
# METABOLOMICS STRATEGY TO COMPREHEND THE INTERACTIONS BETWEEN CIRCADIAN RHYTHMS AND FLAVANOL ACTIVITY ON THE HEPATIC METABOLISM

**Héctor Palacios Jordán**

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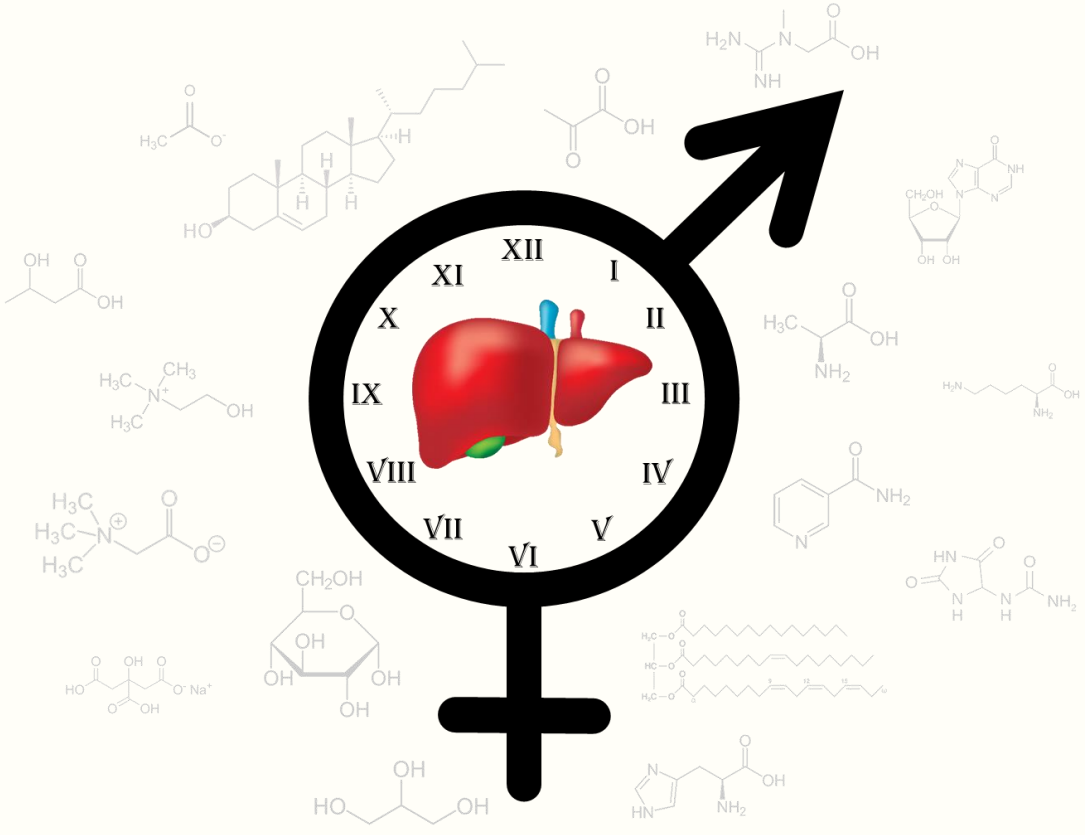


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UNIVERSITAT ROVIRA i VIRGILI

**DOCTORAL THESIS**
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# Metabolomics strategy to comprehend the interactions between circadian rhythms and flavanol activity on the hepatic metabolism

DOCTORAL THESIS

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I STATE that the present study, entitled "**Metabolomics strategy to comprehend the interactions between circadian rhythms and flavanol activity on the hepatic metabolism**", presented by **Hector Palacios Jordan** for the award of the degree of Doctor, has been carried out under my supervision at the **Department of Biochemistry and Biotechnology** of this university and that this thesis is eligible to apply for the International Doctorate Mention.

FAIG CONSTAR que aquest treball, titulat "**Metabolomics strategy to comprehend the interactions between circadian rhythms and flavanol activity on the hepatic metabolism**", que presenta l'**Hèctor Palacios Jordan** per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al **Departament de Bioquímica i Biotecnologia** d'aquesta universitat i que compleix els requisits per poder optar a la Menció Internacional de Doctorat.

HAGO CONSTAR que el presente trabajo, titulado "**Metabolomics strategy to comprehend the interactions between circadian rhythms and flavanol activity on the hepatic metabolism**", que presenta **Héctor Palacios Jordán** para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el **Departamento de Bioquímica y Biotecnología** de esta universidad y que cumple con los requisitos para poder optar a la Mención Internacional de Doctorado.

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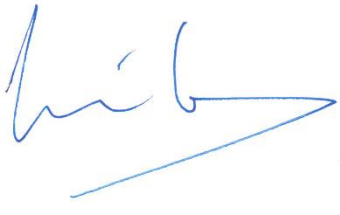

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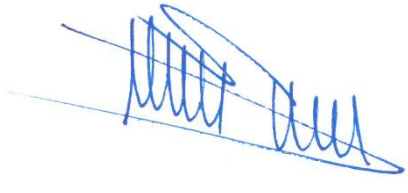

Dr. Miguel Ángel Rodríguez Gómez

*A mi familia y amigos,*

*A Cinta.*

*"Science, my lad, is made up of mistakes,*

*but they are mistakes which it is useful to make,*

*because they lead little by little to the truth."*

*Jules Verne*

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# Summary

A grape seed proanthocyanidin extract (GSPE) has been associated with a widely range of beneficial effects for the prevention and treatment of hepatic metabolic disturbances induced by obesity, such as insulin resistance or steatosis. Moreover, GSPE is capable to modulate the clock system in the liver, which is also disrupted in an obesity status, thus suggesting that GSPE can partially regulate lipid and glucose metabolism by modulating the hepatic circadian rhythms. NMR-based metabolomics strategy is an adequate approach to study the interaction between the proanthocyanidin effects and the circadian rhythmicity of the hepatic metabolism.
In this regard, this thesis aims to evaluate whether a grape seed proanthocyanidin extract (GSPE) has different effects on the hepatic metabolism depending on the administration time, in a metabolic syndrome situation. The circadian rhythm disruption of the hepatic metabolism, caused by obesity, was studied in both genders in order to elucidate whether this disruption is gender-dependent. Female animals showed to be more resistance and flexible against an obesogenic diet.

The chronic administration of GSPE presented different effects in obese male rats depending on its administration time. A large amount of its beneficial effects were found when GSPE was given at the beginning of the light phase. Possible antioxidant effects and an improvement in hepatic insulin sensitivity were only observed in those animals.

The results of this thesis elucidate the importance of the administration time of GSPE. Additionally, this thesis shows the better circadian rhythm flexibility of obese female rats.

# Resumen

El extracto de proantocianidinas procedente de la semilla de uva (GSPE) se ha asociado a un amplio rango de efectos beneficiosos para la prevención o tratamiento de las alteraciones metabólicas hepáticas causadas por la obesidad, p.ej. resistencia a la insulina o esteatosis. Además, el GPSE es capaz de modular los ritmos circadianos hepáticos, los cuales son alterados por la obesidad. Por lo tanto, esto sugiere que el GPSE puede regular parcialmente el metabolismo lipídico y glucídico a través de la modulación de los ritmos circadianos. La metabolòmica basada en RMN es una técnica adecuada para el estudio de las interacciones entre los efectos de las proantocianidinas i los ritmos circadianos del metabolismo hepático.

El objetivo de esta tesis es evaluar si los efectos del GSPE son diferentes dependiendo del momento del día de administración en animales que padecen síndrome metabólico. También se han estudiado las alteraciones en los ritmos circadianos del metabolismo hepático provocadas por una dieta obesogénica para determinar si, dicha alteración, es diferente en función el género. Las ratas hembra obesas mostraron una mayor resistencia y flexibilidad en los ritmos circadianos del metabolismo hepático.

La administración crónica de GSPE presentó diferentes efectos en ratas macho obesas en función del momento de su administración. La mayoría de los efectos beneficiosos fueron hallados cuando el GSPE fue dado al inicio de la fase lumínica. Solo en dichos animales se observó un posible efecto antioxidante y una mejora en la sensibilidad a la insulina en el hígado.

Los resultados de esta tesis eluciden la importancia del momento de administración del GSPE. Además, esta tesis demuestra una mayor flexibilidad en los ritmos circadianos en ratas hembra obesas.

# Resum

Un extracte de proantocianidinas extretes de la llavor del raïm (GSPE) ha estat relacionat amb un ampli rang de efectes beneficiosos per la prevenció i tractament de les alteracions metabòliques hepàtiques induïdes per l'obesitat, p.e., la resistència a la insulina o l'esteatosi. A més, el GPSE és capaç de modular els ritmes circadians del fetge, els quals es troben desajustats a l'obesitat, suggerint que el GSPE pot regular parcialment el metabolisme de lípids i glucosa modulant els ritmes circadians hepàtics. La metabolòmica per RMN pot ser una tècnica adequada per l'estudi de la interacció entre l'efecte de les proantocianidines i els ritmes circadians del metabolisme hepàtic.

En aquest sentit, aquesta tesi te com a objectius avaluar si el GSPE té diferents efectes sobre el metabolisme hepàtic depenent del temps d'administració en animals que pateixen la síndrome metabòlica. Addicionalment, es va estudiar la disrupció dels ritmes circadians del metabolisme hepàtic, causats per l'obesitat, a ambdós gèneres per a eludir si aquesta disrupció és depenent del sexe. Les femelles van mostrar una major resistència i flexibilitat quan eren alimentades amb una dieta obesogénica.

L'administració crònica de GSPE va presentar efectes diferents en rates mascles obeses depenent del temps d'administració. Moltes dels efectes beneficiosos es van trobar en els animals que van ser tractats al principi de la fase lluminosa. A més, també es va observar un possible efecte antioxidant i una millora en la sensibilitat hepàtica a la insulina en aquest animals.

Els resultats d'aquesta tesi ens mostren la importància del temps d'administració del GPSE. A més, aquesta tesi ens ensenya que les rates femelles obeses tenen una millor flexibilitat dels ritmes circadians hepàtics.

# Abbreviations

| | |
|---|---|
| $^{1}H$ | Proton |
| 3-HB | 3-hydroxybutyrate |
| ACC | Acetyl-coA carboxylase |
| Acetyl-CoA | Acetyl coenzyme A |
| AMPK | 5' AMP-activated protein kinase |
| AMPK | Adenosine monophosphate |
| ARA+EPA | Arachidonic acid and eicosapentaenoic acid |
| ATP | Adenosine triphosphate |
| BHMT | Betaine homocysteine S-methyltransferase |
| BMAL1 | Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 |
| CDP-Choline | Cytidine diphosphate-choline pathway |
| CE | Capillary electrophoresis |
| ChREBP | Carbohydrate-response element-binding protein |
| CK | Choline kinase |
| CKIε | Casein kinase Iε |
| CLOCK | Circadian locomotor output cycles kaput |
| CRY | Cryptochrome |
| CVD | Cardiovascular diseases |
| Cyp7a1 | Cholesterol 7α-hydroxylase |
| DAG | Diacylglycerol |
| DAK-δ | Diglycerides kinase delta |
| DHA | Docosahexaenoic acid |
| ELOVL3 | Elongation of very long chain fatty acids protein 3 |
| ER | Endoplasmic reticulum |
| ER | Estrogen receptor |
| FAS | Fatty acid synthase |
| FFA | Free fatty acid |
| FGF15 | Fibroblast growth factor 15 |
| FXR | Nuclear receptor farnesoid X receptor |

| | |
|---|---|
| G6P | Glucose-6-phosphate |
| GC | Gas chromatography |
| GK | Glucokinase |
| GLP1 | Glucagon-like peptide 1 |
| GLUT4 | Glucose transport type 4 |
| GSPE | Grape seed proanthocyanidins extract |
| HIF-1 | Hypoxia-inducible factor 1 |
| HMDB | Human metabolome data base |
| HMG-CoA | β-hydroxy-3-methylglutaryl-CoA reductase |
| HOMA-IR | Homeostasis model assessment-estimated insulin resistance |
| IL-6 | Interleukin 6 |
| IRS1 | Irisin receptor substrate 1 |
| JNK | c-Jun N-terminal kinase |
| KLF10 | Kruppel-like factor 10 |
| LC | Liquid chromatography |
| LPL | Lipoprotein lipase |
| MetS | Metabolic syndrome |
| mLPL | Muscle lipoprotein lipase |
| MS | Mass Spectrometry |
| NAD | Nicotinamide Adenine Dinucleotide |
| NAFLD | Non-alcoholic fatty liver disease |
| NAMPT | Nicotinamide phosphoribosyltransferase |
| NK-kB | Nuclear factor kappa-light-chain-enhancer or activated B cells |
| NMR | Nuclear magnetic resonance |
| NOESY | Nuclear overhauser effect spectroscopy |
| Nrf2 | Nuclear erythroid2-related factor 2 |
| OGTT | Oral glucose tolerance test |
| OGTT | Oral glucose tolerance test |
| PAC | Proanthocyanidin |
| PC | Phosphatidylcholine |

| | |
|---|---|
| PCK1 | Phosphoenolpyruvate carboxykinase |
| PDX1 | Pancreatic and duodenal homeobox 1 |
| PEMT | Phosphatidylethanolamine N-methyltransferase |
| PER | Period |
| PHD3 | Prolyl hydroxylase 3 |
| PKCε | Protein kinase Cε |
| PLS-DAs | Partial Least Square – Discriminant Analysis |
| PPARγ | Peroxisome proliferator-activated receptor γ |
| REV-ERB α/β | Reverse-erythroblastosis |
| ROR | Retinoid-related orpham receptor |
| SAM | S-adenosyl methionine |
| SCN | Suprachiasmatic nucleus |
| SHP | Small heterodimer partner |
| SREBP-1c | Sterol regulatory element-binding transcription factor 1 |
| Srirt1 | Sirtuin 1 |
| T2DM | Type 2 diabetes mellitus |
| TAG | Triglycerides |
| TCA | Tricarboxylic acid cycle |
| TMS | Tetramethylsilane |
| TNK-α | Tumor necrosis factor alpha |
| TSH | Thyroid stimulating hormone |
| TSP | Trisilylpropionic acid |
| TTFL | Transcription/translation feedback loops |
| UCP1 | Uncoupling protein 1 |
| VLDL | Very low-density lipoprotein |
| WHO | World health organization |
| ZT | Zeitgeber |

# INTRODUCTION

# 1. Metabolic syndrome and obesity status

Metabolic syndrome (MetS) is characterized by a set of metabolic disorders comprised by abdominal obesity, insulin resistance, dyslipidemia and hypertension [1]. MetS directly increases the risk of to several metabolic diseases, such as type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVD) [2], thus carrying an increased cardiovascular morbidity and mortality [3]. Obesity is mainly considered to be responsible for the increasing prevalence of MetS, associated to dyslipidemia, with higher plasma triglyceride (TAG) levels and lower high density lipoprotein-cholesterol levels, hyperglycemia and increased CVD risk [4]. As a consequence of a dramatically worldwide increase on the obesity prevalence, which has been nearly tripled from 1975 to 2016, the importance of new therapeutic approaches against MetS is rising. [5].

World Health Organization (WHO) defined obesity as the abnormal or excessive fat accumulation that presents a risk to health [5]. Overweight and obesity are the major risk factors for a number of chronic diseases, including T2DM, cardiovascular diseases and cancer [6–8]. In 2016, WHO estimated that more than 1.9 billion adults aged 18 years and older were overweight, thus representing the 39% (men 39% and women 40%) of the world adult population, and over 650 million of these adults were considered obese, representing the 13% (men 11% and women 15%) of the world adult population.

Obesity is most commonly caused by an energy unbalance in which consumption is in excess of expenditure [9]. Physiological systems are more effective protecting against weight loss than weight gain, which leads to net weight gain as the most common form of energy imbalance [10]. Moreover, heredity plays a significant role in weight gain. In this regard, more than 140 genes sites have been involved in weight

and body size [11]. In addition, gut microbiota has a potential role on obesity development. Different mechanisms have been proposed to explain the role of the gut microbiota in obesity, such as its ability to ferment dietary polysaccharides [12], to decrease liver fatty acid oxidation [13], and to trigger the inflammatory system [14], among others.

## 1.1. Metabolic disorders associated to metabolic syndrome

MetS is a state of chronic low grade inflammation, consequence of a complex interplay between genetic and environment factors such as physical activity, energy dense food intake or stress. It usually causes a positive energy balance which leads to adipose tissue hyperplasia and hypertrophy, thus altering the free fatty acid (FFA) metabolism and the release of adipokines [1]. This ectopic fat accumulation leads to an unusual storage of FFA released by hypertrophic adipocytes in non-adipose tissue such as the liver, skeletal muscle, heart, and pancreas [15, 16].

Adipose tissue regulates a wide array of processes such as fat mass and nutrient balance, immune responses, blood pressure control, haemostasis, bone mass, and thyroid and reproductive functions [17]. Moreover, adipose tissue might act as an endocrine organ secreting molecules which are involved in the regulation of body weight (Leptin, adiponectin), in local inflammations (Tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6)), and in vascular functions (Angiotensin and plasminogen activator inhibitor-1) [18, 19]. In MetS, there is an unusual accumulation of lipids in adipocytes. This lipid augmentation triggers cellular stress and the activation of inflammatory signalling pathways, such as c-Jun N-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer or activated B cells (NK-$\kappa$B) pathways through an increment of macrophages and proinflammatory cytokines [20]. This local inflammation caused by the increase of proinflammatory cytokines secretions like TNF-α, IL-6, leptin, or resistin, can induce different systemic effects

such as insulin resistance in itself, in liver, in muscle and in kidneys, an increase of plasma TAG and glucose, or even atherosclerosis [20–22]. Insulin is a critical regulator of a wide range of aspects of adipocyte biology, including cell differentiation, stimulating glucose transport type 4 (GLUT4), lipogenesis and lipolysis [23]. Hence, insulin resistance in adipose tissue leads to an impaired insulin-mediated suppression of lipolysis. In addition, a reduction on adiponectin secretion by the adipose tissue is related to insulin resistance and lipodystrophy [24].

Besides, MetS can disrupt skeletal muscle function and thereby reduce mobility of individuals [25]. Skeletal muscle is structured by different fibre types, such as type I and type II, that determine their contractile and metabolic properties [26]. As explained before, MetS could be developed by an energy unbalance. In this regard, 5' AMP-activated protein kinase (AMPK) is the principal regulator of energy balance and it is activated by energy depletion, which is signalled by a decrease in the adenosine triphosphate (ATP) and adenosine monophosphate (AMP) ratio [27]. In obesity, AMPK activity is supressed promoting fat deposition [28]. As introduced before, the secretion of adiponectin by adipocytes is reduced as well as their receptors, AdipoR1 and R2, in obesity [29]. The decrease of the adiponectin released by adipose tissue leads to a reduction on insulin sensitivity in the skeletal muscle, and to an increase of its plasma levels, which finally inhibits the AMPK activity in skeletal muscle [30]. Obesity is related with increased diglycerides levels and reduced activity of diglycerides kinase delta (DAK-δ), which are related with fat deposition in the skeletal muscle [31]. Additionally, the reduction of DAK-δ activity alters the AMPK activity and the lipid metabolism [31]. Hence, AMPK signalling pathway might be the causes of impaired muscle function induced by obesity.

Non-alcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of MetS [32]. NAFLD is related with a high risk of developing T2DM, high plasma TAG, and hypertension [32, 33]. Moreover, the TAG accumulation on the liver alters

glucose, FA, and lipoprotein metabolism and causes inflammation [32]. Many complex factors could explain the hepatic accumulation of TAG: an increase on hepatic FA uptake from circulating fatty acids, an increase on its *de novo* synthesis, a reduction on fatty acid oxidation or an alteration on hepatic lipid secretion within very low-density lipoprotein (VLDL) [34]. The major source of FAs in the liver during a post-absorptive condition are the circulating fatty acids released by the adipose tissue, pronominally from the visceral fat [35]. Additionally, the gene expression of hepatic lipoprotein lipase (LPL) is increased in obese subjects leading to NAFLD through the downregulation of miR-467b. This overexpression of hepatic LPL increases the hydrolysis of TAG from chylomicrons and VLDL, and thus, the hepatic TAG uptake [36]. Additionally, liver synthesizes lipids *de novo* through a complex cytosolic polymerization in which acetyl coenzyme A (Acetyl-CoA) is converted to malonyl-CoA by Acetyl-CoA carboxylase and, after several cycles of metabolic reactions, it is converted to palmitate [37]. The *de novo* synthesis of lipids is regulated by insulin and glucose through the activation of sterol regulatory element-binding transcription factor 1 (SREBP-1c) and carbohydrate-response element-binding protein (ChREBP) [38]. In obesity-related T2DM, hyperinsulinemia is associated with a stimulation of *de novo* lipogenesis causing hepatic steatosis [37]. Moreover, the hyperinsulinemia state not only upregulates lipogenesis but also inhibits FFA β-oxidation, thus promoting hepatic fat accumulation [39]. In healthy conditions, the hepatic TAG are assembled into VLDL to be secreted to blood circulation. In NAFLD, the index of TAG content in nascent VLDL is two-fold greater than in a healthy liver, and their secretion is increased in order to provide a mechanism for removing hepatic TAG. However, the rate of secretion does not adequately compensate the rate of TAG production [40]. Additionally, VLDL particles are not able to penetrate sinusoidal endothelial pores to be exported out of the liver [41].

As explained before, T2DM is a metabolic disorder of MetS and affects adipose tissue, skeletal muscle and liver [42]. The cellular mechanisms responsible of insulin resistance are not entirely clear. For example, insulin resistance might be caused by the increase of liver and skeletal muscle FFA uptake due to an augmentation of their release into the circulation from the adipose tissue [43]. In this sense, the excess of intracellular lipid intermediates, particularly diacylglycerol (DAG), could be one reason to develop insulin resistance. In the liver, the link between DAG and insulin resistance is the protein kinase Cε (PKCε). The accumulation of DAG in the liver leads to the activation of PKCε, which consequently inhibits the insulin receptor kinase. Furthermore, insulin resistance can be induced by the endoplasmic reticulum (ER) under stress conditions. The ER stress response might be induced in the liver by saturated FA activating c-Jun N-terminal kinase pathway, which phosphorylates and/or degrades irisin receptor substrate 1 (IRS1) which, in turn leads to insulin resistance [44] (Fig. 1).

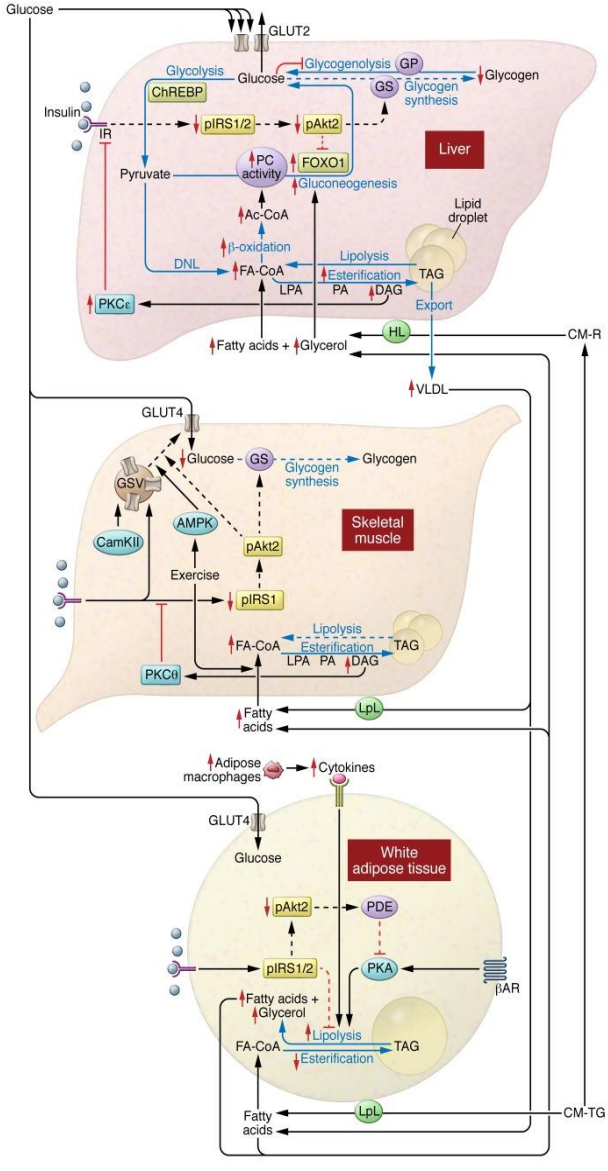


Fig. 1. **Mechanism of insulin resistance in the adipose tissue, skeletal muscle and liver.** Extracted from [42].

## 1.2. Sexual dimorphism of obesity

As described before, obesity is considered to be responsible for the increasing prevalence of MetS. Sexual dimorphism in metabolism is present in obese men and women. Many studies have demonstrated that the propensity to develop obesity differs between the sexes, and this is directly due to sex hormones. Studies using female mice which underwent an ovariectomy presented increased weight gain and impaired metabolism, but when estrogens were replaced, the ovariectomy effects were reversed [45].

The distribution of adipose tissue is significantly different between males and females. While the subcutaneous adipose tissue is higher in females, visceral adipose tissue is higher in males [46]. The augmentation of fat storage in the subcutaneous adipose tissue in women is caused by an increase of the LPL activity, which is a rate limiting step in the FA accumulation derived from circulating TAG. By contrast, this enzyme activation is higher in the visceral adipose tissue in men [47]. In addition, testosterone has a suppressive effect on LPL activity in subcutaneous fat [48].

In obesity, female animals are protected from the promotion of macrophage infiltration in adipose tissue due to an enhancement of adipocyte death [49]. On the contrary, male obese models present an enhanced macrophage infiltration [50]. This effect is related to the adipose tissue distribution, there are more resident macrophages in the visceral adipose tissue, which is higher in male animals [46, 51]. This increase in macrophages leads to an augmentation of the secretion of proinflammatory cytokines, such as TNF-α or IL-6, and a decrease of adiponectin secretion, which induce insulin resistance and endothelial dysfunction [52]. Moreover, female models, through estrogen and its receptor (ER) α, are capable to reduce hypoxia-inducible factor 1 (HIF-1) through an increase of prolyl hydroxylase 3 (PHD3) transcription, which in turn, leads to a reduction of metabolic

inflammations [53]. In addition, there are also sex differences in the induction of brown adipose tissue, which is considered a protective effect against metabolic diseases [54]. In this regard, estrogen have shown to upregulate thermogenesis, which is inversely associated with body weight gain [55].

The molecular metabolism in skeletal muscle is also subjected to gender-specific regulations and it is strongly associated with sex-differences in the adipose tissue [30, 56]. Muscle lipoprotein lipase (mLPL) expression and its activity are higher in female, thus promoting a better TAG clearance from plasma VLDL or chylomicron lipoproteins [57, 58]. Not only the TAG uptake from VLDL is increased in female, but also the FA uptake is increased through an increment of gene and protein expression of fatty acid translocase FAT/CD36, a lipid binding transport protein that facilitates FA transport into skeletal muscle [59]. Consequently, lipid storage is also gender-dependent, presenting a higher intramyocellular TAG content in lean females than in males [60]. Moreover, in females, the storage of TAG is localized in a higher number of lipid droplets, which are bigger compared to the males ones [61, 62]. It might enhance the accessibility of protein lipases to the droplets, thus increasing skeletal muscle TAG oxidation.

Leptin and adiponectin, both secreted by the adipose tissue, are related to fat oxidation in the skeletal muscle. Leptin has been reported to be higher in plasma in females [63]. Higher leptin levels are associated with an increase on fat oxidation in the skeletal muscle. This is explained through the augmentation of phosphorylation of AMPKα2 and the inhibition of acetyl-coA carboxylase (ACC) [64]. Similarly, adiponectin stimulates AMPK activity and inhibits ACC in the skeletal muscle, thus increasing glucose intake and fat oxidation, which is higher in females [65]. Altogether, these findings suggest that skeletal muscle in female models show a better lipid management than males, thus presenting a better lipid uptake, storage and oxidation.

Similarly to lipids, the glucose uptake and its metabolism in the skeletal muscle is increased in female models [66]. Nevertheless, the expression of the insulin-regulated glucose transport GLUT4 expression presents no differences between both genders [67, 68]. Hexokinase II is an important protein involved with the glucose uptake. It might explain why the glucose uptake is increased in female models while the expression of GLUT4 is equal in both genders [69]. The gene expression of hexokinase II has been found to be higher in women than in men [70]. The higher glucose phosphorylation by the hexokinase II in female might lead to an increase of glucose uptake, via reducing the glucose intracellular levels. Glycogen is one of the fates for the phosphorylated glucose [71]. Therefore, an increment on hexokinase expression might lead to an increase in glycogen storage in the muscle, which can influence the glucose uptake. In this sense, the gene expression of glycogen synthase has been demonstrated to be higher in females, although the amount of skeletal muscle glycogen remains similar [72, 73].

In liver, obesogenic diets are associated with the development of NAFLD [74]. As described before, NAFLD is produced by an imbalance in hepatic lipid metabolism, thus leading to a pathological accumulation of TG [75]. The prevalence of NAFLD is lower in women than in men, being 1.6%-24% and 4.3%-42% respectively [76]. The higher prevalence of NAFLD in males correlates with the fact that men have the double hepatic triglyceride content than women, 11% for male and 5% for women [77]. On the contrary, females present a greater rate of hepatic fat oxidation evidenced by a higher production of 3-hydroxybutyrate (3-HB) [97]. In this sense, some studies have demonstrated that plasma glucagon concentration, which is an ketogenesis stimulator, is increased in females, and it might contribute to their increased FA oxidation rate [80]. Although, the VLDL-apo-B-100 secretion has been reported 20% lower in females, the greater molar ratio of VLDL-TAG and VLDL-apo-B100 secretion rates suggests that the feminine liver secretes fewer but TAG-richer

VLDL particles [81]. However, kinetic studies propose that females have an enhanced production and clearance of VLDL-TG compared with males [82]. Furthermore, both genders present significant differences in the one-carbon metabolism pathway, which is strongly related with the VLDL packaging [83–85]. Sadre-Marandi et al. have reported higher choline and betaine levels, and lower homocysteine concentration in females [86]. They suggest that the main reason for the higher choline levels is the upregulation of phosphatidylethanolamine N-methyltransferase (PEMT), while for betaine is the downregulation of betaine homocysteine S-methyltransferase (BHMT) [86]. Altogether, these authors suggest that females are more protected against NAFLD through an increase of FA oxidation and their clearance.

Other researchers have observed sex differences in the development of T2DM, which are characterized by hepatic insulin resistance and elevated hepatic glucose production [87, 88]. In women, the oral glucose tolerance test (OGTT), used to determine T2DM progression [89, 90], has been reported to induce higher plasma glucose levels 2 hours after its administration, thus showing a lower glucose uptake by peripheral tissues and suggesting that women might have higher T2DM prevalence than men [91]. Even so, the risk of developing T2DM is higher in men than in women [92]. The fact that women present higher glucose levels in the OGTT could be due to they have less absolute amount of fat-free muscle mass than men, which is the major metabolically active tissue involved in glucose uptake [91, 92]. On the other hand, the reason why the T2DM prevalence is lower in women might be related to the estrogen effect. Estrogens are capable to increase hepatic insulin sensitivity by decreasing gluconeogenesis and glycogenolysis [93]. In the liver, both ER α and β are expressed in the cell membrane, the cytosol, or the mitochondria in order to regulate the glucose metabolism and the energy balance [94]. A deficiency

of ERα has been related to an increment of fasting insulin concentration, and impaired glucose tolerance [95].

## 1.3. Therapeutic approaches against obesity

Most therapies against obesity are centred on reducing energy intake, increasing the energy expenditure, and/or reducing the inflammatory effects. One of the most important targets in order to reduce the energy intake is the hypothalamus, which is involved in the homeostatic energy metabolism and satiety [96, 97]. Leptin is a satiety hormone secreted by the adipose tissue and binds to its receptor in the arcuate nucleus of the hypothalamus [97]. Leptin resistance is one of the signs of obesity [98]. Hence, leptin resistance might be an important target to develop therapies against obesity reducing the energy intake. Some of the strategies to combat obesity-induced leptin resistance include: leptin reformulation, such as pluronic modified leptin, which improves its blood-brain barrier penetration [99], increasing leptin receptor phosphorylation through the inhibition of protein tyrosine phosphatase [100], or the use of agonists and antagonists of downstream pathways and receptors such as the melanocortin receptor agonist melanotan-II [101]. Another anti-obesity strategy could be to increase the energy expenditure and/or the thermogenesis. Thermogenesis is the result of an increased uncoupling of mitochondrial oxidative phosphorylation raising the consumption of energy and the generation of heat [102]. The uncoupling protein 1 (UCP1) is the mainly mechanism to generate heat, uncoupling the ATP synthesis [102]. It is suggested that UCP1 expression or activity could be a target to develop treatments that might increase the energy expenditure. β3-adrenergic agonists have been used to up-regulated the UPC1-protein, but they need to be well designed for β3-adrenergic receptors, since the activation of β1- and β2-adrenergic receptors have cardiac side effects [103–105]. Other studies have induced the overexpression of UCP1 through the activation of the cold-sensitive calcium channel TRPM8 or the peroxisome proliferator-

activated receptor γ (PPARγ) [106, 107]. Another strategy might be the use of hormones or growth factors to develop and/or differentiate heat-generating brown or beige adipocytes [108]. Beige fat cells positively correlate with insulin sensitivity and hence, the improvement on beige fat cell differentiation might have antidiabetic and anti-obesity effects. Sympathetic activators or arterial natriuretic peptides can also increase the presence of beige fat cells in white depots [109]. All these strategies should be further studied since only caloric restriction and surgery really work.

### 1.3.1. Gender-dependent therapies against obesity

As mention before, obesity is a gender dimorphic disease [110]. Therefore, the response to anti-obesity treatments it is expected to be gender dependent. A meta-analysis of 58 published studies described that men present a higher decrease in body weight in response to diet and lifestyle changes [111]. Likewise, Stroebele-Benschop et al. [112] not only found the body weight more decreased in men due to diet or lifestyle changes, but also in response to bariatric surgeries.

Nevertheless, not only diet and lifestyle changes present different effects depending on the gender, but also the effect of commercial drugs against obesity. For example, glucagon-like peptide 1 (GLP1) derivates are used for the treatment of T2DM and as an anti-obesity drugs [113]. In this sense, Richard et al. [114] showed that GLP-1 effects could be gender-dependent. They determined that female rats were more sensitive than male to the administration of GLP-1 and this differences were revoked by antioestrogens.

# 2.CIRCADIAN RHYTHMS

## 2.1. Molecular machinery of the circadian clock

Circadian rhythms are ~24 h fluctuations of different biological processes in the absence of external cues. They are synchronized to ensure the adaptation of the physiology to external time. Molecular clocks are located along the whole body, whereas its central clock is in the hypothalamic superchiasmatic nuclei. Increasingly, the central clock concept is being substituted by a more "federated" vision since it has been reported that there are others ways to introduce rhythmicity in the biological process in the absence of the hypothalamic superchiasmatic nuclei [115]. It was thought that only the central molecular clock was able to process light inputs from the retina and pass the "external time" to peripheral tissues via endocrine and systemic cues [116], but it has been reported that there are peripheral tissues, such as the pineal gland, liver or adrenal glands, which are directly affected by light [117–119]. One of the most important systemic ways to synchronize routes between the hypothalamus and peripheral tissues is the secretion of hormones by the suprachiasmatic nucleus, such as glucocorticoids, which promote the mobilization of energy [120], or melatonin, which is associated to the circadian regulation of insulin secretion and blood glucose levels [121].

The molecular circadian clock in mammalians is generated by interlocking transcription/translation feedback loops (TTFL) comprised of a set of core clock genes [122]. The TTFL is mainly composed by four integral clock proteins: two activators, brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) [123] and circadian locomotor output cycles kaput (CLOCK) [124]; and two inhibitors, period 1, 2 and 3 (PER 1-3) and cryptochrome (CRY1 and 2) [125, 126]. BMAL1:CLOCK heterodimerize and activate the transcription of PER and CRY

and other genes such as *Reverse-erythroblastosis virus* (*REV-ERB α/β*) or *retinoid-related orpham receptor* (RORs) [127]. PER and CRY proteins heterodimerize in the cytoplasm and are translocated to the nucleus, after that, the dimer is degraded through the ubiquitin-dependent pathways defining the autoregulatory 24 h-loop of the circadian clock [128]. Another important regulator is the ratio oxidized/reduced of Nicotinamide Adenine Dinucleotide (NAD) which modulates the union of the BAML1/CLOCK dimer to the binding sequence site [129]. It is schematized in the Fig.2.

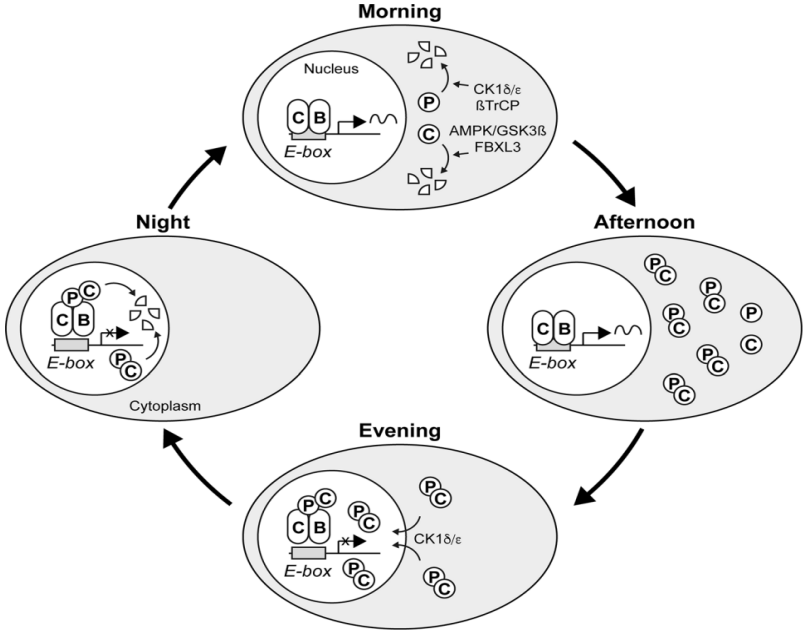


Fig. 2. **Molecular dynamics of the mammalian clock's core TTFL.** Extracted from [145].

## 2.2. Circadian rhythms and obesity

Has been reported that obesogenic diets alter different circadian system outputs, such as locomotor activity, eating patterns or the rhythmicity of the homeostatic neural system activity [130–133]. In normo-caloric feeding conditions, rats and mice present the highest locomotor activity during the night period [134].

However, when these nocturnal animals are fed an obesogenic diet, their locomotor activity is reduced along the nocturnal period and is increased throughout the diurnal period [135, 136]. Moreover, some studies have determined that obesogenic diets also disrupt sleep patterns decreasing awake time, which has been observed to be more fragmented [137]. Furthermore, as mentioned above, eating patterns are altered. Not only there is an increment on calorie ingestion, but also, animals fed with an obesogenic diet eat more frequently [138, 139]. Since food intake is an important signal in the synchronization of peripheral circadian rhythms, changes in eating behaviour might disrupt circadian rhythms [140]. At clock genes expression level, obesogenic diets alter the expression of clock genes in peripheral tissues, whose disruptions depend on the duration of the diet, its energy content and/or the animal species used [136, 141]. Some studies have focused on the effect of obesity in the master clock. For example, Kaneko at. el. found attenuated the expression of BMAL1 and REV-ERBα as well as increased CLOCK gene expression in obese mice fed high fat diet [142]. Moreover, Pivovarova *et. Al.* reported that changes on dietary fat and carbohydrate content altered the diurnal oscillations of the core clock genes *per1, per2,* and *per3* [143].

## 2.2.1. Circadian rhythms in the adipose tissue

Several animal studies have demonstrated that obesogenic diets might induce obesity and insulin resistance through alterations on the oscillation of clock genes in adipose tissue [144]. Different aspects of lipid metabolism, including lipolysis, lipogenesis, and BAT thermogenesis, are regulated by the circadian clocks [145]. The two most important families that connect the lipid metabolism and the core clock mechanism are REV-ERBs and RORs [146]. BMAL1 expression are regulated positively by REV-ERBs and negatively by RORs, and the BMAL1:CLOCK heterodimer regulates the expression of *rev-erb* and *ror* [127, 147]. In this sense, diet-induced obese mice treated with REV-ERB agonist improved dyslipidaemia and

hyperglycaemia [148]. Besides, PPARγ, which expression is mediated by the BMAL1:CLOCK heterodimer, is also involved in lipid and glucose metabolism upon binding of endogenous free fatty acids [149]. Moreover, alterations in adipose clock genes affect the expression of appetite-regulating neuropeptides in the hypothalamus through circadian disruptions on adipokine secretions [150]. In healthy animals, plasma leptin presents circadian rhythms with its higher concentration in the early dark phase in nocturnal animals and in the early mid-light phase in diurnal animals [151]. Obesogenic diets disrupt the leptin circadian variations and it might play a role on leptin resistance and obesity [152]. Another adipokine that presents rhythmicity is the adiponectin, which directly regulates glucose and FA oxidation, and maximum expression occurs during the afternoon [153]. Circadian disruptions in adiponectin may be related with the development of obesity, since there have been observed disruptions in its daily pattern in rats fed an obesogenic diets [153]. Some other adipokines that show circadian rhythms are resistin, visfatin, plasminogen activator inhibitor-1, TNFα and interleukin-6 (IL-6) [154–157].

## 2.2.2. Circadian rhythms in the skeletal muscle

McCarthy et al. [158] found 215 genes whose expressions oscillated over a 24h period in the skeletal muscle. Moreover, most of the them were muscle-specific and involved with metabolism, e.g. *ATP synthases*, *Fbxo32* or *Atrogin-1*, in transcription, *Myod1,* and cytoskeletal organization, e.g. *Myh1*. Physical activity has been identified as a potent zeitgeber. Since the skeletal muscle function and mobility is altered by obesogenic diets, it is expected that changes in physical activity due to obesity might disrupt the circadian rhythms of the skeletal muscle [25, 159]. In order to determine the effects of skeletal muscle in the whole body circadian energy homeostasis, Dyar et al. [160] used a skeletal muscle-specific BMAL1 mutant. They observed reduced the skeletal muscle insulin sensitivity, while the insulin signal

pathway was not found affected. They observed that the translocation of the glucose transporter GLUT4 was reduced. In addition, they also demonstrated that skeletal muscle BMAL1 mutants exhibited a decrease of glucose oxidative rate, probably due to the observed reduction on pyruvate dehydrogenase activity [160]. Furthermore, another important factor that contributes to the deregulation of circadian rhythms in the skeletal muscle is AMPK. It regulates the expression of several clock genes and its activity is suppressed in presence of obesity [28, 161].

## 2.2.3. Circadian rhythms in the liver

Disruptions in hepatic circadian rhythms might lead to NAFLD [162]. Kohsaka et al. [163] determined that in mice fed a high-fat diet, the amplitude of BMAL1 expression was reduced in the liver in both, during the light and dark periods, whereas PER2 expression was decreased only during the dark period. Most of the liver metabolism pathways are under circadian control, such as glucose metabolism, lipid metabolism or bile acid metabolism [164–166] (Fig. 3).

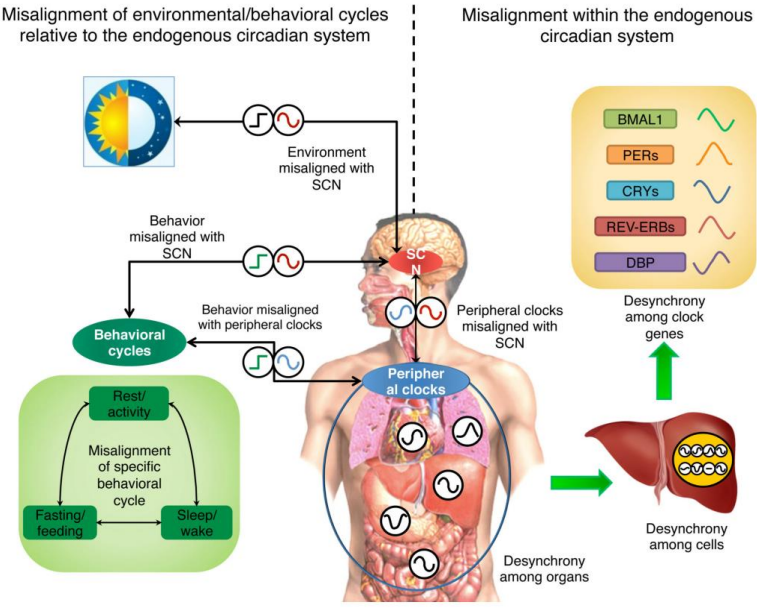


Fig. 3. **The misalignment of behavioural cycles and environment misalignment with the suprachiasmatic nucleus desynchronized among hepatic clock genes**. Extracted from [133]

### 2.2.3.1 Circadian rhythms of carbohydrates metabolism

The liver is the principal gluconeogenic organ in the maintenance of homeostatic blood glucose levels [167]. In BMAL1 mutant mice, hepatocytes decrease glucose transporter GLUT2 expression thus reducing the post-absorptive glucose uptake [168]. Once glucose has entered the hepatocyte, the circadian clock controls physiological glucose levels through diverse mechanisms. Inside the hepatocyte, glucose is phosphorylated to glucose-6-phopshate and can be used or stored as glycogen [169]. Both, glycolysis and glycogen synthesis, are controlled by the glucokinase enzyme, whose expression is under circadian control and its zenith appears during the transition from the rest phase to the active phase [170, 171]. Moreover, glucose metabolism is regulated by external cues, such as insulin and glucagon, both presenting rhythmicity [172, 173]. Hence, the insulin and glucagon rhythmicity is transferred to the liver through their signalling effect [171]. For example, insulin controls the activity of glycogen synthase kinase 3 in a circadian manner which determines the activity of glycogen synthase [174], or the stability of REV-ERB [175], which in turn dictates the rhythmicity of other metabolic processes [176].

### 2.2.3.2. Circadian rhythms of lipid and bile acid metabolism

In several studies, it has been stablished the rhythmicity of lipids in plasma, which have been shown to be generated by liver [177, 178]. Most of the enzymes related to the lipid metabolism are under circadian control. Malonyl-CoA is generated by ACC through the carboxylation of the cytosolic acetyl-CoA, which is an important step in fatty acids synthesis [179]. This step is under circadian control through AMPK, which inhibits fatty acid synthesis and promotes fatty acid oxidation by the inactivation of ACC activity [180]. Besides, AMPK drives the phosphorylation of casein kinase Iε (CKIε), which then phosphorylates protein PER and facilitates its

degradation [181]. Moreover, AMPK is capable of interact directly with the core clock phosphorylating CRY [181]. Furthermore, fatty acid synthesis is also under circadian control by the transcription of *Elovl3* and *Fas* [162, 182]. Additionally, β-oxidation and ketone-bodies production are regulated in a circadian manner though the expression of genes, such as *Cpt*1/2 or *Hmgcs*2 [183, 184]. But also, the expression of enzymes involved in hepatic TG synthesis from glycerol-3-phosphate, such as *Gpat*2, *Asgpat*1/2, *Lpin*1/2, and *Dgat*2, present rhythmicity [185]. The zenith of TAG appears during the rest phase, while their lowest concentration is found during the active phase [185]. Additionally, REV-ERBα/β regulates hepatic choline metabolism through the expression of choline kinase (CK) α [186]. CKα is the first enzyme in the CDP-Choline pathway in which, at the end, produces phosphatidylcholine (PC). PC is the most abundant phospholipid in VLDL and its production is necessary for VLDL secretion, so is conceivable that the rhythmicity in VLDL is linked to the circadian rhythms of PC [186]. In this sense, previous studies have demonstrated that deficiency in both REV-ERBs causes hepatic steatosis [187].

Bile acids are synthetized from cholesterol in the liver [188]. Their synthesis is controlled by the nuclear receptor farnesoid X receptor (FXR) and the small heterodimer partner (SHP) in the liver, and by the hormone fibroblast growth factor 15 (FGF15), which is secreted by the intestine [189, 190]. The hepatic expression of both, FXR and SHP, and the intestinal FGF15 secretion are under circadian control [191]. Altogether drive the circadian transcription of cholesterol 7α-hydroxylase (Cyp7a1), which is a rate-limiting enzyme in the bile acid synthesis pathway [191].

## 2.2.4. Hormones: Linker between circadian clocks

The circadian expression or secretion of hormones, such as cortisol, insulin, ghrelin, adiponectin or leptin to the main bloodstream are used to link the rhythmicity of the different tissues and organs. As described before, most of these

hormones are dysregulated by obesity. Cortisol production peaks in the morning preparing the system for the waking-associated stress [192]. Some studies link the dysregulation of cortisol with obese-related metabolic disorders [193]. In the case of insulin, it presents its zenith during the active phase to stimulate anabolism [194]. Furthermore, the lack of PER and CRY results in hyperinsulinemia, while the loss of CLOCK and BMAL1 leads to hypoinsulinemia [195–197]. Moreover, insulin can also act as circadian clock regulator. In this sense, Yamajuku et al. [198] showed that insulin acts as a synchronizer for the liver clock. The stomach secretes ghrelin with a peak in the inactive phase, which can regulate directly the expression of clock genes in the suprachiasmatic nucleus, thus increasing food intake [199, 200]. On the other hand, as explained before, adiponectin is secreted by the adipose tissue and presents a peak during the afternoon [153]. Mice with metabolic syndrome and hypoadiponectinemia have showed alterations on their circadian gene expressions in liver and skeletal muscle. Additionally, the expression of clock genes in the liver were ameliorated when the adiponectin gene expression was recovered [201]. Another hormone also secreted by adipose tissue is leptin, which, in this case, peaks during the active phase. Disruptions in circadian clocks lead to leptin resistance and shifts in energy balance and weight gain [202].

## 2.2.5. The role of gender in circadian rhythms

As explained above, circadian rhythms depend on external cues such as light, food or activity. There have been suggested habit differences between genders, such as the sleeping time. For instance, women present earlier timing, longer duration of sleep and more slow-wave sleep [203]. However, there are not too many reports showing the differences or similitudes of the central clock gene expressions between both genders. Although the neurobiological mechanism underlying the sex difference in circadian alignment is unknown, other physiological parameters, such as plasma melatonin and body temperature, are used to find out these differences.

Duffy et al. [204] found a shorter inherent circadian period of melatonin and lower body temperature in women, and those findings were observed even when both, man and women, maintained identical bedtime and wake times. As described before, internal cues, such as sex hormones, which are secreted on a circadian basis, could target suprachiasmatic nucleus [205]. It has been shown that circulating estrogen and androgen regulate circadian locomotor rhythms through their interaction with the suprachiasmatic nucleus [206]. Hence, this disparity between both genders suggests differences in the circadian rhythms of the peripheral tissues.

As explained before, insulin and leptin regulate lipid and carbohydrate metabolism. Moreover, both insulin and leptin exhibit circadian rhythms and present different behaviours depending on the gender. In this sense, Ahren et al. [207] compared the rhythmicity of plasma leptin, insulin and glucose in mice. In the case of leptin, it showed to oscillate in both genders peaking during the dark period, but females presented a higher peak than males. Plasma insulin showed to peak at the beginning of the dark period, but, in this case, male mice exhibited a highest concentration. These gender differences on both, plasma insulin and leptin, suggest different effects on their targeted peripheral tissues.

Xiaoxia et al. [208] showed gender differences in the expression of clock genes including *clock*, *bmal*1, *per* 1-3 and *cry* 1-2 in the liver of mice. They found circadian rhythms in both genders for these genes, but, most of these hepatic clock genes presented their mRNA peaks 30 min earlier in female than in male mice. Another study suggests that the clock genes *cry*1 and *cry*2 are determinants for the sexual dimorphism in liver metabolism [209]. Additionally, Perez-Mendoza et al. [210] said that not only CRY1 was important to determine the sexual dimorphism, but also REV-ERB$\alpha$ and ROR$\gamma$. In the same study, they found that the daily expression of hepatic BMAL1 was more altered in female mice fed a high-fat diet than in male mice. Since BMAL1 regulates PPARs and they play a key role in the transcription of genes

involved in lipid metabolism [149], it is expected that the gender-specific dysregulations by obesogenic diets in BMAL1 expression, alter the rhythmicity of the lipid metabolism in a circadian manner, depending on the gender.

# 3. POLYPHENOLS

## 3.1. Structure and classification

Polyphenols are secondary plant metabolites produced in metabolic pathways, which are generally involved in chemical defense against pathogens, reproductive processes, and plant-plant communication [211]. Polyphenols are a group of phenolic compounds characterized by at least two phenyl rings and one or more hydroxyl substituents [212]. Polyphenols can be simply classified into flavonoids and non-flavonoids [213]. All flavonoids share the basic structure of diphenyl propanes (C6-C3-C6 abbreviated carbon structure), in which phenolic rings are usually connected by a heterocyclic ring, commonly a closed pyran. Flavonoids are classified in a wide range of compounds depending on the variations of the hydroxylation pattern and the oxidation state of the central ring: flavanols, flavanones, flavonols, flavones, isoflavones, and anthocyanins [214] (Fig. 4.).

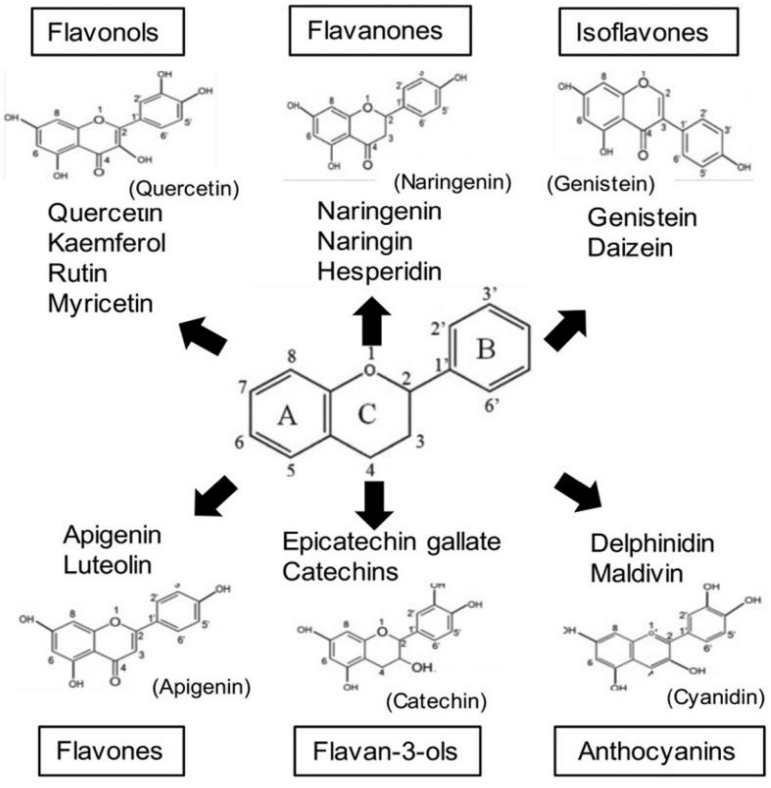


Fig. 4. **Classification of flavonoids according to their chemical structures**. Extracted from [214].

Flavanols are found in food mainly as aglycones and in the form of monomeric units, catechins and epicatechins, or polymeric forms, proanthocyanidins (PACs). PACs are polymers of catechins that are bound together by links between C4 and C8 or C6 [215]. They have been demonstrated to be the responsible for the astringent character of fruits, such as grapes, peaches, or apples, and beverages like wine, tea or beer [216].

## 3.2. Metabolism of flavanols and their mechanisms of action

Flavanols, depending on their structure, can be absorbed as low-molecular weight molecules or after being metabolised by the microbiota. In the enterocytes, flavanol monomers can enter through passive diffusion [217]. Nevertheless, the polymeric forms must be metabolized to become sulfate, glucuronide, and

methylated sulfate/glucuronide metabolites to be catalized by sulfotransferases, uridine-5'-monophosphate glucuronosyltransferases and catechol-O methyltransferases [217, 218]. It has been demonstrated that the large amounts of flavanols are metabolised mainly in the liver, and small amounts can be metabolised by the intestinal mucosa, playing the liver a secondary role modifying them [219]. Although flavanol structures are an important factor for their bioavailability, another external factor, such as the food matrix, food processing and the ingestion duration, are relevant [220, 221]. Furthermore, the internal factors related with the individual, such as sex, age, physiological condition or pathology, might exert effects on the flavanol bioavailability [221]. Regarding their secretion, Stoupi et al. [222] administrated B2 procyanidin and, approximately a 76% was secreted via urine indicating renal clearance. Around the 24% of the dose was excreted with the faeces through biliary secretions and enterohepatic recycling.

The capability of binding to proteins and epigenetic modifications are the two major mechanisms that have been suggested to clarify the biological effects of PACs. They are translated into a multitude of biological activities, such as antioxidant properties, cancer chemoprevention or anti-inflammatory and anti-diabetic effects [223]. Most of these beneficial effects appear to be due to specific interactions with proteins and enzymes. In this sense, a recent study showed a PAC antioxidant effect against β-hydroxybutyrate-induced oxidative damage through the activation of the nuclear erythroid2-related factor 2 (Nrf2) signalling pathway, and increased activities of catalase and glutathione peroxidase in bovine endometrial cells [224]. In another study, Toden et al. [225] used PACs to investigate their anti-tumorigenic effect. They demonstrated that their cancer chemoprevention was carried out through the inhibition of cellular proliferation, induced apoptosis and cell cycle arrest. Another PACs mechanism of action, is the capacity to induce the transactivation of some nuclear receptors, such as RORα, which regulates the molecular clock in the liver

[226]. As mention before, PACs can also modulate epigenetics. In this regard, it has been shown that the PACs present in green tea were capable to inhibit histone acetyltransferase and histone deacetylase [227]. Moreover, PACs can directly interact with miRNAs [228].

## 3.3. Effects of flavanols against metabolic syndrome

A wide range of studies have demonstrated the ability of PACs to reduce body weight and fat depots [229–231]. It makes them a good nutritional supplement to act against obesity. Nevertheless, this PAC effects seem to depend on the dose, administration days, animal species and the experimental approach [231].

In addition to their body weight effect, PACs have shown to modulate the functionality of adipose tissue, skeletal muscle and liver. In the adipose tissue, Pascual-Serrano et al. [232] found that a grape seed proanthocyanidins extract (GSPE), increased the number of adipocytes and reduced their size. At molecular level, GSPE induced adipogenesis through the upregulation of PPAR$\gamma$ in a Sirtuin 1 (Sirt1)-dependent manner. In another study, PACs activated *Prkaa* gene, which up-regulated lipolysis in the adipose tissue [233]. Moreover, the authors also reported anti-inflammatory effects through a decrease of TNF-$\alpha$ and IL-6 in mesenteric white adipose tissue of rats fed a high fat diet [234]. PACs may also affect the skeletal muscle, and some of their effects have been assigned to AMPK$\alpha$ activations. Casanova et al. [235] showed that PACs increase AMPK phosphorylation rising the muscle gene expression of UCP2, which results in increased thermogenesis and energy expenditure. The activation of AMPK also inhibits lipogenesis and stimulates fatty acid oxidation [161].

Regarding to the effect of flavanols in the liver, recent studies with diet-induced obese mice, rats, chickens or zebrafish demonstrated that green tea, which is rich in flavanols, might downregulate the expression of genes involved in fat synthesis, such

as malic enzyme or glucose-6-phosphatase, and upregulate the mRNA levels of genes related to fat β-oxidation like carnitine palmitoyltransgerase-1, acyl-CoA dehydrogenase or PPARα [236–239]. In this sense, Baiges et al. [240] observed, by proteomics, a reduction on the translation of proteins involved in hepatic glycogenesis, glycolysis, and fatty acid and triglyceride synthesis in obese diet-induced female rats treated with GSPE. Another study concluded that green tea was able to prevent obesity through the stimulation of the mitochondrial complex chain, thus increasing the energy expenditure, particularly from the oxidation of lipid substrates [241]. Moreover, Liu et al. [242] also found decreased the intrahepatocellular lipid content and the hepatic TAG levels, which were associated with the downregulation of mTOR/SREBP-1-mediated *de novo* lipogenesis. In addition, Baselga-Escudero et al. [243] observed that the chronic administration of PACs restored the increase of miR-122 and miR-33a in obese rats. Both, miR-122 and 33a, play a critical role in lipid metabolism in the liver, and their disruption can contribute to the development of obesity and metabolic syndrome.

There are a lot of studies that suggest that flavanols may attenuate insulin resistance in obese models. Insulin is secreted by the pancreas and more specifically, by the β-cells. PACs have been related to a reduction of insulin production, gene expression and content, and pancreatic and duodenal homeobox 1 (PDX1) gene expression [244]. Additionally, PACs have shown to reduce the characteristic accumulation of lipids by obesogenic diets through the increase of lipid β-oxidation and downregulating genes related with lipid synthesis, such as *fasn* or *SREBF*1 [244, 245]. In addition, other studies showed significant decreases in glucose and insulin levels after treatments with PACs, in animals fed cafeteria diet. In these cases, their effect is related to an increased glucose uptake and energy expenditure in insulin-sensitive tissues [246, 247]. The increase of glucose uptake by peripheral tissues has been related to the antihyperglycemic effect of the PACs, whose common target for their

effect is AMPK [248–250]. Its activation induces GLUT4 translocation to the plasma membrane, thus enhancing the glucose uptake [251–253]. Additionally, enzymes related to glucose metabolism, such as glucokinase, glucose-6-phosphatase, or phosphoenolpyruvate, are target of PACs, which improve insulin sensitivity [254].

## 3.4. Modulation of circadian rhythms by flavanols

PACs have shown to present chronobiological properties [255, 256]. In this regard, our research group has observed that PACs modulate melatonin in plasma and the expression pattern of clock genes in the hypothalamus [257]. Moreover, in this study, Ribas-Latre et al. [257] found that PACs, administrated at zeitgeber 0 (ZT0), at the beginning of the light period, increased plasma melatonin concentration in the middle of the light period and modified the rhythmicity of several plasma metabolites. They suggested two different mechanisms to explain these GPSE effects. On one side, they proposed that PACs may act as a nonphotic cue, triggering the central clock system during the light period. Alternatively, PACs may act directly in the intestine, which can send cues to the brain through neuronal and/or hormonal mechanisms. At molecular levels, PACs were able to alter the BMAL1 pattern expression, which is defined as the master regulator of the molecular clock system [258].

In addition to their effect on the master clock, PACs might also modify circadian rhythms in peripheral tissues, such as the liver. In this regard, Ribas-Latre et al. determined the effect of PACs in the rat livers [226]. Interestingly, PACs modulated nicotinamide phosphoribosyltransferase (NAMPT) and NAD in the liver. NAMPT is the rate-limiting enzyme in the synthesis of NAD in its salvage pathway [259]. Furthermore, NAD activated SIRT 1 and 3, which control many cellular processes, in a circadian manner, by cycling the activation of proteins, through exchanging their acetylated and deacetylated forms.

PACs not only have the capacity to entrain peripheral molecular clock in healthy models, but also are able to mitigate clock disruptions induced by obesogenic diets. PACs administration nearly correct all of disruptions in the clock genes induced by obesity in liver and gut, but their effect is less effective in white adipose tissue [260]. Altogether, these findings suggest that it makes the time of administration of PACs crucial to understand their beneficial effects against obesity.

# 4. METABOLOMICS

The set of metabolites that characterize the phenotype of a living organism is called metabolome. One of the most common approaches to study the metabolome is the analysis of its constituent metabolites through metabolomics. Metabolomics is defined as the study of small molecular or metabolites (<1500 Da) in living systems, which provides information with a high potential for precisely characterization of the physiological state of a cell, tissue, organ or organism [261]. In 1999, Nicolson and colleagues originally introduced the term metabonomic defined as “the quantitative measurement of multiparametric metabolic responses of a living system to pathophysiological stimuli or genetic modification” [262]. Moreover, metabolomics can be also described as “the non-biased identification and quantification of all metabolites in a biological system” [263]. Despite of having two definitions, in real life, these two expressions are used interchangeably and they have the same purpose: characterize the metabolome [264]. The human metabolome is influenced by the environment, e.g., food, drink, or medication (exogenous metabolites) as well as by the “in-vironment” such as the gut microbiota (endogenous metabolites) [265]. The fact that metabolome is influenced by the environment, which is ever-changing and extremely dynamic, it makes the obtaining of an understandable characterization of the metabolome, a very difficult and challenging task [266]. Nevertheless, metabolomics offers the best trade-off and has become very popular

for the purpose of metabolic discoveries and phenotype characterization. In biological systems, the number of small molecules is far greater than those currently represented on the metabolic charts of the analytic platforms. The difference between the known and the unknown metabolites is described as “metabolic dark matter” [267]. In order to reduce the among of unknown metabolites found, metabolic databases have been openly designed and are in constant development together with analytical and technical improvements. The Human Metabolome Data Base (HMDB) [268] lists 156.223 of non-redundant metabolites with their spectres. Only 18.598 can be detected and quantified, and nothing but a few are routinely subjected to quantitative analysis. Metabolomic approaches can be targeted or non-targeted [269]. Targeted metabolic approaches refer to the quantitative measurement of a specific group of known metabolites and it requires *a priori* knowledge [270]. In contrast, the non-targeted approaches have been applied in metabolic studies which want to focus on the global metabolic profiling, and it is usually performed to generate hypothesis and/or classification purposes [271]. A critical step to obtain the maximum information from a biological sample is the extraction procedure, since the number of metabolites and their chemical nature depend on it [272]. There are a wide range of different metabolic platforms to perform metabolomics, the two most widely applied are Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS), the last one is usually coupled with gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE) [273–276].

## 4.1. Metabolomics technologies

NMR spectrometry and MS are the most relevant approaches in order to carry out metabolomics. Both technologies give different and complementary information, useful to extend the coverage of the metabolome [277].

### 4.1.1. Mass Spectrometry

MS is an analytical technique used to determine the mass of atoms or molecules, which also gives structural information through ions fragmentation [278]. MS is usually used in targeted metabolomics [279–281]. Isotopically labelled compounds or external standards are used to generate calibration curves to both, identify and quantify metabolites. In order to reduce the complexity of identifying analytes, different separation chromatographic techniques, such as GC, LC and CE are coupled to MS. GC-MS is used to separate and analyse volatile compounds. In this sense, the samples become vaporized and pass through a column, in which are separated by their different retention times [282]. In LC-MS the sample is mixed with water and organic solvents, and it is passed through a column that contains a solid absorbent material which differentiates the retention time inside the column depending on the interaction between the compounds and the column [283]. Finally, in CE-MS the metabolite separation is based on the electrophoretic mobility of each compound using an electric field between the electrodes [284].

### 4.1.2. Nuclear Magnetic Resonance (NMR)

NMR spectroscopy has been defined as "…the study of molecular structure through measurement of the interaction of an oscillating radiofrequency electromagnetic field with a collection of nuclei immersed in a strong external magnetic field" [285]. In its simplest form, NMR experiments consist of three steps: to place the sample in a static magnetic field, to apply a radio frequency pulse to

excite the sample nuclei and to measure the signal frequency emitted by the sample. The analyst can determine the bonding and arrangements of atoms in the sample from the emitted frequency. Additional information about the chemical nature and the spatial configuration can be also inferred. In metabolomics, the NMR signals are usually plotted as spectra and analysed respected to two features: intensity and frequency [286].

One of the most used nuclei in metabolomic is the proton ($^1$H), although its chemical shift range is smaller compared to other nuclei. Hence, the peaks plotted from a complex mixture in a $^1$H experiment are too much closer or overlapped, which makes the spectral assignment a challenging task. In order to solve the overlapping problem in the traditional $^1$H NMR spectroscopy, a two-dimensional (2D) $^1$H NMR spectroscopy is applied to assist the identification of metabolites [287, 288]. Other tools and strategies, such us low volume probes [289], standardization procedures [290] or different software for analysis [291], could be used to deal with the high degree of complexity in biological samples of metabolomic studies.

The metabolomic datasets are usually acquired by either, NMR or MS, despite their fundamental complementarity. Nevertheless, their combination could reduce their weaknesses and limitations. MS has more sensitivity than NMR, since its detection limit reaches a nanomolar scale. Furthermore, while NMR is more focused on non-targeted experiments, MS is more used on targeted studies. However, NMR is non-destructive and sample can be recovered and stored for further analysis. Actually, using HRMAS NMR the sample tissues can be used directly, without being processed [292]. On the contrary, MS is a destructive technique, although a small amount of sample is needed. The number of detectable metabolites in NMR are lesser than in MS, but the reproducibility in NMR is higher [293].

### 4.1.2.1. NMR-based metabolomic applications.

NMR-based metabolomics have a wide range of applications. For example, NMR metabolomic approaches can be used to detect urine or plasma biomarkers from different pathologies [294]. Furthermore, metabolomics by NMR can be used to characterize a specific biological matrix. In this sense, Song et. al. characterized rice gran from various *Oryza sativa L.* cultivars using $^{1}$H HR-MAS NMR-based [295]. Besides, NMR is also capable to track the metabolic fate of a specific metabolite, labelling this molecule with $^{13}$C [296, 297]. In this regard, Zhou et. al. determined the metabolic fate of glucose in brains of APP/PS1 transgenic mice [298]. In hospitals, NMR-based metabolomics are used in order to diagnosticate diseases, such as gastric cancer, only doing a simple $^{1}$H NMR urinary metabolomic profiling [299]. Toxicity studies are also performed with NMR [300, 301]. Finally, NMR has also proved to be a potential tool to determine the effects of supplementations in nutritional interventions [265, 302].

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# HYPOTHESIS AND OBJECTIVES

# Hypothesis and objectives

Metabolic syndrome (MetS) is one of the most prevalent pathologies in the world. The International Diabetes Federation (IDF) estimates that around the 25% of the world's population presents MetS. It represents a clustering of different metabolic abnormalities, such as abdominal obesity, hypertension or insulin resistance. In fact, obesity is mainly considered to be the responsible for the increased MetS prevalence. However, it does not affect in the same way both sexes.

In addition to all the metabolic disorders associated to obesity, the circadian mechanisms are also disrupted in obese subjects. Circadian rhythms are 24h fluctuations of different biological processes in the absence of external cues. The central clock is manly synchronized by the light, while peripheral tissues are regulated by the diet. Since obesogenic diets affect in a gender manner, it is expected that the circadian disruptions are also gender dependent.

Polyphenols have been used during the last decades as a treatment to act against the pathologies related to obesity. In this thesis, it has particularly been used a grape seed proanthocyanidin extract (GSPE) which possesses a large number of benefits, such as antioxidant properties, ameliorated dyslipidemia, decreased chronic inflammation, reduced hypertension and improved insulin sensitivity, among others. Additionally, proanthocyanidins are potential circadian rhythms modulators, thus improving the circadian disruptions in obese subjects.

Liver is an essential metabolic organ, and most of its metabolic processes are altered in obesity, thus leading to Non-Alcoholic Fatty Liver Diseases. Several studies have demonstrated that metabolomics is a powerful tool to determine the metabolic status of biofluids, tissues and/or organs. In the sense, nuclear magnetic resonance (NMR) is a robust technology to analyse the liver metabolome.

Hence, considering all this information, we hypothesize that **the beneficial effects of GSPE against the hepatic metabolic disruptions, present in MetS, are time dependent, particularly when they are induced by an obesogenic diet**. In other words, the time of GSPE administration might change its beneficial effects against diet-induced MetS.

To verify the confidence of this hypothesis, the main objective of this thesis was **to determine whether GPSE has different effects on the hepatic metabolism depending on the administration time, when obesity and MetS are taking place.**

Different specific objectives were proposed to reach the general aim:

1. **To assess the impact of diet-induced obesity on the circadian rhythms of the hepatic metabolome depending on the gender.**

   To archived this objective, Fischer 344 male and female rats were fed a chow or a cafeteria diet, and subsequently sacrificed at two different times, ZT3 and ZT15 (3h after the lights turn on and turn off). Results revealed that the obesogenic diet disrupted the circadian rhythms of the hepatic metabolome in both genders. Nevertheless, female rats were more resistant to the obesogenic diet effects, since the improved circadian rhythms of most of the metabolites related to the VLDL synthesis and packaging (**Manuscript 1**).

2. **To determine if the beneficial effects of a grape seed proanthocyanidins extract (GSPE) are time-dependent.**

   To archive this objective, Wistar rats fed with a cafeteria diet were orally supplemented with GSPE at two different times, ZT0 and ZT12 (when the lights turn on and turn off). In order to avoid the beneficial effects attributed to the female gender against obesity, only male rats were used. Our results revealed that the administration of GSPE is time-dependent. It presented

better antioxidant effect and insulin sensitive in the liver when it was administrated at ZT3 **(Manuscript 2)**.

# Hipòtesis i objectius

La MetS (síndrome metabòlica) és una de les patologies amb més prevalença del món. La International Diabetes Federation (IDF) estima que aproximadament el 25% de la població mundial presenta MetS. Aquesta patologia està formada per un conjunt de diferents anomalies metabòliques, com per exemple l'obesitat abdominal la hipertensió o la resistència a la insulina. De fet, l'obesitat és considerada la responsable principal de l'increment de la prevalença de la MetS. A més, la MetS no afecta de la mateixa manera ambdós sexes.

Addicionalment a tots els desordres metabòlics associats a l'obesitat, els ritmes circadians també es troben alterats en subjectes obesos. Els ritmes circadians són fluctuacions de 24h de diferents processos biològics en absència de senyals externes. Els ritmes del rellotge central es sincronitzen per la llum, mentre els ritmes del teixits perifèrics ho fan principalment per la dieta. Ja que les dietes obesogèniques afecten de manera diferent segons el sexe, és d'esperar que les disrupcions del ritmes circadians siguin dependents del gènere.

El polifenols s'han fet servir durant les últimes dècades com a tractament per pal·liar les patologies associades a l'obesitat. En aquesta tesi s'ha fet servir particularment un extracte de proantocianidines de pinyol de raïm (GSPE), el qual posseeix un gran nombre d'efectes beneficiosos, com per exemple: propietats antioxidant, disminució de la dislipèmia, reducció de la inflamació crònica, disminució de la hipertensió i millora de la sensitivitat a la insulina. Addicionalment, les proantocianidines són potencials moduladors dels ritmes circadians, i per tant, poden millorar la disrupció dels ritmes circadians donats a l'obesitat.

El fetge és un òrgan metabòlic essencial i molts del seus processos metabòlics són alterats a l'obesitat, desenvolupant malalties de fetge gras no associat a l'alcoholèmia. Molts estudis han demostrat que la metabolòmica és una eina

adequada per la determinació de l'esta metabòlic de biofluids, teixits i/o òrgans. En aquest sentit, la ressonància magnètica nucel·lar (RMN) és un tècnica robusta adequada per la anàlisis del metabolisme hepàtic.

Per tant, considerant tota aquesta informació, la hipòtesis plantejada és que **els efectes beneficiosos del GSPE contra les disrupcions metabòliques del fetge, presents a la MetS, són dependents del temps, particularment quan la MetS és induïda per una dieta obesogénica.** En altres paraules, el temps d'administració del GSPE pot canviar els seus efectes contra la MetS induïda per una dieta obesogénica.

Per verificar la confiança d'aquesta tesi, l'objectiu d'aquesta va ser **determinar si el GSPE té diferents efectes sobre el metabolisme hepàtic depenent del temps d'administració, quan hi ha obesitat i MetS.**

Diferents objectius específics van ser proposats per obtenir l'objectiu principal:

1. **Valorar si la disrupció dels ritmes circadians del metabolisme hepàtic provocada per l'obesitat és depenent del sexe.**

   Per aconseguir aquest objectiu, es van alimentar rates Fischer 344 mascles i femelles amb una dieta estàndard o de cafeteria, i posteriorment sacrificar a dos temps diferents, ZT3 i ZT15 (3h després de que les llums s'encenen o s'apaguen). El resultats mostren que la dieta obesogénica altera els ritmes circadians d'ambdós gèneres. No obstant, les rates femelles van ser més resistents als efectes de la dieta de cafeteria, ja que van millorar els ritmes circadians de molts dels metabòlits relacionats amb la síntesi i l'empaquetament de les VLDL (**Article 1**).
2. **Determinar si l'efecte beneficiós d'un extracte de proantocianidines del pinyol de raïm (GSPE) depèn del seu temps d'administració.**

Per aconseguir aquest objectiu, rates Wistar mascle alimentades amb una dieta de cafeteria van ser suplementades amb GSPE a dos temps diferents, ZT0 i ZT12 (quan

les llums s'encenen i quan s'apaguen). Per evitar els efectes beneficiosos atribuïts al gènere femení contra l'obesitat, només es van fer servir mascles. Els nostres resultats van revelar que l'administració del GSPE és depenent del temps. Aquests presenta un possible millor efecte antioxidant i una millora en la sensibilitat a la insulina al fetge quan és administrat pel matí, ZT0 (**Article 2**).

# RESULTS

# CHAPTER 1

## To assess the impact of diet-induced obesity on the circadian rhythms of the hepatic metabolome depending on the gender.

# Manuscript 1

## The disruption of liver metabolic circadian rhythms by an obesogenic diet is sex-dependent in Fischer 344 rats.

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## Abstract

Circadian rhythms are ~24 h fluctuations of different biological processes that are regulated by the circadian clock system which is localized in the superchiasmatic nucleus of the hypothalamus. Circadian rhythms exert a major influence on most cell processes, such as hepatocyte metabolism. In the liver, glucose and lipid metabolism is regulated by insulin and glucagon, whose secretion present rhythmicity. Moreover, cholesterol metabolism, and thus, bile acid synthesis is under circadian rhythms. Obesity diets are capable of disrupting the liver metabolome circadian rhythms, and circadian misalignment is a risk factor for metabolic diseases. However, obesogenic diets do not affect both genders in the same manner. We hypothesized that the disruption of circadian rhythms by obesity in the hepatic metabolism is gender dependent. Male and female Fischer 344 rats were fed either a standard diet or an obesogenic diet and sacrificed at two different moments, either when the light was turned on or turned off. Female rats fed an obesogenic diet showed changes in a circadian manner in most of the metabolites related to VLDL packaging in the liver, such as choline, phosphatidylcholine, betaine, phosphatidylethanolamine, diglycerides and triglycerides, while in male rats, these metabolites did not present oscillations. In addition, choline and triglycerides were found to be lower in females in the liver. These findings suggest that females are protected against the obesogenic diet-induced increase of lipids through the improvement of the circadian rhythms of metabolites related to VLDL packaging.

**Keywords**

Circadian rhythms; obesity; gender; liver; metabolomics

# 1. Introduction

Almost all mammalian cell types express clock genes and are synchronized by the circadian clock system, which is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and ensures that internal physiology is synchronized with the external environment [1]. A ~24 h rhythm is produced by a core transcription and translation feedback loop comprising the clock genes brain and muscle ARNT-like (BMAL1), clock circadian regulator (CLOCK), period circadian regulator 1 (PER1) and PER2, and cryptochrome circadian regulator 1 (CRY1) and CRY2. BMAL1 and CLOCK proteins form a heterodimer that activates the transcription of PER and CRY genes and other clock-controlled genes by binding to E-box response elements within their promoters. CRY and PER proteins oligomerize after reaching an appropriate concentration and translocate into the nucleus to inhibit CLOCK-BMAL1-mediated transcription until they are posteriorly phosphorylated by Casein Kinase I (CKI) ε or δ, ubiquitinated and subsequently degraded by the proteasome system [2,3].

Circadian rhythm exerts a major influence on liver metabolism [4]. Glucose and lipid metabolism is subjected to timed circadian control [5–7]. Glucagon and insulin, synthesized in and released from pancreatic $\alpha$ and $\beta$ cells, respectively, regulate these pathways, and daily plasma rhythms in these hormones have been identified in rodents and humans [8]. Cholesterol is also synthesized in a circadian manner in the liver through the circadian expression of β-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA) [9]. Bile acids are synthesized from cholesterol exclusively in the liver by the rate-limiting enzyme cholesterol 7$\alpha$-hydroxylase (CYP7A1), and it exhibits a well-documented rhythm of mRNA expression in rodents and enzyme activity in human serum [10,11]. Moreover, enzymes that regulate fatty acid synthesis, such as elongation of very long chain fatty acids protein 3 (ELOVL3), ELOVL6, and fatty acid synthase (FAS), have rhythmic expression patterns [12]. Furthermore, core clock and clock-controlled genes have additional roles in glucose

metabolism in the liver. Zhang at all. [13] showed that the overexpression of *Cry1* decreases blood glucose levels and increases insulin sensitivity in diabetic mice. Thus, in a healthy state, the concentration of metabolites oscillates depending on the time, and liver metabolomics is a useful tool to quantify these 24-hour oscillations and the internal time [14].

Obesogenic diets are capable of disturbing the master circadian clock, and circadian misalignment has been identified as a risk factor for metabolic disease [15,16]. High-fat diets disrupt clock gene expression within the hypothalamus, the liver, and adipose tissue, as well as the rhythmicity of hormones and nuclear hormone receptors involved in fuel utilization, such leptin and thyroid stimulating hormone (TSH), and testosterone in mice, rats and humans [17–19].

Notably, obesogenic diets do not act in the same manner in both genders [20]. Van Nas et al. [21] observed a strong correlation between body fat and lipids with a sex-specific group of genes in adipose tissue and liver. In addition, a study performed by Carmona-Alcocer et al. [22] demonstrated that plasma parameters related to metabolic syndrome, such as leptin and insulin, are modulated in a sex-dependent manner by obesogenic diets. Circadian rhythms could participate in this differential behaviour between genders. Supporting this idea, the interaction between melatonin, which regulates the circadian rhythms [23], and sex hormones might influence sleep and body temperature rhythms across the menstrual cycle. [24]. Regarding carbohydrate metabolism, kruppel-like factor 10 (KLF10) is a transcription factor regulated by CLOCK and BMAL which regulates the expression of genes involved in glycolysis and gluconeogenesis [25]. Interestingly, the loss of KLF10 affects glucose metabolism in a sex-specific manner, inducing postprandial and fasting hyperglycaemia in male mice, whereas female mice remained normoglycemic [26].

In this study, we tested whether the homeostatic equilibrium of the hepatic metabolism, which is managed by the circadian clock system, is disrupted differently by an obesogenic diet depending on the gender in Fischer 344 rats. The hepatic metabolites were quantified at Zeitghbert time (ZT) 0, when the light was turned on, and at ZT12, when the light was turned off. This strategy allowed us to determine metabolite oscillations at two important points of the 24 h cycle, when the day and the night begin, thus when rats go to sleep or become active, respectively. Our results revealed a preservation of the homeostatic equilibrium on the circadian oscillations of the hepatic metabolism only in female rats fed an obesogenic diet, particularly in the variations of those metabolites related to the VLDL packaging, which was accompanied by a healthier liver and plasma metabolome. In contrast, the hepatic metabolism of male rats fed an obesogenic diet lost the capacity to oscillate, which was associated with a worse metabolic profile in the liver of male rats.

## 2. Materials and Methods

### 2.1. Animal Experimental Procedure

The investigation was carried out in accordance with the ethical standards and the Declaration of Helsinki and was approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira I Virgili (reference number 9495 by Generalitat de Catalunya).

Eight-week-old female and male Fisher 344 rats were purchased from Charles River Laboratories (Barcelona, Spain). Female and male rats were housed separated in animal quarters at 22˚C with a light/dark period of 12 h (light from 08:00 to 20:00 hours). The animals were randomly divided into eight groups (n=8) depending on the diet, gender and the sacrifice hour: 11:00 (ZT3) or 23:00 (ZT15). The control groups (STD) were fed a standard chow diet (STD Panlab A04, Panlab, Barcelona, Spain) and

water ad libitum. A cafeteria diet consisting of biscuits with pate, biscuits with cheese, bacon, carrots and sweetened milk (20% sucrose w/v) in addition to the standard diet was used as an obesogenic diet in the obese groups (CAF). After nine weeks, the rats were fasted for 3 h after ZT0 or ZT12 and then sacrificed by decapitation. Blood from the saphenous vein was collected, and serum was obtained by centrifugation (1500x g, 20 min, 4˚C). The liver was harvested and immediately frozen in liquid nitrogen. Both serum and liver were stored at -80 ˚C until further use.

### 2.2. Biometric parameters and serum analysis

Lean and fat measurements (in grams and percentage of body weight) were performed 1 week before the sacrifice using an EchoMRI-700TM device (Echo Medical Systems, L.L.C., Houston, TX, United States). Body weigh was monitored weekly until the end of the experiment. Body weight gain was calculated by subtracting the initial body weight from the final one. Food intake was determined weekly weighting the food used as obesogenic diet, and the caloric intake was calculated according to the caloric content of each food provide by the manufacturer. These variables were evaluated as physiological indicator of the obesity grade in the experimental animals (Table 1).

Triglycerides (QCA, Barcelona, Spain) in serum were analysed by an enzymatic colorimetric assay kit following the instruction provided by the manufacturer.

### 2.3. Liver Extraction Procedure for 1H NMR-Based Metabolomics Assays

Liver extraction was performed following the procedure described by Vinaixa et al. with slight modifications [27]. A portion of hepatic tissue (50 mg) was removed, flash-frozen, and manually homogenized using a micropestle in 1 mL of H2O/$CH_3CN$ (1/1). The homogenates were centrifuged at 15000× *g* for 30 min at 4 °C. Supernatants (hydrophilic metabolites) and pellets (lipophilic metabolites) were separately lyophilized overnight to remove water for better NMR performance and, once dried,

stored at -80 °C until further analysis. The lipophilic pellet extracts were subsequently extracted with 1 ml of a solution of $CHCl_3/CH_3OH$ (2:1) and then vortexed, homogenized for 20 min, and centrifuged for 15 min at 6000× *g* at room temperature. The new lipophilic supernatant was separated and dried.

**2.4. NMR analyses**

For NMR measurements, the hydrophilic extracts were reconstituted in 600 µl of $D_2O$ phosphate buffer (PBS 0.05 mM, pH 7.4, 99.5% $D_2O$) containing 0.73 mM trisilylpropionic acid (TSP). The dried lipophilic extracts were reconstituted with a solution of $CDCl_3/CD_3OD$ (2:1) containing 1.18 mM tetramethylsilane (TMS) and then vortexed. Both extracts were transferred into 5-mm NMR glass tubes for NMR measurement. The $^1H$ NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz using a 5 mm PBBO broadband gradient probe. For aqueous extracts, one-dimensional $^1H$ pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY) presaturation sequence (RD–90°–t1–90°–tm–90° ACQ) to suppress the residual water peak, and the mixing time was set at 100 ms. Solvent presaturation with an irradiation power of 75 Hz was applied during recycling delay (RD = 5 s) and mixing time. The 90° pulse length was calibrated for each sample and varied from 9.95 to 10.06 µs. The spectral width was 12 kHz (20 ppm), and a total of 256 transients were collected into 64 k data points for each 1H spectrum. In the case of lipophilic extracts, a 90º pulse with a presaturation sequence (*zgpr*) was used to suppress the residual water signal of methanol. An RD of 5.0 s with an acquisition time of 2.94 s was used. The 90° pulse length was calibrated for each sample and varied from 9.92 to 10.04 µs. After 4 dummy scans, a total of 128 scans were collected into 64K data points with a spectral width of 18.6 ppm.

The exponential line broadening applied before Fourier transformation was 0.3 Hz. The frequency domain spectra were phased, baseline-corrected and referenced to TSP or TMS signal ($\delta$= 0 ppm) using TopSpin software (version 2.1, Bruker).

### 2.5. NMR Data Analysis.

The acquired $^{1}$H NMR spectra were compared to references of pure compounds from the metabolic profiling AMIX spectra database (Bruker), HMDB, Chenomx NMR suite 8.4 software (Chenomx Inc., Edmonton, AN, Canada) and databases for metabolite identification. In addition, we assigned metabolites by $^{1}$H–$^{1}$H homonuclear correlation (COSY and TOCSY) and $^{1}$H–$^{13}$C heteronuclear (HSQC) 2D NMR experiments and by correlation with pure compounds run in-house. After pre-processing, specific $^{1}$H NMR regions identified in the spectra were integrated using the AMIX 3.9 software package.

### 2.6. Data Processing and Multivariate Analysis.

Absolute concentrations derived from both lipophilic and hydrophilic extracts were arranged together in one single data matrix. Previously, data were scaled to unit variance to give all the identified metabolites the same weighing into the model. Data analysis and statistical calculation were performed with IBM Corp. (released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). For the Partial Least Square – Discriminant Analysis (PLS-DAs), MetaboAnalyst software 4.0 was used (https://www.metaboanalyst.ca/).

## 3. Results

### 3.1. Biometric parameters validated the obesogenic effect of the diet

The biometric parameters confirmed that the obesogenic diet used in this experiment caused the obese status in the Fischer 344 rats. The initial body weight

were no differences between group being the male rats more heavy than female ones. After 9 weeks of obesogenic diet feeding, the final body weight and body weight gain of the cafeteria groups were significantly higher than the standard ones. Moreover, the caloric intake was also increased in the group fed an obesogenic diet (Supplementary table 1).

### 3.2. Multivariate chemometric analysis of NMR data

The PCA methodology was used to detect putative outliers. The PCA was performed using the whole spectra, removing the TSP or TMS, methanol, chloroform and water regions in both phases, aqueous and lipid. After alignment and normalization of the spectra, a total of 45 metabolites were found. In the aqueous phase, 28 metabolites were identified and integrated, and 17 metabolites in the lipid phase (Supplementary tables 2, 3, 4 and 5).

### 3.3. Multivariant analyses showed a gender-dependent dysregulation of the circadian oscillations of the hepatic metabolism induced by an obesogenic diet.

First, we analysed whether metabolites oscillate between ZT3 and ZT15 in the liver. To this end, a supervised PLS-DA model was applied to determine how the diet influenced the homeostatic equilibrium of the hepatic metabolites depending on gender. The PLS-DAs were performed by comparing both times and diets for females (Figure 1A) and males (Figure 1B) separately.

In females, clear grouping according to the diet was observed using the PLS-DA approach. Moreover, the control female rats showed a separation by ZT. Notably, ZT separation was also very evident in female rats fed an obesogenic diet. In the constructed PLS-DA model, the $R^2$ and $Q^2$ values were 0.990 and 0.942, respectively, employing the 4th principal component. To validate the model, permutation testing was performed allowing 1000 permutations. The significance of the model was $p < 0.001$, verifying the validity of the model. Therefore, although the metabolites that

oscillated in the livers of female rats fed control or obesogenic diet were different, female rats maintained their oscillation when fed an obesogenic diet.

In males, as well as in females, a clear clustering by diet was found. Furthermore, there was also grouping in the control male rats according to the ZT, as in females. However, in contrast to female rats fed an obesogenic diet, male rats fed an obesogenic diet did not present a significant difference between both ZTs, as it did in standard-fed male animals. The $R^2$ and $Q^2$ values were 0.963 and 0.839, respectively, employing the 3rd principal component. The significance for the model was $p < 0.001$, which confirms the model validity. These results indicate that male rats were more susceptible to the disruption of the hepatic metabolites oscillation induced by obesogenic diets than female rats.

Altogether, the separation between both ZTs in the PLS-DA robustly indicates that the hepatic metabolism exhibited rhythmicity in the animals fed a control diet between ZT3 and ZT15, but only female rats were capable of maintaining oscillations under an obesogenic diet.

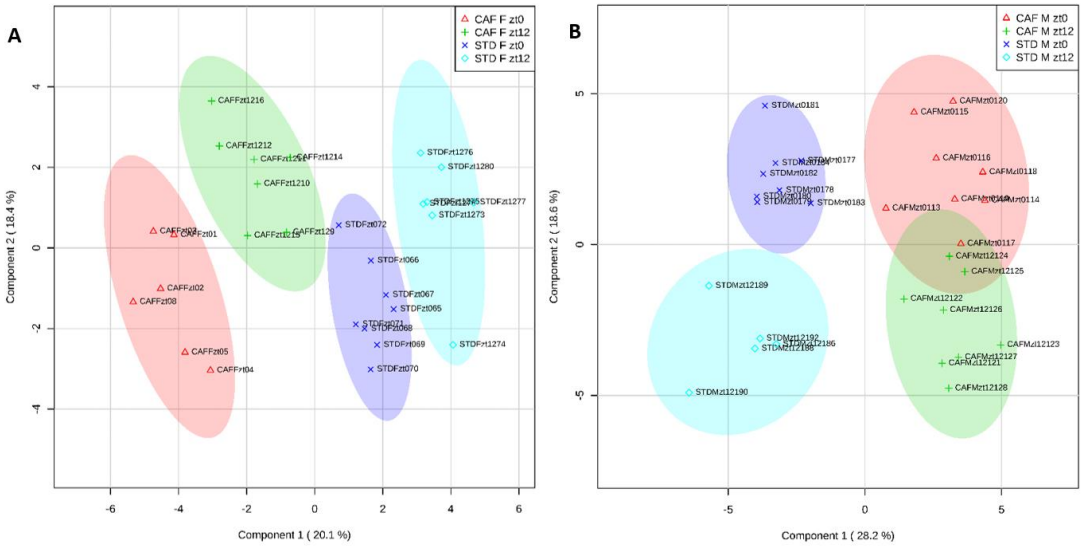


**Figure 1**. The 45 liver metabolites were used to set up a PLS-DA predictive model. It was done in both, females (A) and males (B).

### 3.4. The oscillation of the metabolites across time is gender-dependent.

Once we determined that the hepatic metabolism has circadian oscillations, we further studied which specific metabolites showed oscillations between ZT3 and ZT15 and whether these characteristic metabolite oscillations were specific to gender and/or could be modified by an obesogenic diet (Supplementary tables 2, 3, 4 and 5).

In control rats, the metabolites shared in both genders that were found to be higher at ZT3 were fumarate and ascorbate, while choline and lysine were increased at ZT15. In males, inosine and alanine had higher concentrations at ZT3, and 3-hydroxybutyrate (3-OHB), creatinine and sphingomyelin were increased at ZT15. In contrast, females presented lower levels of isoleucine, omega-3 and docosahexaenoic acid (DHA) at ZT3. Therefore, some metabolites that oscillate were specific for each gender in normal and healthy rats.

Feeding rats an obesogenic diet strongly modified the metabolites that oscillate in control rats. Notably, the metabolite oscillations shared by both genders in control rats were lost. However, 3-OHB, glutamine, free cholesterol, esterified cholesterol, phosphatidylethanolamine and the sum of arachidonic acid and eicosapentaenoic acid (ARA+EPA) showed oscillations between ZT3 and ZT15 in rats fed an obesogenic diet, with high concentrations of these metabolites at ZT15. Additionally, the number of metabolites that oscillated in females fed an obesogenic diet was higher than those that oscillated in control females. The female group exhibited oscillations in isoleucine, omega-3 and DHA, such as in control female rats, but also oscillations in fumarate, choline, betaine, phenylalanine, tyrosine, valine, TAG, DG, total phospholipids, phosphatidylserine, phosphatidylcholine, sphingomyelin and plasmalogen, metabolites that showed higher concentrations at ZT15. In contrast, male rats fed an obesogenic diet showed a reduced number of oscillating

metabolites. In this sense, only total cholesterol and creatine showed oscillations in their concentrations in obese male rats, with low levels at ZT3. These results indicated that an obesogenic diet altered the metabolite oscillation characteristics of healthy rats. Moreover, as for normal rats, metabolic oscillations were specific for each gender. In addition, the male rats fed an obesogenic diet presented low levels of PC and choline at ZT15, which are indicative of steatosis [28].

Since the PLS-DA exhibited a clustering by ZT only in female animals fed an obesogenic diet and the analysis of variance showed that the variation in time of metabolites was gender specific, we considered metabolites that oscillate in female obese rats as relevant metabolites maintaining circadian rhythms in the hepatic metabolism. These metabolites were 3-OHB, fumarate, choline, betaine, phenylalanine, valine, TAG, DG, sphingomyelin, plasmalogen, total phospholipids, PC, phosphatidylserine, omega-3, DHA and ARA+EPA. Notably, most of the metabolites are related to the metabolism of the VLDL packaging, indicating that VLDL synthesis and secretion in the liver is a key process to maintain the metabolite homeostasis under the cafeteria diet (Figure 2).

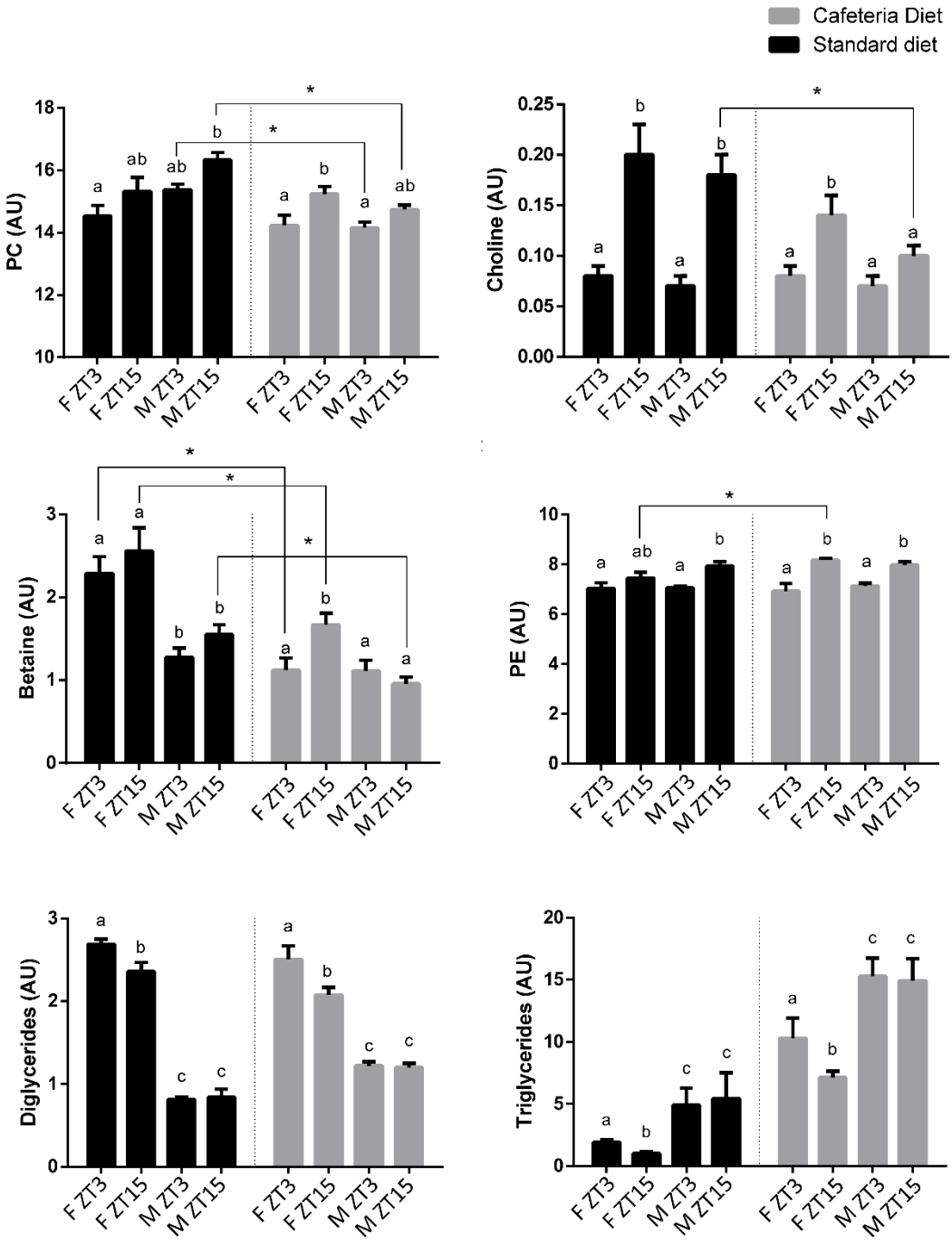


**Figure 2**. The concentration of the metabolites in the liver of female and male Fischer 344 rats fed with cafeteria or standard diet. Data are expressed as the mean ± SEM (n=8). [abc]Mean values with unlike letters were significant different among groups (one-way ANOVA and Duncan's *post hoc* test). PC, phosphatidylcholine; PE, phospatidylethanolamine.

### 3.5. PC oscillations in the livers of female rats fed an obesogenic diet were negatively correlated with plasma TAG oscillations.

VLDL secretion by the liver is directly related to plasma TAG levels. Thus, we further analysed plasma TAG levels. There was no significant difference in plasma TGAs between ZT3 and ZT15 in any gender. The TAG concentrations in obese females were 205.72 ± 39.81 mg/dL and 156.65 ± 19.11 mg/dL at ZT3 and ZT15, respectively. In obese males, the values were 360.79 ± 25.37 mg/dL at ZT3 and 397.5 ± 23.52 mg/dL at ZT15.

PC in the liver is strongly related to VLDL packaging and, therefore, with TAG secretion [29]. To determine whether PC oscillations in the liver are related to VLDL secretion, correlation analysis between PC in liver and plasma TAG was performed in both genders. Figure 3(A) exhibits a negative correlation between the PC levels in the liver and the plasma TAG concentration in CAF-fed female animals. These results suggest a biological role for the liver PC levels in the clearance of plasma TAG. Moreover, in Figure 3(B), CAF-fed male rats did not show any correlation between both parameters.

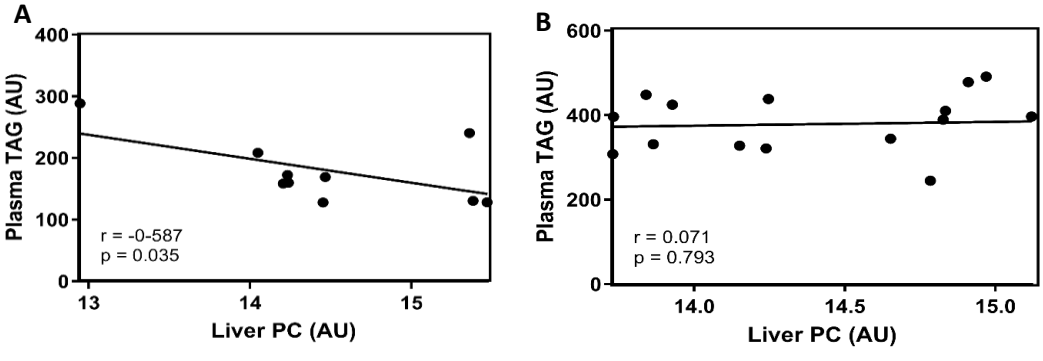


**Figure 3**. Plot of the significant associations between the live phosphatidylcholine and plasmatic triglycerides in female (A) and male(B). Each plot presents each Pearson r correlation value and the corresponding p-value. P-value < 0.05 were considered statistically significant.

## 4. Discussion

In the present study, we showed that the homeostatic oscillations between two ZTs of the 24 h cycle, modulated by circadian rhythms, were affected by an obesogenic diet in a different way in female and male rats. Multivariate analyses were carried out with 45 metabolites in both genders at ZT3 and ZT15. The results showed a clear clustering depending on the diet and the ZT in the female rats on both diets. However, the male rats exhibited a clear clustering depending only on the diet with a slight clustering between ZTs in control males that was lost in males fed an obesogenic diet. These results suggested that males, in contrast with the females, were not able to tolerate an obesogenic diet, thus exhibiting a loss of the homeostatic equilibrium due to a total or partial dysregulation of circadian rhythms on the hepatic metabolism. During the day, CLOCK and BMAL1 heterodimerize to drive rhythmic expression of downstream target genes, which in turn regulate diverse metabolic processes and their homeostasis [30]. Unbalanced diets might cause disruptions on the internal clock system, which constitute risk factors for the metabolic syndrome disorders such as type 2 diabetes mellitus, cardiovascular diseases, thrombosis and inflammation [31]. In addition, the loss of circadian rhythmicity has previously been observed in peripheral tissues in animals subjected to an obesogenic diet [32,33]. Previous studies have demonstrated that the chronic consumption of high-calorie diets disrupt the correct functionality of the mammalian circadian clock [17]. Whereas central clock genes are mainly synchronized by the light, peripheral clock genes are principally regulated by the diet and the feeding time. [34].

As mentioned previously, our results indicated that female rats could maintain the differences between both ZT3 and ZT15 in the hepatic metabolism, presenting better metabolic flexibility under an obesogenic diet. The protective effect of oestrogens

against obesogenic diets might be one reason to explain why the obese female animals conserved the circadian oscillation on the hepatic metabolites [35,36].

Most of the metabolites that showed differences between both genders and, specifically, those that helped to explain the capacity of the female rats fed an obesogenic diet to keep the circadian rhythms of the hepatic metabolism, were both directly and/or indirectly related to VLDL packaging. The most abundant phospholipid in the VLDLs is PC, representing 70% of phospholipids [37]. PC is not only the most abundant phospholipid in VLDL but is also required for its assembly [38]. Hence, reduced levels of hepatic PC are associated with an impairment in VLDL secretion from the liver [39]. In control animals of both genders, there was no difference between the ZTs in PC. Otherwise, the obese female rats exhibited circadian variations in PC with higher concentrations at ZT15, whereas the obese male rats did not show oscillations at all. Furthermore, the PC concentration in CAF-fed male rats was lower than that in STD-fed male rats. Since low levels of PC have been associated with fatty acid liver disease [40], our results suggest that obese male rats have a higher degree of steatosis-lipid accumulation than obese female animals, which can maintain the homeostatic rhythmicity of PC.

PC could be derived either from the phosphorylation of choline, through the cytidine diphosphate-choline pathway (CDP-Choline), or from the phosphatidylethanolamine N-methyltransferase pathway (PEMT), and several metabolites of these pathways showed rhythmicity in this study [41,42] (Figure 4).

For the CDP-choline pathway, choline is the first metabolite of the pathway, which is converted to CDP-choline after a set of reactions, and it is esterified with diglycerides (DAG) by choline phosphotransferase to produce PC [41]. In control rats, the levels of choline in the liver were significantly higher at ZT15 than at ZT3 in both genders. Notably, this choline oscillation was only maintained in the female animals fed an

obesogenic diet, which had a higher concentration at the same time than in the control animals, i.e., ZT15. DAG, which are necessary to esterify CDP-choline, also exhibited circadian rhythms in both diets in the female rats but presented lower levels at ZT15. The fact that females fed an obesogenic diet presented higher levels of choline and PC and lower levels of DAG at ZT15 than at ZT3 suggested that PC production is increased through the CDP-choline pathway at ZT15 in females.

In addition to the CDP-choline pathway, the PEMT pathway generates PC from PE through 3 transmethylation reactions using S-adenosyl methionine (SAM) as a methyl donor and is catalysed by phosphatidylethanolamine N-methyltransferase (PEMT) [43]. Choline is oxidized to betaine, which acts as a methyl donor for the synthesis of methionine and dimethylglycine from homocysteine [44,45]. Finally, the enzyme methionine adenosyltransferase transforms methionine into SAM [46]. In this study, betaine presented oscillations only in female rats fed an obesogenic diet, with the highest concentration at ZT15. Additionally, PE levels in the liver oscillated between ZT3 and ZT15 control males and in males and females fed an obesogenic diet. In all these cases, PE concentration was higher at ZT15 than at ZT3. Notably, female rats fed an obesogenic diet presented oscillations in choline, betaine and PE levels in the liver, with higher levels at the same time, at ZT15. Thus, females fed an obesogenic diet also showed clear rhythmicity in the metabolites related to the PEMT pathway, while the rest of the groups presented very few or no circadian rhythms on the metabolites of this pathway. This fact strongly suggests that females fed an obesogenic diet increased the production of PC at ZT15 through the activation of both choline pathways at this ZT, as revealed by the increase in several metabolites related to both the methyl donor and the PEMT pathways. This finding indicates that PC was not only increased at ZT15 by the CDP-choline pathway but also by the PEMT pathway in female animals fed an obesogenic diet.

In contrast to females, the choline levels at ZT15 in the liver of males fed an obesogenic diet were lower than in control males, indicating a low choline disposal at this ZT. Choline deficiency has been reported to be related to decreased methylation capacity, perturbed phosphatidylcholine synthesis, impaired VLDL secretion, decreased PPARα signalling and altered lipid metabolism, influencing the progression from a healthy liver to fatty liver [28]. Therefore, this choline scarcity in the liver could be relevant for the fragility shown by male rats to resist an obesogenic diet.

The VLDLs are the primary vehicle for the transport of TAG [47]. TAG may be assembled from FAs derived from plasma NEFAs, chylomicron remnants, or from *de novo* lipogenesis [48]. Once the VLDLs are assembled with the TAG, they are secreted to the blood [49,50]. Hence, the ability to keep or generate daily variation in the metabolites related to the packaging of VLDL by the female rats under an obesogenic diet might directly affect the liver TAG concentration and its daily variation [51]. Only the females presented circadian oscillations in the concentration of TAGs in the liver under both diets with lower levels at ZT15 than at ZT3 in both diets. Based on the results of this study, the ability of female rats to maintain the rhythmicity of VLDL packaging through PC synthesis may be essential to maintain the rhythmicity of TAG in the livers of female rats fed an obesogenic diet.

Since rats present nocturnal feeding, it is expected that the major intake of TAG from the diet occurs during the dark period [52]. The appearance of circadian rhythms in the metabolites related to VLDL suggests their synthesis in a circadian manner. Thus, we propose that the female rats start to increase the VLDL level at ZT15 through the metabolites involved in the CDP-choline and PEMT methylation pathways to respond to the excess TAG derived from the obesogenic diet, increasing their secretion packaged in VLDL during the dark period.

In blood, previous studies have shown a positive correlation between VLDL and plasma TAG [53]. Our results have shown a negative correlation between both the liver PC and the plasma TAG in CAF-fed female animals. The lowest PC concentration was found at ZT3; consequently, the highest plasma TAG levels should be at the same time. This finding suggested that PC was used during the dark period to package the TAG derived from the diet in VLDL, which was finally secreted into the blood.

In summary, our results demonstrated that the metabolism related to the synthesis and packaging of VLDL in the liver changes in a circadian manner, and these variations are affected by gender and diet. Notably, gender emerges as an essential factor in the capacity of the liver to manage an obesogenic diet, with females being more resistant to an obesogenic diet. In this sense, female animals respond to the increased lipids derived from an obesogenic diet through the improvement of the circadian rhythm in most of the metabolites related to the synthesis of VLDL. This could allow an increase in TAG exit from the liver during the dark period, reducing the possibility of suffering fatty liver disease. All these findings highlight the need to use both genders in nutritional interventions because the existence of different rhythmicity in the hepatic metabolism in each gender could significantly condition the effect of a specific diet.

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# Supplementary Material

**Supplementary table 1.** Biometric parameters in both male and female Fischer 344 rats fed either standard or obesogenic diet for 9 weeks.

| | Female | | | | Male | | | |
|---|---|---|---|---|---|---|---|---|
| | Standard | | Cafeteria | | Standard | | Cafeteria | |
| | ZT3 | ZT15 | ZT3 | ZT15 | ZT3 | ZT15 | ZT3 | ZT15 |
| **Biometic parameters** | | | | | | | | |
| Inital body weight (g) | 151,35 ± 2,58 | 154,15 ± 2,39 | 153 ± 2,8 | 158,29 ± 2,19 | 228,48 ± 4,53 | 228,5 ± 9,63 | 207,46 ± 11,24 | 226,58 ± 2,45 |
| Final body weight (g) | 201,25 ± ,.72$^{a}$ | 203,75 ± 4,27$^{a}$ | 262,81 ± 5,84$^{b}$ | 260,69 ± 7,57$^{b}$ | 354,81 ± 6,54$^{a}$ | 349,63 ± 15,24$^{a}$ | 418,5 ± 6,78$^{b}$ | 410,75 ± 4,68$^{b}$ |
| Body weight gain (g) | 49,9 ± 1,89$^{a}$ | 49,6 ± 3,35$^{a}$ | 109,81 ± 3,74$^{b}$ | 102,4 ± 6,06$^{b}$ | 126,33 ± 3,17$^{a}$ | 121,13 ± 7,97$^{a}$ | 211,04 ± 12,48$^{b}$ | 184,17 ± 3,96$^{c}$ |
| Caloric intake (KJ) | 158,06 ± 2.52$^{a}$ | 158.06 ± 2.42$^{a}$ | 511,99 ± 39.11$^{b}$ | 449,34 ± 7.39$^{b}$ | 253.75 ± 4.04$^{a}$ | 253.5 ± 7.48$^{a}$ | 664,20 ± 14.86$^{b}$ | 630,89 ± 23,15$^{b}$ |

*Female and male F344 rats were fed either a standard or obesogenic diet during 9 weeks. Data are expressed as the mean ± SEM (n = 8). One-way ANOVA and Duncan's post hoc tests were performed to compare differences between groups. Significant differences are represented by different letters (a, b).*

**Supplementary Table 2.** Concentration of representative aqueous liver metabolites analysed by Nuclear Magnetic Resonance in female and male Fisher 344 rats fed with a chow diet and sacrificed in two different times, ZT3 or ZT15.

| | **Female** | | **Male** | |
|---|---|---|---|---|
| **Aqueous metabolites concentration (AU)** | ZT3 | **ZT15** | **ZT3** | **ZT15** |
| 3-Hydroxybutyrate | 0.16 ± 0.01$^{a}$ | 0.2 ± 0.03$^{a}$ | 0.22 ± 0.01$^{a}$ | 0.32 ± 0.05$^{b}$ |
| Formate | 0.04 ± 0.003 | 0.05 ± 0.003 | 0.05 ± 0.003 | 0.05 ± 0.003 |
| Fumarate | 0.08 ± 0.01$^{a}$ | 0.05 ± 0.01$^{bc}$ | 0.06 ± 0.003$^{b}$ | 0.04 ± 0.004$^{c}$ |
| Glucose 6-Phosphate | 15.68 ± 1.3$^{ab}$ | 14.73 ± 1.3$^{b}$ | 20.22 ± 0.8$^{c}$ | 18.38 ± 1.2$^{ac}$ |
| Lactate | 6.67 ± 0.7 | 6.81 ± 1.1 | 6.09 ± 0.7 | 5.80 ± 0.7 |
| Pyruvate | 0.04 ± 0.02 | 0.05 ± 0.02 | 0.07 ± 0.01 | 0.03 ± 0.01 |
| Succinate | 0.77 ± 0.1 | 0.77 ± 0.1 | 0.88 ± 0.1 | 0.98 ± 0.1 |
| Acetate | 0.23 ± 0.02$^{a}$ | 0.26 ± 0.02$^{a}$ | 0.74 ± 0.2$^{b}$ | 0.87 ± 0.1$^{b}$ |
| Choline | 0.08 ± 0.01$^{a}$ | 0.20 ± 0.03$^{b}$ | 0.07 ± 0.01$^{a}$ | 0.18 ± 0.02$^{b}$ |
| Betaine | 2.29 ± 0.2$^{a}$ | 2.56 ± 0.3$^{a}$ | 1.28 ± 0.1$^{b}$ | 1.55 ± 0.1$^{b}$ |
| Creatine | 0.25 ± 0.02 | 0.22 ± 0.01 | 0.21 ± 0.01 | 0.19 ± 0.01 |
| Creatine Phosphate | 0.05 ± 0.002 | 0.06 ± 0.01 | 0.04 ± 0.001 | 0.04 ± 0.003 |
| Creatinine | 0.02 ± 0.002$^{a}$ | 0.03 ± 0.003$^{a}$ | 0.03 ± 0.003$^{a}$ | 0.04 ± 0.01$^{b}$ |
| NAD+/NADH | 0.17 ± 0.01 | 0.16 ± 0.02 | 0.19 ± 0.03 | 0.22 ± 0.02 |
| Niacinamide | 0.20 ± 0.01 | 0.19 ± 0.01 | 0.21 ± 0.01 | 0.21 ± 0.01 |
| Inosine | 0.69 ± 0.1$^{a}$ | 0.70 ± 0.1$^{a}$ | 1.56 ± 0.1$^{b}$ | 1.32 ± 0.2$^{c}$ |
| Alanine | 2.64 ± 0.1$^{a}$ | 2.24 ± 0.2$^{a}$ | 3.40 ± 0.2$^{b}$ | 2.52 ± 0.2$^{a}$ |
| Glutamate | 0.96 ± 0.1 | 0.99 ± 0.1 | 1.31 ± 0.1 | 1.21 ± 0.1 |
| Glutamine | 3.30 ± 0.2 | 4.11 ± 0.4 | 3.36 ± 0.2 | 3.92 ± 0.3 |
| Glycine | 1.44 ± 0.1$^{a}$ | 1.38 ± 0.1$^{a}$ | 1.04 ± 0.1$^{b}$ | 0.99 ± 0.1$^{b}$ |
| Histidine | 0.04 ± 0.002 | 0.05 ± 0.003 | 0.05 ± 0.004 | 0.04 ± 0.003 |
| Isoleucine | 0.19 ± 0.01$^{a}$ | 0.22 ± 0.01$^{b}$ | 0.19 ± 0.01$^{ab}$ | 0.22 ± 0.01$^{b}$ |
| Leucine | 0.60 ± 0.02 | 0.70 ± 0.1 | 0.59 ± 0.02 | 0.68 ± 0.03 |
| Lysine | 0.44 ± 0.02$^{ab}$ | 0.64 ± 0.1$^{c}$ | 0.38 ± 0.01$^{a}$ | 0.50 ± 0.03$^{b}$ |
| Phenylalanine | 0.38 ± 0.02 | 0.40 ± 0.03 | 0.34 ± 0.02 | 0.38 ± 0.01 |
| Tyrosine | 0.13 ± 0.01 | 0.12 ± 0.01 | 0.15 ± 0.004 | 0.17 ± 0.01 |
| Valine | 0.28 ± 0.01 | 0.32 ± 0.02 | 0.30 ± 0.01 | 0.35 ± 0.02 |
| Ascorbate | 0.56 ± 0.02$^{a}$ | 0.47 ± 0.04$^{b}$ | 0.65 ± 0.02$^{c}$ | 0.54 ± 0.03$^{ab}$ |

Standard female and male Fischer 344 rats were sacrificed at ZT3 and ZT15 fed with a chow diet for 9 weeks. Data are expressed as the mean ± SEM (n=8). All the metabolites were obtained by performing a nuclear magnetic resonance (NMR) analysis. One-way ANOVA and Duncan's post hoc test were performed to compare the values between groups and significant differences were represented with letters (a, b, c).

**Supplementary Table 3.** Concentration of representative lipid liver metabolites analysed by Nuclear Magnetic Resonance in female and male Fisher 344 rats fed with a chow diet and sacrificed in two different times, ZT3 or ZT15.

| | Female | | Male | |
|---|---|---|---|---|
| **Metabolites concentration (AU)** | **ZT3** | **ZT15** | **ZT3** | **ZT15** |
| Total cholesterol | 3.73 ± 0.1 | 3.78 ± 0.1 | 3.55 ± 0.1 | 3.86 ± 0.2 |
| Free cholesterol | 3.58 ± 0.1 | 3.65 ± 0.1 | 3.40 ± 0.1 | 3.44 ± 0.2 |
| E. cholesterol | 1.49 ± 0.1 | 1.55 ± 0.1 | 0.26 ± 0.01 | 0.30 ± 0.1 |
| Triglycerides | 1.91 ± 0.2$^{a}$ | 1.03 ± 0.1$^{b}$ | 4.92 ± 1.4$^{c}$ | 5.456 ± 2.1$^{c}$ |
| Diglycerides | 2.68 ± 0.1$^{a}$ | 2.36 ± 0.1$^{b}$ | 0.81 ± 0.03$^{c}$ | 0.80 ± 0.1$^{c}$ |
| Monoglycerides | 1.12 ± 0.2 | 0.85 ± 0.1 | 0.65 ± 0.02 | 0.71 ± 0.04 |
| Total phospholipids | 15.15 ± 0.4$^{a}$ | 16.15 ± 0.6$^{ab}$ | 17.84 ±0.2$^{bc}$ | 18.32 ± 0.9$^{c}$ |
| PEA | 7.05 ± 0.2 | 7.46 ± 0.2 | 7.06 ± 0.1 | 7.94 ± 0.2 |
| Phosphatidylserine | 0.23 ± 0.01$^{a}$ | 0.23 ± 0.01$^{a}$ | 0.17 ± 0.01$^{b}$ | 0.17 ± 0.01$^{b}$ |
| Phosphatidylcholine | 14.54 ± 0.3$^{a}$ | 15.32 ± 0.5$^{ab}$ | 15.4 ± $^{ab}$ | 16.3 ± 0.24$^{b}$ |
| Sphingomyelin | 0.95 ± 0.02$^{a}$ | 0.98 ± 0.04$^{a}$ | 1.1 ± 0.03$^{a}$ | 1.18 ± 0.1$^{b}$ |
| Plasmalogen | 0.50 ± 0.03$^{a}$ | 0.53 ± 0.03$^{a}$ | 0.41 ± 0.02$^{b}$ | 0.59 ± 0.1$^{a}$ |
| Omega-3 | 5.00 ± 0.1$^{a}$ | 5.48 ± 0.2$^{b}$ | 4.40 ± 0.1$^{c}$ | 4.66 ± 0.3$^{bc}$ |
| ARA+EPA | 8.26 ± 0.4$^{ab}$ | 9.05 ± 0.4$^{b}$ | 6.88 ± 0.2$^{c}$ | 7.56 ± 0.6$^{ac}$ |
| Oleic acid | 9.94 ± 0.7 | 8.08 ± 0.3 | 14.47 ± 2.2 | 14.61 ± 3.5 |
| DHA | 3.10 ± 0.1$^{a}$ | 3.49 ± 0.2$^{b}$ | 2.81 ± 0.1$^{a}$ | 3.10 ± 0.3$^{a}$ |
| Linoleic acid | 2.80 ± 0.2$^{a}$ | 2.41 ± 0.2$^{a}$ | 7.16 ± 0.8$^{b}$ | 6.65 ± 0.5$^{b}$ |

Standard female and male Fischer 344 rats were sacrificed at ZT3 and ZT15 fed with a chow diet for 9 weeks. Data are expressed as the mean ± SEM (n=8). All the metabolites were obtained by performing a nuclear magnetic resonance (NMR) analysis. One-way ANOVA and Duncan's post hoc test were performed to compare the values between groups and significant differences were represented with letters (a, b, c). PE, phosphatidylethanolamine; E.cholesterol, esterified cholesterol.

**Supplementary Table 4.** Concentration of representative aqueous liver metabolites analysed by Nuclear Magnetic Resonance in female and male Fisher 344 rats fed with a cafeteria diet and sacrificed in two different times, ZT3 or ZT15.

| | Female | | Male | |
|---|---|---|---|---|
| **Metabolites concentration (AU)** | **ZT3** | **ZT15** | **ZT3** | **ZT15** |
| 3-Hydroxybutyrate | 0.22 ± 0.04$^{a}$ | 0.41 ± 0.1$^{b}$ | 0.18 ± 0.01$^{a}$ | 0.34 ± 0.04$^{b}$ |
| Formate | 0.05 ± 0.004 | 0.05 ± 0.003 | 0.05 ± 0.004 | 0.05 ± 0.004 |
| Fumarate | 0.11 ± 0.01$^{a}$ | 0.08 ± 0.01$^{b}$ | 0.05 ± 0.01$^{b}$ | 0.05 ± 0.001$^{b}$ |
| Glucose 6-Phosphate | 16.07 ± 1.01$^{a}$ | 17.43 ± 1.5$^{a}$ | 21.50 ± 1.5$^{b}$ | 20.85 ± 0.1$^{b}$ |
| Lactate | 5.67 ± 0.7 | 6.44 ± 0.6 | 5.85 ± 0.3 | 5.75 ± 0.5 |
| Pyruvate | 0.11 ± 0.02 | 0.07 ± 0.001 | 0.06 ± 0.01 | 0.05 ± 0.01 |
| Succinate | 0.71 ± 0.1$^{a}$ | 0.78 ± 0.1$^{ab}$ | 1.15 ± 0.1$^{c}$ | 1.12 ± 0.1$^{bc}$ |
| Acetate | 0.22 ± 0.02 | 0.46 ± 0.1 | 0.34 ± 0.1 | 0.30 ± 0.001 |
| Choline | 0.08 ± 0.01$^{a}$ | 0.14 ± 0.02$^{b}$ | 0.07 ± 0.01$^{a}$ | 0.10 ± 0.01$^{ab}$ |
| Betaine | 1.12 ± 0.2$^{a}$ | 1.67 ± 0.1$^{b}$ | 1.11 ± 0.1$^{a}$ | 0.96 ± 0.1$^{a}$ |
| Creatine | 0.27 ± 0.02$^{a}$ | 0.22 ± 0.02$^{a}$ | 0.22 ± 0.02$^{a}$ | 0.16 ± 0.01$^{b}$ |
| Creatine Phosphate | 0.05 ± 0.01$^{ab}$ | 0.06 ± 0.01$^{b}$ | 0.04 ± 0.002$^{ac}$ | 0.03 ± 0.001$^{c}$ |
| Creatinine | 0.02 ± 0.002$^{a}$ | 0.03 ±0.002$^{b}$ | 0.04 ± 0.002$^{c}$ | 0.03 ± 0.001$^{b}$ |
| NAD+/NADH | 0.12 ± 0.02$^{a}$ | 0.16 ± 0.02$^{a}$ | 0.24 ± 0.03$^{b}$ | 0.25 ± 0.02$^{b}$ |
| Niacinamide | 0.20 ± 0.02$^{ab}$ | 0.21 ± 0.01$^{a}$ | 0.19 ± 0.01$^{b}$ | 0.2 ± 0.01$^{b}$ |
| Inosine | 1.04 ± 0.1$^{a}$ | 0.98 ± 0.1$^{a}$ | 1.57 ± 0.1$^{b}$ | 1.75 ± 0.1$^{b}$ |
| Alanine | 3.01 ± 0.2$^{ab}$ | 2.53 ± 0.2$^{a}$ | 3.41 ± 0.1$^{b}$ | 2.98 ± 0.2$^{ab}$ |
| Glutamate | 1.07 ± 0.1$^{a}$ | 1.17 ± 0.1$^{a}$ | 1.84 ± 0.1$^{b}$ | 1.72 ± 0.1$^{b}$ |
| Glutamine | 3.16 ± 0.3$^{a}$ | 3.89 ± 0.2$^{b}$ | 3.21 ± 0.2$^{a}$ | 4.10 ± 0.2$^{b}$ |
| Glycine | 1.25 ± 0.1$^{a}$ | 1.34 ± 0.1$^{ab}$ | 0.99 ± 0.1$^{b}$ | 1.05 ± 0.1$^{b}$ |
| Histidine | 0.05 ± 0.004 | 0.05 ± 0.01 | 0.04 ± 0.002 | 0.05 ± 0.002 |
| Isoleucine | 017 ± 0.01$^{a}$ | 0.21 ± 0.02$^{b}$ | 0.17 ± 0.01$^{a}$ | 0.18 ± 0.004$^{a}$ |
| Leucine | 0.63 ± 0.1$^{ab}$ | 0.72 ± 0.04$^{a}$ | 0.55 ± 0.03$^{b}$ | 0.65 ± 0.03$^{ab}$ |
| Lysine | 0.44 ± 00.03$^{ab}$ | 0.51 ± 0.04$^{a}$ | 0.33 ± 0.01$^{c}$ | 0.37 ± 0.01$^{bc}$ |
| Pehnylalanine | 0.33 ± 0.02$^{a}$ | 0.41 ± 0.03$^{b}$ | 0.31 ± 0.02$^{a}$ | 0.33 ± 0.01$^{a}$ |
| Tyrosine | 0.10 ± 0.01$^{a}$ | 0.12 ± 0.01$^{ab}$ | 0.13 ± 0.01$^{b}$ | 0.13 ± 0.003$^{b}$ |
| Valine | 0.23 ± 0.01$^{a}$ | 0.29 ± 0.02$^{b}$ | 0.24 ± 0.01$^{a}$ | 0.27 ± 0.01$^{ab}$ |
| Ascorbate | 0.57 ± 0.1$^{ab}$ | 0.50 ± 0.02$^{a}$ | 0.71 ± 0.1$^{c}$ | 0.62 ± 0.04$^{cb}$ |

Cafeteria female and male Fischer 344 rats were sacrificed at ZT3 and ZT15 fed with a chow diet for 9 weeks. Data are expressed as the mean ± SEM (n=8). All the metabolites were obtained by performing a nuclear magnetic resonance (NMR) analysis. One-way ANOVA and Duncan's post hoc test were performed to compare the values between groups and significant differences were represented with letters (a, b, c).

**Supplementary Table 5.** Concentration of representative lipid liver metabolites analysed by Nuclear Magnetic Resonance in female and male Fisher 344 rats fed with a cafeteria diet and sacrificed in two different times, ZT3 or ZT15.

| | Female | | Male | |
|---|---|---|---|---|
| **Metabolites concentration (AU)** | **ZT3** | **ZT15** | **ZT3** | **ZT15** |
| Total cholesterol | 4.33 ± 0.1$^{a}$ | 4.66 ± 0.1$^{ab}$ | 5.04 ± 0.3$^{b}$ | 6.23 ± 0.3$^{c}$ |
| Free cholesterol | 3.66 ± 0.09 | 4.10 ± 0.07 | 3.51 ± 0.08 | 3.86 ± 0.06 |
| Esterified cholesterol | 2.88 ± 0.17 | 4.10 ± 0.07 | 1.52 ± 0.19 | 2.54 ± 0.30 |
| Tryglicerides | 10.30 ± 1.6$^{a}$ | 7.14 ± 0.5$^{b}$ | 15.29 ± 1.5$^{c}$ | 14.92 ± 1.8$^{c}$ |
| Diglycerides | 2.51 ± 0.1$^{a}$ | 2.08 ± 0.1$^{b}$ | 1.12 ± 0.1$^{c}$ | 1.20 ± 0.1$^{c}$ |
| Monoglycerides | 0.87 ± 0.1$^{a}$ | 0.90 ± 0.03$^{a}$ | 0.69 ± 0.03$^{b}$ | 0.71 ± 0.03$^{b}$ |
| Total phospholipids | 14.99 ± 0.3$^{a}$ | 16.71 ± 0.3$^{b}$ | 16.75 ± 0.2$^{b}$ | 17.57 ± 0.2$^{b}$ |
| Phosphatidylethanolamine | 6.93 ± 0.3$^{a}$ | 8.18 ± 0.1$^{b}$ | 7.14 ± 0.1$^{a}$ | 7.98 ± 0.1$^{b}$ |
| Phosphatidylserine | 0.36 ± 0.01$^{a}$ | 0.47 ± 0.03$^{b}$ | 0.29 ± 0.04$^{a}$ | 0.38 ± 0.03$^{ab}$ |
| Phosphatidylcholine | 14.23 ± 0.3$^{a}$ | 15.24 ± 0.2$^{b}$ | 14.15 ± 0.2$^{a}$ | 14.74 ± 0.1$^{ab}$ |
| Sphingomyelin | 0.89 ± 0.03$^{a}$ | 1.11 ± 0.02$^{b}$ | 1.09 ± 0.03$^{b}$ | 1.19 ± 0.04$^{b}$ |
| Plasmalogen | 0.44 ± 0.03$^{a}$ | 0.54 ± 0.03$^{b}$ | 0.42 ± 0.01$^{a}$ | 0.40 ± 0.02$^{a}$ |
| Omega-3 | 4.26 ± 0.1$^{a}$ | 4.87 ± 0.1$^{b}$ | 3.75 ± 0.1$^{a}$ | 4.14 ± 0.2$^{a}$ |
| ARA+EPA | 9.52 ± 0.4$^{ab}$ | 11.44 ± 0.4$^{c}$ | 8.16 ± 0.3$^{a}$ | 9.54 ± 0.1$^{b}$ |
| Oleic acid | 27.05 ± 2.9 | 23.33 ± 2.2 | 35.72 ± 2.5 | 35.56 ± 3.3 |
| DHA | 2.92 ± 0.1$^{a}$ | 3.51 ± 0.1$^{b}$ | 2.22 ± 0.1$^{c}$ | 2.49 ± 0.1$^{ac}$ |
| Linoleic acid | 3.79 ± 0.3$^{a}$ | 2.87 ± 0.1$^{a}$ | 13.17 ± 1.2$^{b}$ | 13.63 ± 1.3$^{b}$ |

Cafeteria female and male Fischer 344 rats were sacrificed at ZT3 and ZT15 fed with a chow diet for 9 weeks. Data are expressed as the mean ± SEM (n=8). All the metabolites were obtained by performing a nuclear magnetic resonance (NMR) analysis. One-way ANOVA and Duncan's post hoc test were performed to compare the values between groups and significant differences were represented with letters (a, b, c).

# CHAPTER 2

**To determine if the beneficial effects of a grape seed proanthocyanidins extract (GSPE) are time-dependent.**

# Manuscript 2

## The beneficial effects of a grape seed proanthocyanidin extract on the liver metabolism is time-dependent in obese Wistar rats.

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**In preparation**

## Abstract

Flavanols, the most abundant polyphenols in the human diet, have demonstrated to improve the disturbances associated with diet-induced obesity. A grape seed proanthocyanidin extract (GSPE) has been used against many metabolic obesity-related dysfunctions, such as insulin resistance or steatosis. In addition, GSPE has been shown to be able to modulate the hepatic clock system, which regulates the lipid and glucose metabolism. Hence, GPSE might adjust the hepatic lipid and glucose metabolism disruptions through the GSPE clock system modulation capability, altered in the obesity status. Taken together, these evidences propose that the GSPE beneficial effects on the hepatic metabolism might depend on its administration time. Male Wistar rats were fed a cafeteria diet and treated with GSPE at two different times, when the lights turned on (ZT0), or turned off (ZT12). Independently from the administration time, the treated groups presented reduced hepatic lipid concentrations. In contrast, only GSPE administrated at ZT0 might increase its antioxidant effects through an increment on the glutathione levels, and only those rats treated at ZT0 exhibited lower concentration of metabolites related to gluconeogenesis. Furthermore, this group of animals presented lower plasma insulin levels, HOMA-IR and a better response to a glucose oral charge than those animals treated at ZT12. Taken together, these results suggested an improvement on hepatic insulin sensitivity. The different effects between both administration times might be related to the increased hepatic niacinamide levels found at ZT0, which is associated with the glucose metabolism and the circadian rhythms.

**Keywords**

Proanthocyanidins; obesity; time-dependent; liver; metabolomics

# 1. Introduction

World Health Organization (OMS) defined obesity as the abnormal or excessive fat accumulation that presents a risk to health [1]. Overweight and obesity are the most important factor for a number of diseases such as type 2 diabetes mellitus (T2DM), cardiovascular diseases or cancer [2–4]. Obesity is commonly caused by an energy unbalance in which the consumption is in excess of expenditure [5]. Obesogenic diets are capable of disrupting a wide range of metabolisms, playing the liver a central role in the nutrient metabolisms [6]. Non-alcoholic fatty liver disease (NAFLD) has been linked with obesity [7]. NAFLD is characterized by hepatic lipid accumulations and an impaired carbohydrate metabolism [8]. Additionally, insulin resistance is one of the hallmarks of NAFLD [9]. Furthermore, obesogenic diets are able to disrupt circadian rhythms, which have been identified as a risk factor for metabolic diseases [10,11].

Circadian rhythms are synchronized by the central circadian clock system, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, which is mainly regulated by the light [12]. The central clock genes are generally composed by the brain and muscle ARNT-like (BMAL1), circadian locomotor output cycles kaput (CLOCK), period circadian regulator 1 (PER1) and PER2, and cryptochrome circadian regulator 1 (CRY1) and CRY2 [13]. Briefly, BMAL1 and CLOCK heterodimerize to activate the transcription of CRY and PER which are accumulated in the cytosol. These then are translocated into the nucleus inhibiting the dimer BMAL1:CLOCK transcription function. PER and CRY complexes are then degraded by casein kinase 1 (CK) $\varepsilon$, CK1$\delta$, and F-box/LRR-repeat protein 3 [13]. The transcriptional rhythmicity of these genes is generated approximately along 24 hours [13]. These circadian rhythms exert a major influence on the liver metabolism [14]. The hepatic carbohydrate metabolism is under circadian control [14]. Part of its regulation is performed through the circadian synthesis and release of glucagon and insulin by the pancreatic $\alpha$ and $\beta$ cells [15]. In the same way as the carbohydrate metabolism, some enzymes involved

in the synthesis of FA, such as the elongation of very long chain fatty acids protein 3 (ELOVL3), ELOVL6, and the fatty acid synthase (FAS), are expressed in a circadian manner [16]. Moreover, the synthesis of cholesterol and bile acids present circadian rhythms through the circadian expressions of β-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA) and cholesterol 7$\alpha$-hydroxylase (CYP7A1) respectively [17–19].

Proanthocyanidins (PACs) are a group of polyphenols widely distributed in fruits and vegetables [20]. In this sense, many studies have been developed using a grape seed proanthocyanidin extract (GSPE) due to its capacity to enhance the different complications related to obesity [21,22]. Particularly, GSPE presents a large amount of benefits against NAFLD. In this sense, GSPE ameliorates dyslipidaemia and inhibits genes related to the lipogenesis and VLDL packaging in rats fed an obesogenic diet [23]. Moreover, GSPE consumption restores the hepatic glucose homeostasis after its disruption caused by obesogenic diets [24]. Additionally, GSPE is capable to activate the glutathione metabolism, thus increasing the antioxidant capacity of the hepatocyte [25]. Although the light is considered the main signal to synchronize the external environment with the central clock system, dietary compounds, such as proanthocyanidins, might also act as an external cue to synchronize biological rhythms [26]. In this sense, GSPE alters the oscillation of the expressions of BMAL1 and Nampt, as well as, some metabolites present in the plasma of rats fed a standard chow diet [26].

Therefore, the aim of the current study was to determine whether beneficial GSPE effects on the liver were time dependent. Wistar rats fed obesogenic diet were treated with GPSE at two different Zeitgeber (ZT), when the light turned on and off. This strategy allowed us to determine the GSPE effects on the hepatic metabolism depending on the administration time.

# 2. Materials and Methods

## 2.1. Grape Seed Proanthocyanidin Extract

The grape seed proanthocyanidins extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The composition of the GSPE has been previously characterized by Margalef et al. [27].

## 2.2. Animal Experimental procedure

The investigation was carried out in accordance with the ethical standards and the Declaration of Helsinki and was approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 9495 by Generalitat de Catalunya).

Eight-week-old male Wistar rats were purchased from Charles River Laboratories (Barcelona, Spain). The animals were housed individually in animal quarters at 22˚C with a light/dark period of 12h (light from 09:00 to 21:00 hours). The animals were divided into four groups randomly (n=8). A cafeteria diet consisting of biscuits with pate, biscuits with cheese, bacon, carrots and sweetened milk (20% sucrose w/v) in addition to a standard diet (STD Panlab A04, Panlab, Barcelona, Spain) was used as obesogenic diet (CAF). The CAF control groups were fed an obesogenic diet and water ad libitum, one CAF group received the vehicle at the beginning of the light period (ZT0), when the light turned on (CAF-ZT0), and the other one at the beginning of the dark period (ZT12), when the light turned off (CAF-ZT12). The other two CAF groups were supplemented with GSPE (25 mg/kg BW) at ZT0 and ZT12, GSPE-ZT0 and GSPE-ZT12 respectively. The treatment was administrated after five weeks of cafeteria diet during four weeks. Blood from saphenous vein was collected, and serum was obtained by centrifugation (1500 x g, 20 min, 4˚C). After nine weeks, the animals were fasted for 3h after ZT0 and then sacrificed by decapitation. The liver

was harvested and immediately frozen in liquid nitrogen. Both serum and liver were stored a -80 ˚C until further use.

### 2.3. Quantification of Plasma Parameters

The levels of insulin in plasma were measured by ELISA (ELISA kit EZRMI-13K and ELISA kit EZRL-83K, Millipore Ibérica, Madrid, Spain). Glucose (GLU) in plasma was measured with enzymatic colorimetric kits (QCA, Barcelona, Spain) according to the manufacturer's instructions. Homeostasis model assessment-estimated insulin resistance (HOMA-IR) values were calculated from insulin and glucose levels in plasma.

### 2.4. Oral glucose tolerance test (OGTT)

At 3 weeks after the initiation of the treatment, rats were fasted overnight, and then were given a gavage of 4mg glucose/g body weight. Glucose levels were measured before the glucose load at time 0, and at 15, 30, 60 and 120 min after the glucose administration.

### 2.5. Gene expression analysis

The total RNA was extracted from the livers using TRIzol Reagent (Thermo Fisher Scientific, Barcelona, Spain) according to the manufacturer's protocol. The RNA was quantified on NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Barcelona, Spain). A Labnet MultiGene Gradient PCR Thermal Cycler (Sigma-Aldrich, Madrid, Spain) was used for the reverse transcription. The reaction was performed according to the manufacturer's instruction. The cDNA was subjected to a quantitative reverse transcriptase polymerase chain reaction

amplification using iTaq Universal SYBR Green Supermix (Bio-Rad, Madrid, Spain). The primers used for the different genes are described in the Table 1 and were obtained from Biomers.net (Ulm, Germany). The fold changes in the mRNA levels were calculated as a percentage of the CAF-ZT0 group using the $-2^{\Delta\Delta Ct}$ method [28] with Ppia gene as endogen control.

**Table 1**. Gk, Pck1 and Ppia primer sequences.

| Gene | Forward primer (5' to 3') | Reverse primer (5' to 3') |
|---|---|---|
| GK | CTGTGAAAGCGTGTCCACTC | GCCCTCCTCTGATTCGATGA |
| PCK1 | GCAAACCAGCAAGCACAATG | CTCGAAGTGGAACCAAACCC |
| PPIA | CCAAACACAAATGGTTCCCAGT | ATTCCTGGACCCAAAACGCT |

Gk (*Gk*), Glucokinase; *Pck1*, Phosphoenolpyruvate carboxylase 1; *Pppia*, Peptidylprolyl isomerase A.

### 2.6. Liver Extraction Procedure for 1H NMR Based Metabolomics Assays

Liver extractions were performed following the procedure described by Vinaixa et al. with slight modifications [29]. Briefly, a portion of hepatic tissue (50 mg) was removed, flash-frozen, and manually homogenized using a Micropestle in 1 mL of $H_2O/CH_3CN$ (1/1). The homogenates were centrifuged at 15000 × *g* for 30 min at 4 °C. Supernatants (hydrophilic metabolites) and pellets (lipophilic metabolites) were separately lyophilized overnight to remove water for NMR experiments and, once dried, stored at -80 °C until further analysis. The lipophilic pellet extracts were subsequently extracted with 1 ml of a solution $CHCl_3/CH_3OH$ (2:1) and then vortexed, homogenized for 20 min, centrifuged for 15 min at 6000× *g* at room temperature. The new lipophilic supernatant was separated and dried under $N_2$ stream.

### 2.7. NMR analyses

For NMR measurements, the hydrophilic extracts were reconstituted in 600 µl of D2O phosphate buffer (PBS 0.05 mM, pH 7.4, 99.5 % D2O) containing 0.73 mM trisilylpropionic acid-d6 (TSP-d6). The dried lipophilic extracts were reconstituted with a solution of $CDCl_3/CD_3OD$ (2:1) containing 1.18 mM tetramethylsilane (TMS) and then vortexed. Both extracts were transferred into 5-mm NMR glass tubes for NMR measurement. $^1H$ NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz using a 5 mm PBBO broadband gradient probe. For the aqueous extracts one-dimensional $^1H$ pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY) presaturation sequence (RD–90°–t1–90°–tm–90° ACQ) to suppress the residual water peak, and the mixing time was set at 100 ms. Solvent presaturation with irradiation power of 75 Hz was applied during recycling delay (RD = 5 s) and mixing time. The 90° pulse length was calibrated for each sample. The spectral width was 12 kHz (20 ppm), and a total of 256 transients were collected into 64 k data points for each $^1H$ spectrum. In the case of lipophilic extracts, a 90º pulse with presaturation sequence (*zgpr*) was used to supress water residual signal of methanol. A RD of 5.0 s with acquisition time of 2.94 s were used. The 90° pulse length was calibrated for each sample. After 4 dummy scans, a total of 128 scans were collected into 64K data points with a spectral with of 18.6 ppm.

The exponential line broadening applied before Fourier transformation was of 0.3 Hz. The frequency domain spectra were phased, baseline-corrected and referenced to TSP or TMS signal ($\delta$= 0 ppm) using TopSpin software (version 3.6 Bruker).

### 2.8. NMR Data Analysis

The acquired $^1H$ NMR were compared to references of pure compounds from the metabolic profiling AMIX spectra database (Bruker), HMDB, Chenomx NMR suite 8.4

software (Chenomx Inc., Edmonton, AN, Canada) and databases for metabolite identification. In addition, we assigned metabolites by $^{1}H–^{1}H$ homonuclear correlation (COSY and TOCSY) and $^{1}H–^{13}C$ heteronuclear (HSQC) 2D NMR experiments and by correlation with pure compounds run in-house. After pre-processing, specific $^{1}H$ NMR regions identified in the spectra were integrated using the AMIX 3.9 software package.

### 2.9. Data Processing and Multivariate Analysis

Absolute concentrations in the NMR tube derived from both lipophilic and hydrophilic extracts were arranged together in one single data matrix. Previously, data were scaled to unit variance to give all the identified metabolites the same weighing into the model. Data analysis and statistical calculation were performed with IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. For the orthogonal partial least squares – discriminant analysis (OPLS-DA) the MetaboAnalyst software 4.0 was used (https://www.metaboanalyst.ca/).

## 3. Results

### 3.1. Multivariate chemometric analysis of NMR data

The PCA methodology was used to detect putative outliers. The PCA was performed using the whole spectra removing the TSP, methanol, chloroform and water regions in both phases, aqueous and lipid. After the alignment and the normalization of the spectres, 26 metabolites were identified and integrated in the aqueous phase and 16 metabolites in the lipid phase (Figure 1).

### 3.2. Multivariant analyses showed that the GSPE effects on the live metabolome is time-dependent.

Pairwise OPLS-DA models were constructed to compare the effect of GSPE administrated at two different moments (GSPE-ZT0 and GSPE-ZT12), with respect to their respective control groups (CAF-ZT0 and CAF-ZT12). A model with a predictive ability [$Q^2Y$= 0.289, P=0.04] was obtained comparing the liver metabolites of CAF-ZT0 and GSPE-ZT0. The metabolites that were significantly altered in this model and found lower at GSPE-ZT0 were plasmalogen, linoleic acid, monoglycerides, esterified cholesterol, total cholesterol, glycerol, alanine, succinate, lactate and glucose-6-phosphate. In contrast, glutathione was increased in GSPE-ZT0 animals (Figure 1-A).

Significant differences were also shown when the liver metabolites of the CAF-ZT12 group were compared with the metabolites of the GSPE-ZT12 group. The model presented a good predictive ability [$Q^2Y$= 0.3742, P=0.02]. Plasmalogen, ARA+EPA, linoleic acid and free cholesterol shown lower concentrations at the GSPE-ZT12 group. In contrast, ascorbate, tyrosine, phenylalanine, isoleucine, creatinine, creatine and glucose-6-phosphate were increased in GSPE-ZT12 animals (Figure 1-B).

Altogether these results suggested that the effects of GSPE in the liver metabolome depends on the time of administration, since most of the aqueous metabolites were affected differently. The reduction of alanine, lactate and glucose-6-phoshpate concentrations in GSPE-ZT0 suggested a reduction of hepatic gluconeogenesis, thus an improving the insulin sensitivity (Figure 1).

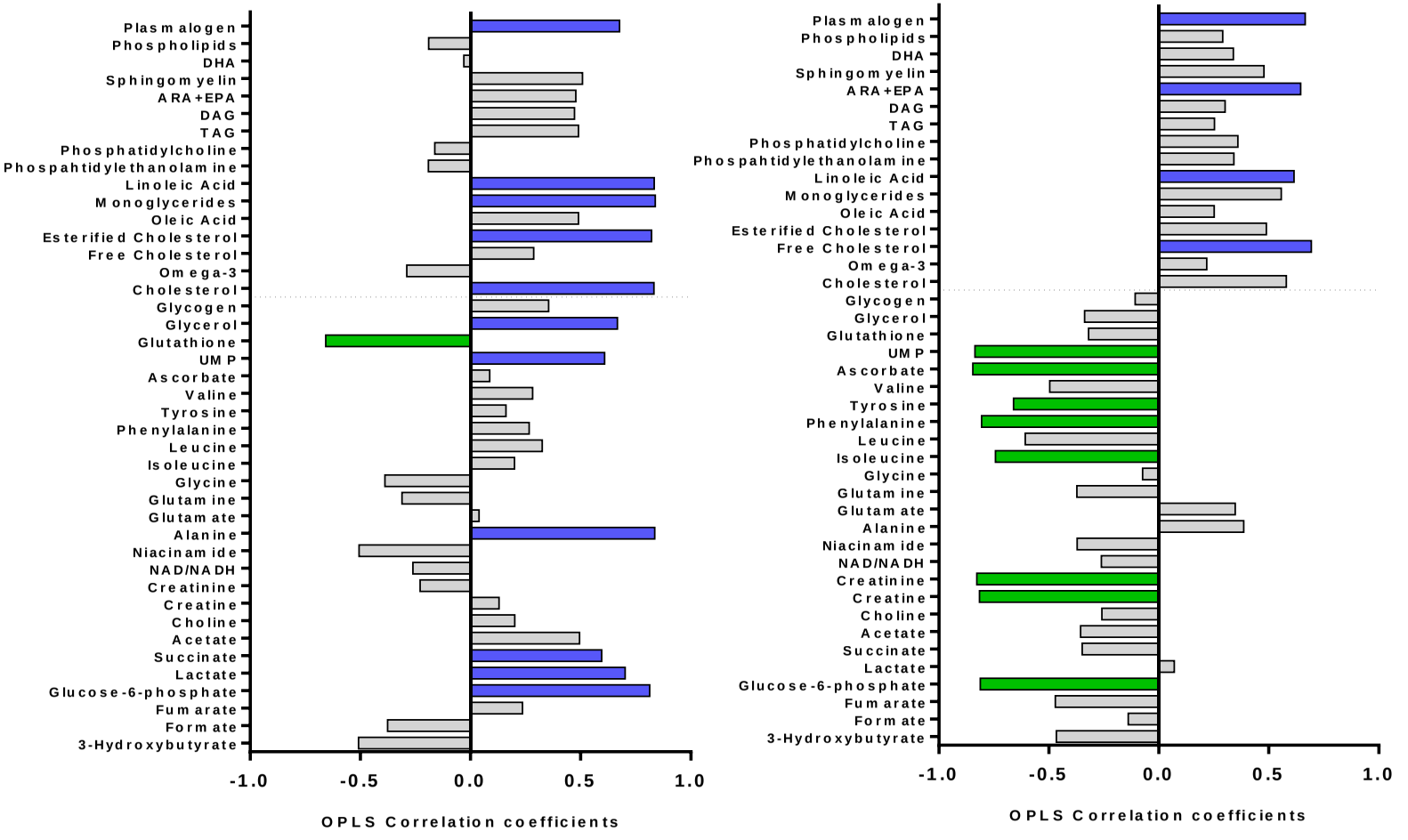


**Figure 1**. The 42 liver metabolites were used to set up OPLS-DA models. It was done in both, ZT0 (A) and ZT12 (B) comparing the cafeteria groups with their zeitgeber treated groups. The significative values were represented in blue for those that were increased in the CAF group and green for those increased in the GSPE groups ($p < 0.05$).

### 3.3. GSPE administrate at ZT0 improves insulin resistance.

In order to determine the insulin resistance degree, plasma insulin and glucose levels were quantified. As depicted in Table 1, GSPE-ZT0 animals presented decreased insulin levels in plasma. This effect on the plasma insulin concentration did not appear in the group treated at ZT12. Furthermore, the plasma glucose levels were higher in GSPE-ZT12 (Table 2). In addition, in the OGTT assay, glucose levels were significantly reduced at 0 and 15 min in the GSPE-ZT0 animals (Figure 2 A), while no differences were observed between the CAF-ZT12 and GSPE-ZT12. (Figure 2 B).

In order to evaluate insulin resistance, the HOMA-IR value was calculated. Notably, the HOMA-IR value in the GSPE-ZT0 group exhibited lower levels than in the CAF-ZT0 group while there was any difference at ZT12 indicating an improvement on insulin sensitivity by GSPE only when it is administrated at ZT0.

**Table 2**. Plasmatic parameters in Wistar rats fed with a cafeteria diet treated with GSPE at two different times, ZT0 and ZT12

| Plasma parameters | CAF-ZT0 | GSPE-ZT0 | CAF-ZT12 | GSPE-ZT12 |
|---|---|---|---|---|
| Glucose (mM) | 7.72 ± 0.5 | 7.67 ± 0.2 | 8.32 ± 0.6 | 7.68 ± 0.24 |
| Cholesterol (mg/dL) | 78.15 ± 6.16 | 76.95 ± 6.44 | 73.5 ± 1.29 | 71.38 ± 5.71 |
| TAG (mg/dL) | 141.81 ± 14.02 | 123.09 ± 11.64 | 190.13 ± 24.01 | 235.57 ± 43.27 |
| Insulin (ng/mL) | 8.6 ± 1.08 | 5.53 ± 1.06* | 7.94 ± 0.78 | 8.3 ± 1.88 |
| HOMA-IR | 134.77 ± 19.41 | 84.56 ± 15.92* | 126.53 ± 11.58 | 112.85 ± 14.65 |

Male Wistar rats were fed with a cafeteria diet for 11 weeks and treated with GPSE at two different time the last 4 weeks. Data expressed as the mean ± SEM (n=8). TAG, triglycerides; HOMAR-IR, homeostatic model assessment for insulin resistance. Student's t-tests were performed to compere the differences between the groups treated at the same time. Significant differences (p-Value < 0.05) between the CAF and its treated ZT group are represented by an *.

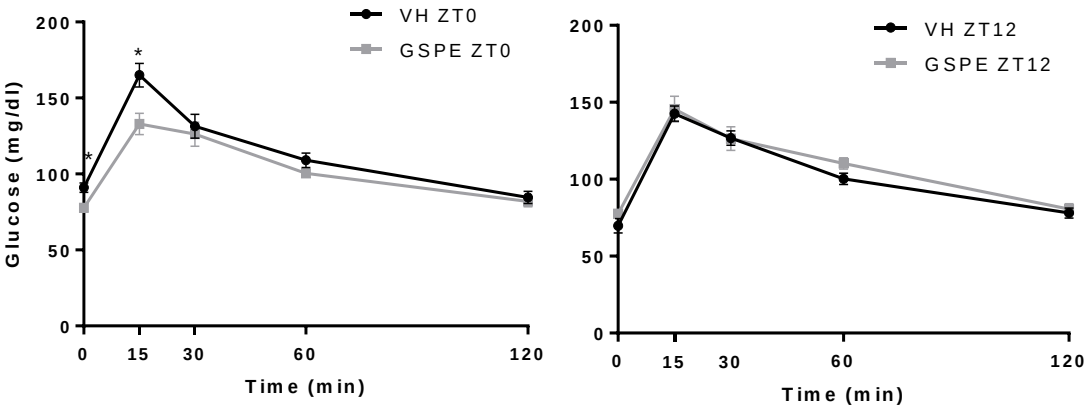


**Figure 2**. Plasma glucose concentration during the OGTT. (A) CAF-ZT0 group compared with GSPE-ZT0. (B) CAF-ZT12 group compared with GSPE-ZT12. Data are expressed as the mean ± SEM (n=8). P-value < 0.05 were considered statistically significant (*).

### 3.4. GSPE did not show significative changes in gluconeogenesis and glycolysis gene expression.

The changes observed in the insulin resistance and in the metabolites related to gluconeogenesis prompted us to study the gene expression of the *glucokinase* (*Gk*) and *phosphoenolpyruvate carboxykinase* (*Pck1*). However, we did not observe significant differences in *Gk* and *Pck1* relative gene expression between the CAF groups and their respective GSPE groups.

### 3.5. Plasmatic insulin correlated negatively with liver glycogen and niacinamide levels in the ZT0 groups.

In order to assess how the insulin sensitivity improvement affected the hepatic metabolism, correlations between insulin and the hepatic metabolites levels were performed. Figure 3 showed a negative correlation between plasma insulin values with niacinamide and glycogen levels in the liver at ZT0. At ZT0, glycogen tended to be higher when the levels of plasmatic insulin were decreased. At ZT12, there was not a significative correlation between plasma insulin and glycogen (Figure 3).

In case of niacinamide, its levels at ZT0 were found higher when plasma insulin concentration was low. Any correlation was found at ZT12 between niacinamide and plasma insulin. Moreover, the niacinamide levels in the liver tended to be reduced by GSPE when it was administrated at ZT0 (Figure 3).

The Insulin correlation with glycogen verified the improvement on insulin sensitivity hypothesized. Moreover, the correlation between niacinamide and insulin at ZT0 might explain the reason by why the hepatic effects of GSPE were time-dependent.

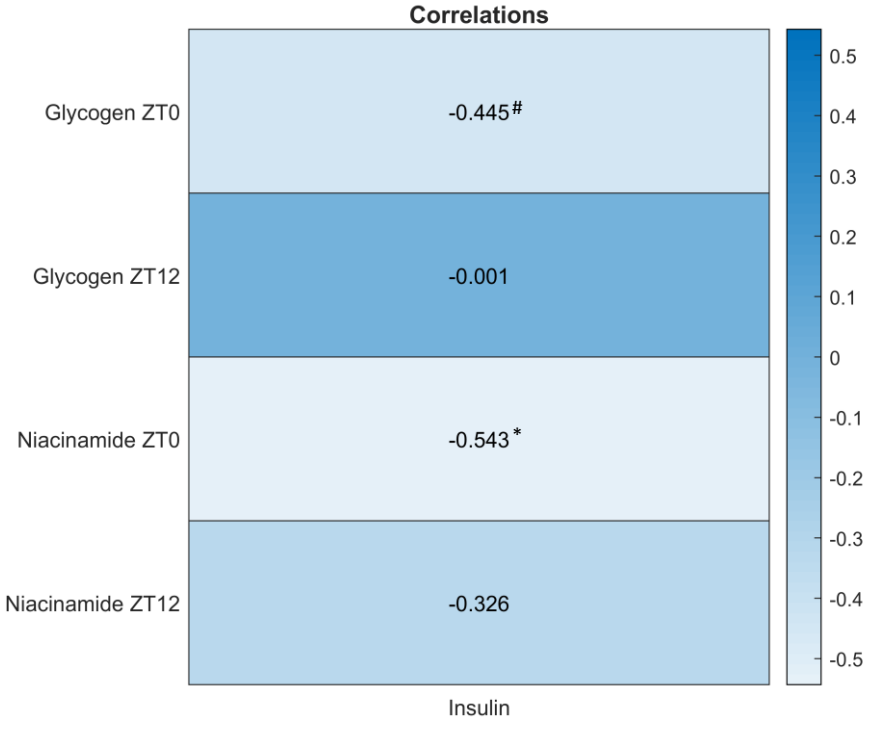


**Figure 3.** Correlation plot between plasma insulin and hepatic glycogen at all times. Each plot presents each Pearson r correlation value. Significant correlation between Niacinamide ZT0 and Insulin is represented by an * (p-value < 0.05); The tendency between Glycogen ZT0 and insulin is represented by an # ( 0.05 < p-Value < 0.1).

## 4. Discussion

In the present study, we have shown that the effects of GSPE against the metabolic syndrome were different depending on the its administration time. To this end, multivariate analyses were performed with 42 metabolites in two different times, ZT0 and ZT12. The OPLS-DA model showed that the effects of GSPE on the hepatic metabolism were time-dependent. In both administration times, the lipid profiling was improved by the GSPE treatment presenting lower lipid concentration. Previous studies have demonstrated the beneficial effects of GSPE on the hepatic lipids in rats fed a obesogenic diets [23,30]. In these sense, Baiges *at al.* showed a decrease of proteins related to the fatty acids (FA) and triglycerides (TAG) synthesis in the liver, induced by GSPE [23]. In our study, we found decreased the hepatic TAG but not significantly in both times, although the monoglycerides levels were significantly reduced by GSPE at ZT0. Additionally, the cholesterol management was differently altered by GSPE depending on the administration time. In this sense, GPSE is capable of modify the Rev-Erbα amplitude [31]. In this regard, the bile acids and cholesterol biosynthesis are under circadian control through Rev-Erbα, which regulates the expression of SREBP, and consequently, the genes related with the cholesterol and bile acids metabolism [32].

The increase on glutathione levels found only in those animals treated at ZT0 suggested a better antioxidant effect only in this group, since glutathione is the most important low-molecular-weight antioxidant synthesized in cells [33]. A previous study demonstrated that the oxidative stress induced by an obesogenic diet, which increased reactive oxygen species and/or decreased glutathione antioxidant metabolism, was reduced by GSPE through the improvement of the hepatic glutathione metabolism [25]. The GSPE antioxidant effects are caused by an increased on the glutathione/ oxidized glutathione ratio [34]. The differences between both GSPE administration time groups might be caused by their enzymatic

regulations, since most of the enzymes related to the glutathione metabolism, such as glutathione peroxidase, glutathione reductase and glutathione S-transferase, present circadian rhythms [35].

The reduction of the metabolites related to the gluconeogenesis, glucose-6-phosphate (G6P), lactate and alanine [36,37], suggested an increase of the insulin sensitivity in the liver. In a healthy liver, after food intake, insulin is secreted to reduce the plasma glucose, which is metabolized by the liver, which will further synthetize glycogen or FA, or use it as energy source through the TCA cycle [38]. Moreover, insulin inhibits gluconeogenesis and promotes glycolysis in the liver [39,40]. Insulin resistance is one of the symptoms of the metabolic syndrome [41]. Hence, the hepatic gluconeogenesis inhibition by insulin is lost in presences of insulin resistance [42]. Our results suggested that there was a possible improvement against insulin resistance only in those animals treated with GSPE at ZT0, since an improvement on insulin gluconeogenesis inhibitory effect was observed. The enhancement on insulin sensitivity at GSPE-ZT0 was corroborated with the insulin levels, HOMA-IR and the OGTT values. β-cells, in subjects with insulin resistance, increase three to four times the insulin secretion trying to balance the lack of insulin sensitivity [43]. Hence, the reduction of metabolites related to gluconeogenesis, simultaneously with the lower concentration of plasmatic insulin, suggested an improvement on insulin sensitivity [39]. In the GSPE-ZT0, the reduction of plasmatic glucose after 15 min in the OGTT suggested that the liver was capable to uptake glucose more efficiently than the CAF-ZT0 animals, effect that might be explained by an enhancement of insulin sensitivity. In addition, the reduction of HOMA-IR, which is a simplified measure of insulin resistance [44], in the GSPE-ZT0 group supported the insulin sensitivity improvement hypothesis.

We also further studied the gene expression of both GK and PCK1. Both are key enzymes of glycolysis and gluconeogenesis respectively, and are regulated by insulin

in the liver [45,46]. Despite the fact that we did not see differences on gene expressions, these results do not mean that there were no significant differences on their protein concentrations or enzymatic activities of GK and PCK1. There is a whole system of post-transcriptional regulations, such as microRNAs, and post-translational, phosphorylation and acetylation, that can have a direct impact on the concentration or activity of these enzymes [47–49].

In order to determine how the improvement of insulin sensitivity was related to the liver metabolome, some correlations between the plasmatic insulin and the metabolites found in the liver were performed. Niacinamide and glycogen correlated significantly with plasmatic insulin at ZT0. After food intake, insulin activates the enzymes related to the glycogenesis to store plasma glucose, derived from the diet, into hepatic glycogen [50]. The negative correlation between glycogen and insulin found in the ZT0 groups was in consonance with our previous results. In this sense, plasm insulin was not being capable to interact with the liver in the CAF-ZT0 group, since higher plasma insulin levels were not able to modulate the hepatic glycogen. On the other hand, in the GSPE-ZT0 group, this interaction was improved, since it showed an increase of glucose storage with lower insulin levels in plasma, possibly due to the improvement on insulin sensitivity. The fact that the ZT12 groups there were no correlations between insulin and glycogen robustly indicated that both groups presented insulin resistance, since plasma insulin was incapable to modify glycogen levels in the liver, effect that is in consonance with our previous results.

Not only glycogen showed a negative correlation with plasma insulin at ZT0, but also niacinamide in the liver presented lower levels when the insulin concentration was higher. Niacinamide, used predominantly for the $NAD^+$ biosynthesis in mammals [51], consists of two enzymatic processes by nicotinamide phosphoribosyltransferase (Nampt) and niacinamide mononucleotide adenylyltransferase [52,53]. Glucose and lipid metabolism is regulated by sirtuins

(Sirt), which are NAD-dependent deacetylases, related with aging, diabetes, and hepatic steatosis [54–56]. Niacinamide might act as a favourable $NAD^+$ donor as well as an effective Sirt1 inhibitor [57]. S.J. Yang et al. [58] treated rats fed a high fat diet with different doses of niacinamide. They showed lower area under the curve (AUC) values of the OGTT, serum fasting glucose, serum fasting insulin, HOMA-IR values, and higher serum adiponectin levels. Furthermore, Ribas-Latre et al. [26] observed that GSPE modulates the diurnal expression pattern of BMAL1 and Nampt in rats fed a standard chow diet. In addition, a previous study demonstrated that dietary proanthocyanidins raised hepatic $NAD^+$ metabolism, SIRT expression and its activity in healthy rats [59]. Hence, the improvement on the insulin sensitivity and glucose metabolism observed in the liver at ZT0 might be due to an increase of niacinamide levels in the liver. The hepatic niacinamide levels tended to be increased at GSPE-ZT0, while there were no differences between both ZT12 groups. Despite the fact that niacinamide levels at ZT12 were not significantly different between them, the GSPE-ZT group exhibited a higher niacinamide concentration than the CAF-ZT12 group. Since niacinamide is capable to act as a $NAD^+$ donor, as well as a Sirt1 inhibitor, and both are strongly related with circadian rhythms, it is expected that the effect of niacinamide on the hepatic $NAD^+$ metabolism, caused by PACs, is time-dependent. The effect of niacinamide at ZT0 seemed to be the boosting of the $NAD^+$ metabolism, while, at ZT12, the niacinamide effect might be associated with the Sirt1inhibition, thus producing the opposite effect on the glucose metabolism. Hence, this might be a reason why glucose-6-phosphate in GSPE-ZT12 was found higher than in CAF-ZT12.

In summary, our results have demonstrated that the effects of GSPE in the hepatic metabolism is time-dependent. GSPE administrated at ZT0 and ZT12 improved similarly the lipid profiling, and had different effects in the cholesterol management. Additionally, GSPE at ZT0 might present antioxidant effects through an increase of glutathione. At the same time, the GSPE administration is capable to improve the

glucose metabolism in the liver through the enhancement of its insulin sensitivity. This effect might be related to an increment of niacinamide, which boosts the $NAD^+$ metabolism and Sirt1 activity. On the other hand, the results observed in the animals treated at ZT12 suggested that niacinamide might act as a Sirt1 inhibitor, thus impairing the glucose metabolism in the liver. In conclusion, GSPE presented better beneficial effects on the hepatic metabolism when it is administrated at the beginning of the light period.

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# GENERAL DISCUSSION

# General discussion

Natural polyphenols have been identified as powerful agents for the prevention and treatment of liver diseases [1]. In this sense, it has been widely demonstrated the beneficial effects of proanthocyanidins (PAC) on the hepatic metabolic disturbances induced by obesity, such as insulin resistance or steatosis [2]. Additionally, PAC, particularly a grape seed proanthocyanidin extract (GSPE), is capable to modulate the clock system in the liver, which is also disrupted in an obesity status [3]. It proposes that GSPE can regulate lipid and glucose metabolism by adjusting the hepatic circadian rhythms. Altogether, these evidences suggest that the beneficial GSPE effects on the hepatic metabolism might be different depending on its time of administration on light/dark cycle.

Therefore, the first objective of this thesis was to assess the impact of diet-induced obesity on the circadian rhythms of the hepatic metabolome depending on the gender (**Manuscript 1**). To the best of our knowledge, this is the first manuscript in which it is determined how similar and/or different are the hepatic disruption of the metabolic circadian rhythms in diet-induced obese rats depending on the gender. In our study, the hepatic metabolome characterization, through non-targeted NMR, demonstrated that female rats were more resistant than male animals to deleterious effects of an obesogenic diet, since the clear metabolic clustering depending on the zeitgeber (ZT) observed in animals fed a standard diet were only conserved in female fed a cafeteria diet. It suggested that female rats presented a better metabolic flexibility under an obesogenic diet, effect that might be associated to the protective effect of oestrogens against obesity.

Most of the metabolites that differed between obese males and females, that might help to maintain the differences between both zeitgeber, showed to be related with the VLDL packaging. Phosphatidylcholine is the most abundant phospholipid in VLDL

and is required to their assembly [4]. Only female fed a cafeteria diet presented rhythmicity in phosphatidylcholine and in metabolites related with its synthesis such as choline, betaine, diglycerides, and phosphatidylethanolamine. These metabolites belong to two different pathways, CDP-choline and phosphatidylethanolamine N-methyltransferase pathways, and both presented higher phosphatidylcholine synthesis at ZT15, at the beginning of the dark period [5,6]. Additionally, obese male rats showed lower choline and phosphatidylcholine levels than standard male rats, which were related to decreased methylation capacity, perturbed phosphatidylcholine synthesis, impaired VLDL secretion, and altered lipid metabolism [7,8]. All these repercussions influence the progression from a healthy liver to a fatty one [7]. Taken together, our results suggested that females responded to the obesogenic diet through the improvement of the circadian rhythms in most of the metabolites related to the synthesis of VLDL. In this sense, an improvement on the management of hepatic and plasma TAG was expected, since VLDL carry them out from the liver to the circulating blood [9]. In this regard, only female animals presented circadian rhythms in hepatic TAG in both diets. Moreover, plasma TAG correlated negatively with the hepatic phosphatidylcholine levels, which suggested that phosphatidylcholine was being incorporated into VLDL and secreted into the circulating blood, thus increasing plasma TAG levels.

Once the sexual dimorphism in the circadian rhythms disruption by diet-induced obesity was studied, the effect of a grape seed proanthocyanidin extract (GSPE) was evaluated depending on the administration time. The understanding of the circadian rhythm disruptions in the hepatic metabolism, caused by diet-induced obesity, was important in order to comprehend the probable different GSPE effects on the hepatic metabolism when it was administrated at two different zeitgebers. The hypothesis of having different GSPE effects depending on the administration time is supported by previous studies performed in our research group. In this sense, Ribas-Latre et al. [10] have demonstrated that GPSE was able to modulate central and

peripheral clock genes. To check the possible time-dependent GSPE effects, we proposed the second aim of this thesis, which consisted on verifying the impact of GSPE time administration on the liver metabolism, only in obese male rats, since female rats presented an intrinsic hormonal protection which might mask the GSPE beneficial effects (**Manuscript 2**).

GSPE is known to cause a large amount of benefits against pathologies related with diet-induced obesity [11,12]. Our results showed, independently of the GSPE time administration, a subtle improvement on the hepatic lipid profiling. Moreover, GPSE altered differently the cholesterol management. The animals treated at the beginning of the light period (ZT0) presented reduced esterified cholesterol, and those treated at the dark onset (ZT12) showed decreased free cholesterol. It makes sense since GPSE is able to modify the Rev-Erb$\alpha$ amplitude, thus altering the expression of SREBP, and consequently the circadian rhythm of genes related with the cholesterol and bile acids metabolism [13]. GSPE has been also associated with a reduction of oxidative stress induced by obesogenic diets [14]. On this subject, a previous study has demonstrated the GSPE antioxidant effects are caused by an increased total glutathione/oxidized glutathione ratio. In our study, the glutathione levels were significantly higher only in those rats treated at ZT0. The differences between both GSPE administration time groups might be explained by the fact that most of the enzymes related to the glutathione metabolism, such as glutathione peroxidase, glutathione reductase and glutathione S-transferase, present circadian rhythms [15].

In addition to these findings, alanine, lactate and glucose-6-phosphate presented different concentrations depending on the GSPE administration time, since a reduction on their concentrations was found in those rats treated at ZT0. These metabolites are related with the hepatic gluconeogenesis, thus suggesting a possible alteration in this pathway. In the liver, gluconeogenesis is regulated by both insulin,

inhibiting it, and glucagon, activating it [16,17]. In this sense, obesity is strongly related with insulin resistance, and thus, an impaired gluconeogenesis inhibition [18]. Therefore, a reduction on the gluconeogenesis-related metabolites might be explained by an insulin sensitivity enhancement. In order to verify the insulin sensitivity improvement, plasma insulin, HOMAR-IR and an oral glucose tolerance test (OGTT) were performed. All these essays supported that GPSE administrated only at ZT0 ameliorated the insulin resistance.

In the interest of elucidate how the insulin sensitivity improvement affected the liver metabolome, correlations between plasma insulin and the hepatic metabolites found were performed. As expected, plasma insulin correlated with glycogen in animals treated at ZT0 due to the insulin sensitivity improvement. In addition to glycogen, a correlation between plasma insulin and niacinamide was also observed at ZT0. In mammals, niacinamide is used predominantly for the biosynthesis of $NAD^+$ [19]. In addition, niacinamide acts as a favourable NAD+ donor as well as inhibiting Sirt1. Sirtuins are NAD-dependent deacetylases related with aging, diabetes and hepatic steatosis [20,21]. Moreover, glucose and lipid metabolism are also regulated by sirtuins. A previous study has demonstrated that different doses of niacinamide improved the area under the curve in OGTT, serum fasting glucose, serum fasting insulin and the HOMAR-IR value [22]. Taken together, the increased niacinamide observed could be a reason for the improvement on the hepatic insulin sensitivity. Moreover, niacinamide might also help to explain the time-dependent effect of GSPE, since it is capable to act as a NAD+ donor as well as Sirt1 inhibitor, both strongly related with circadian rhythms [23].

Considering all the results of this thesis, we could confirm that the time administration of GSPE had an impact on its beneficial effects. As expected, the effects of GSPE administrating it at two zeitgeber were different being more effective against the pathologies associated to obesity when it was administrated at the

beginning of the light phase, which it might partly be possible, by its capacity to modulate clock genes. Nevertheless, there are necessary further studies to elucidate the different effects of GSPE on different tissues and at different molecular levels depending on the time administration. In addition, it would also be needed to determine if these differences remain in female animals, since the circadian disruptions in diet-induced obesity are gender dependent.

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# CONCLUSIONS

# Conclusions

1. The circadian rhythms disruptions of the hepatic metabolism induced in animals subjected to a 9-weeks obesogenic diet varies depending on the gender:

    a. Female animals fed an obesogenic diet were more metabolic flexible since they were able to preserve the differences in the metabolism of two different zeitgebers.
    b. Obese female animals were more protected against obesogenic diet due to their improvement on the rhythmicity of metabolites related to the VLDL packaging, thus enhancing the hepatic triglyceride secretion into the circulating blood.

2. The effects of a 25 mg GPSE/kg BW dose in male animals subjected to a 9-week obesogenic diet are different depending on the time of administration:

    a. The studied beneficial effects of GSPE on the hepatic lipid content are independent of its administration time. Only the cholesterol management was found time-dependent.
    b. The antioxidant effects associated to GPSE depend on the administration time, since it is more effective when the animals are treated at the beginning of the light phase.
    c. GPSE administration at ZT0 ameliorate hepatic insulin resistance, thus reducing the hepatic gluconeogenesis.

d. The hepatic improvement of insulin sensitivity obtained only when GSPE is administrated at ZT0, might be related to the increase of niacinamide levels, since niacinamide presents beneficial effects on the glucose metabolism and it is strongly related with the regulation of circadian rhythms.

# Conclusions

1. La disrupció dels ritmes circadians del metabolisme hepàtic, induïts en animals alimentats amb una dieta obesogénica durant 9 setmanes, varien segons el gènere:

    a. Les rates femelles alimentades amb una dieta obesogénica van ser molt més flexibles, ja que van ser capaces de mantenir les diferències en el metabolisme entre els dos temps.
    b. Les femelles obeses estaven més protegides dels efectes de la dieta obesogénica degut a la millora en els ritmes circadians dels metabòlits relacionats amb l'empaquetament de les VLDL, i d'aquesta forma, millorant la secreció dels triglicèrids hepàtics a la circulació sanguínia.

2. Els efectes d'una dosis de 25 mg GSPE/kg pes en mascles, alimentats amb una dieta obesogénica durant 9 setmanes, van ser diferents depenent del temps d'administració:

    a. L'estudiat efecte beneficiós del GSPE sobre el perfil lipídic del fetge és independent del temps d'administració. Només la gestió del colesterol es va trobar dependent del temps.
    b. L'efecte antioxidant associat al GSPE és depenent del temps d'administració, ja que aquest és més efectiu quan els animals són tractats al principi de la fase lumínica.
    c. El GPSE administrat a ZT0 redueix la resistència a la insulina, i per tant, hi ha una reducció de la gluconeogènesis al fetge.

d. La millora en la sensibilitat a la insulina obtinguda només quan el GSPE es administrat a ZT0 pot estar relacionada amb un augment en els nivell de niacinamide, ja que aquest presenta efectes beneficiosos en el metabolisme glucídic i està fortament relacionat amb la regulació dels ritmes circadians.

# List of publications

## Papers included in the thesis

### A. Submitted papers

**Héctor Palacios-Jordan**, Miguel Z. Martin-Gonzalez, Manuel Suárez, Gerard Aragonès, Begoña Mugureza, Miguel A. Rodríguez, Cinta Bladé. The disruption of liver metabolic circadian rhythms by an obesogenic diet is sex-dependent in Fischer 344 rats. [Submitted to Journal of Nutritional Biochemistry].

**Héctor Palacios-Jordan**, Albert Gibert-Ramos, Miguel Martin-Gonzalez, Gerard Aragonès, Begoña Mugureza, Lluis Arola, Cinta Bladé, Miguel A. Rodríguez. The beneficial effects of a grape seed proanthocyanidin extract on the liver metabolism is time-dependent in obese Wistar rats. In preparation.

## Other papers

### A. Published papers

Gibert-Ramos A, **Palacios-Jordan H**, Salvadó MJ, Crescenti A. Consumption of out-of-season orange modulates fat accumulation, morphology and gene expression in the adipose tissue of Fischer 344 rats. Eur J Nutr. 2019 Feb 20. doi: 10.1007/s00394-019-01930-9. Impact factor: 4.449. SI Journal Citation Reports © Ranking: (Q1). (European Journal of Nutrition).

# List of conference papers

**NuGoWeek. Molecular Nutrition-Understanding how food influence health.** 2017. Bulgaria. *$^{1}$H-NMR metabolomic approach reveals that conjugated linoleic acid supplementation improves the liver metabolome profile in rats with metabolic syndrome.* **Palacios-Jordan H**, Pascual-Serrano A, Arola-Arnal A, Rodríguez MA, Bladé C. Poster Presentation.

**IX Seminario sobre Alimentación y Estilos de Vida Saludables.** 2017. Spain. *Efecto de la hora del día en la que se administran las proantocianidinas o un hidrolizado de pata de pollo sobre la homeostasis de la glucosa.* **Héctor Palacios**, Miguel Martín-González, Andreu Gual, Miguel Ángel Rodríguez, Cinta Bladé. Poster Presentation.

**XVII Congreso de la Sociedad Española de Nutrición (SEÑ) y X Jornada de l'Associació Catalana de Ciències de l'Alimentació (ACCA**). Spain. *Low dose of conjugated linolenic acid reduces weight gain, increasing energy expenditure without enhancing hepatic steatosis in cafeteria diet-fed rats.* Miguel Martín-González, Héctor Palacios, Gerard Aragonès, Begoña Muguerza

**X Seminario sobre Alimentación y Estilos de Vida Saludables 1r curso de actualización sobre NUTRIGENÓMICA Y NUTRICIÓN PERSONALIZADA Reuniones colaterales: CIBEROBN y ESFRI-ES**. Spain. *Low dose of conjugated linoleic acid supplementation improves serum lipid profile of cafeteria- diet induced obese rats without inducing hepatic steatosis*. Miguel Martín, Héctor Palacios, Miguel A Rodríguez, Cinta Bladé, Begoña Muguerza, Gerard Aragonès

**NuegoWeek2018, Mitochondria, Nutrition and Health**. United Kingdom. *Low dose of conjugated linolenic acid induces thermogenesis, without generating a pathological inflammation status in cafeteria diet-fed rats.* Miguel Martín, Héctor Palacios, Cinta Bladé, Begoña Muguerza, Gerard Aragonès.

A grape seed proanthocyanidin extract (GSPE) has been associated with a widely range of beneficial effects for the prevention and treatment of hepatic metabolic disturbances induced by obesity, such as insulin resistance or steatosis. Moreover, GSPE is capable to modulate the clock system in the liver, which is also disrupted in an obesity status, thus suggesting that GSPE can partially regulate lipid and glucose metabolism by modulating the hepatic circadian rhythms. NMR-based metabolomics strategy is an adequate approach to study the interaction between the proanthocyanidin effects and the circadian rhythmicity of the hepatic metabolism.

In this regard, this thesis aims to evaluate whether a grape seed proanthocyanidin extract (GSPE) has different effects on the hepatic metabolism depending on the administration time, in a metabolic syndrome situation. The circadian rhythm disruption of the hepatic metabolism, caused by obesity, was studied in both genders in order to elucidate whether this disruption is gender-dependent. Female animals showed to be more resistance and flexible against an obesogenic diet.

The chronic administration of GSPE presented different effects in obese male rats depending on its administration time. A large amount of its beneficial effects were found when GSPE was given at the beginning of the light phase. Possible antioxidant effects and an improvement in hepatic insulin sensitivity were only observed in those animals.

The results of this thesis elucidate the importance of the administration time of GSPE. Additionally, this thesis shows the better circadian rhythm flexibility of obese female rats.