	55	55	55	56	56	56
57	57	57	58	58	58	59	59	59	60	60	60	61	61	61	62	62	62	63	63	63	64	64	64
65	65	65	66	66	66	67	67	67	68	68	68	69	69	69	70	70	70	71	71	71	72	72	72
73	73	73	74	74	74	75	75	75	76	76	76	77	77	77	78	78	78	79	79	79	80	80	80
81	81	81	82	82	82	83	83	83	84	84	84	85	85	85	86	86	86	87	87	87	88	88	88
89	89	89	90	90	90	91	91	91	92	92	92	93	93	93	94	94	94	95	95	95	96	96	96
49	49	49	50	50	50	51	51	51	52	52	52	53	53	53	54	54	54	55	55	55	56	56	56
57	57	57	58	58	58	59	59	59	60	60	60	61	61	61	62	62	62	63	63	63	64	64	64
65	65	65	66	66	66	67	67	67	68	68	68	69	69	69	70	70	70	71	71	71	72	72	72
73	73	73	74	74	74	75	75	75	76	76	76	77	77	77	78	78	78	79	79	79	80	80	80
81	81	81	82	82	82	83	83	83	84	84	84	85	85	85	86	86	86	87	87	87	88	88	88
89	89	89	90	90	90	91	91	91	92	92	92	93	93	93	94	94	94	95	95	95	96	96	96

Annex 4: Plate 96 layout for performing the efficiency test

Table A4. Example of efficiency test layout in a 96-well plate. *First, a pool of 8 STD samples was prepared and serial dilutions were made. Samples were added vertically in triplicate. Primers were added horizontally: Ppia is represented in purple and Sucnr1 is represented in pink.*

		1/5	1/5	1/5			1/5	1/5	1/5		
		1/10	1/10	1/10			1/10	1/10	1/10		
		1/20	1/20	1/20			1/20	1/20	1/20		
		1/40	1/40	1/40			1/40	1/40	1/40		

Annex 5: Reagents used in the RT-qPCR *Mastermix*

Table A5. Example of the calculation of the reagents needed for PCR. *The reagents that make up the MasterMix, the amount of each reagent per sample, and the total volume of MasterMix needed are specified.*

Reagents (MasterMix)	Volume (µL/sample) 96 well plate	Total Volume (µL) Nº Samples = 15 96 well plate	Volume (µL/sample) 384 well plate	Total Volume (µL) Nº Samples = 156 384 well plate
SYBR Green Supermix	5	75	2.5	390
Forward primer	0.5	7.5	0.25	39
Reverse primer	0.5	7.5	0.25	39
Nuclease-free water	2	30	-	-
Total Volume	8	120	3	468