Paula Fort Acosta

FINAL DEGREE PROJECT

BIOCHEMISTRY AND MOLECULAR BIOLOGY DEGREE

Role of proanthocyanidins on metabolic homeostasis and hepatic oxidative stress in rats subjected to different photoperiods in an obesogenic context

Academic tutor: Dr. Josep Gómez Álvarez, Biochemistry and Biotechnology Department

In cooperation with: Nutrigenomics Research Group, Rovira i Virgili University

ROVIRA I VIRGILI UNIVERSITY

TARRAGONA 2022

Project based on the results obtained in “Pràctiques Externes” subject, carried out at the Nutrigenomics research group (Biochemistry and Biotechnology Department, Rovira i Virgili University) and supervised by Dr. Miquel Mulero Abellán and Mr. Antonio Jesús Cortés Espinar.

INDEX

1. ABSTRACT

Fifty-four 12-week-old male Fisher 344 rats were housed under 3 different photoperiods (L18, L12 and L6). For each photoperiod, animals were divided in animals fed with standard diet and animals received cafeteria diet for 5 weeks. Then, an oral GSPE daily dose or vehicle were administered to cafeteria groups, while standard group was given only vehicle, for 4 weeks, generating 9 final groups (n=6).

Results showed that GSPE (proanthocyanidins) was able to ameliorate the effect of this diet by decreasing body weight gain at L18 and by decreasing TAG levels much more on L12 than in the other two photoperiods.

Keywords: Metabolic syndrome (MetS); non-alcoholic fatty liver disease (NAFLD); oxidative stress; grape seed proanthocyanidin extract (GSPE); cafeteria diet (CAF); photoperiod.

2. ABBREVIATIONS

ANOVA	Analysis of variance
AUC	Area Under Curve
CAF	Cafeteria diet
CAT	Catalase
CVD	Cardiovascular diseases
FFA	Free fatty acids
FIS1	Mitochondrial fission 1
GPx	Glutathione peroxidase
GSPE	Grape seed proanthocyanidin extract
GSR	Glutathione reductase
HDL	High-density lipoprotein
HF	High-fat diet
IR	Insulin resistance
LDL	Low-density lipoprotein
L18	Large photoperiod (18 light hours : 6 dark hours)
L12	Normal photoperiod (12 light hours : 12 dark hours)
L6	Short photoperiod (6 light hours : 18 dark hours)
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MetS	Metabolic syndrome
MFN2	Mitofusin 2
NAFLD	Non-alcoholic fatty liver disease
NAFL	Non-alcoholic fatty liver
NASH	Non-alcoholic steatohepatitis
NOS2	Nitric Oxide Synthase 2
PAC	Proanthocyanidin
PPIA	Peptidylprolyl Isomerase A
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
SOD	Superoxide dismutase
STD	Standard diet
T2D	Type 2 diabetes
TAG	Triacylglycerides
VH	Vehicle

3. INTRODUCTION

3.1. Metabolic syndrome

The metabolic syndrome (MetS) is a cluster of cardiometabolic risk factors with high prevalence among adult populations and elevated costs for public health systems worldwide. According to the most used definition, the revised Adult Treatment Panel-III (ATP-III), the MetS is diagnosed when at least three of five of the following alterations are present: visceral obesity, dysglycemia, raised arterial blood pressure, hypertrygliceridemia and small, dense LDL cholesterol, together with low HDL-cholesterol [1], [2].

As one study published in 2021 [3], the International Diabetes Federation (IDF) estimates that approximately a quarter of the world population is affected by the pathology. A similar incidence was presented by the National Cholesterol Education Program (NCEP), which estimates that MetS prevalence covers more than 20 % of the adult population in Western countries [4]. However, there is variation in the prevalence of MetS under different diagnostic criteria and across ethnic groups. Factors such as age, sex, ethnicity, and environmental and socio-cultural aspects can completely change these estimates.

The presence of MetS leads to an increased risk of type 2 diabetes (T2D) and cardiovascular disease (CVD), in the form of coronary or peripheral atherosclerosis and heart failure [3]. Moreover, MetS is associated with some other systemic complications that affect different organs and systems, such as respiratory disease, osteoarticular disease, and cancer and non-alcoholic fatty liver disease (NAFLD). The so-called "deadly quartet" composed of obesity, glucose intolerance, hypertriglyceridemia, and hypertension, has a multifactorial origin that is not entirely understood. Nonetheless, genetic predisposition and environmental factors have assumed the leading role in establishing the syndrome [1], [3].

3.1.1. Non-alcoholic fatty liver disease

As the epidemics of obesity, type 2 diabetes mellitus, cardiovascular disease and metabolic syndrome increase worldwide, the prevalence of NAFLD is increasing proportionately, and 25% of the global adult population is potentially affected by the disease. Given the rapidly growing global burden of NAFLD, efforts must continue to find accurate non-invasive diagnostic and prognostic biomarkers, as advanced liver fibrosis. Traditionally, liver biopsy was used to characterize and quantify histological features of

steatosis, inflammation and so on, but this invasive procedure was not suitable for widespread use to assess disease stage or determine progression or response to therapy [5], [6].

In a healthy liver, if there is presence of a free fatty acids (FFA) accumulation, this leads to the generation of hepatic steatosis. This reversible process, if it is not caused by the alcohol consumption, ends up by the development of NAFLD with or without mild inflammation. NAFLD can turn into non-alcoholic steatohepatitis (NASH), a potentially progressive liver disease known for having more severe molecular hallmarks, such as inflammation, oxidative stress and lipotoxicity [5], [7].

Interestingly, the step from NAFLD to NASH is also reversible. Nonetheless, if a long-term activation of the inflammation and both necrotic and apoptotic processes take place, it can turn out to cirrhosis, which is an irreversible pathway. At this point, the liver transplant is the only option. *Figure 1* shows the progression from a healthy liver to a liver with cirrhosis, with its causes and the risk factors that can trigger the disease. Cirrhosis can be developed by cholestasis, an accumulation of bile acid in the liver, too. After some years, if the liver is not transplanted, there is a high probability of liver failure or hepatocellular carcinoma outcome, with an increased death probability [6], [7].

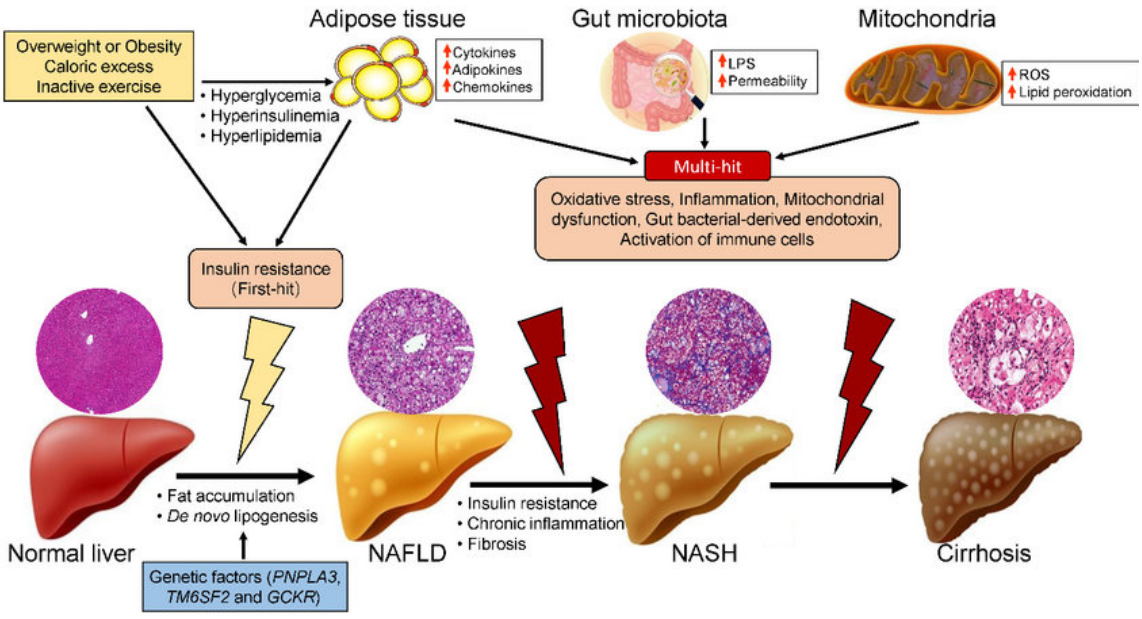

Figure 1. *Non-alcoholic fatty liver disease progression from normal liver to cirrhosis showing the causes and risk factors inboxes. NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis. Obtained from* [8].

NAFLD and NASH carry a large economic concern and create poor health-related quality of life. That is because there is a well-known association between NAFLD, metabolic

syndrome and insulin resistance (IR), some of the most common and prevalent conditions worldwide [9]. Nevertheless, it remains unknown if it is considered a cause or an effect of MetS. Despite of this fact, cardiovascular disease is the leading cause of death in people with NAFLD [10].

3.1.2. Obesity

Obesity is closely associated with MetS, and it is the result of an individual complex interaction of factors as genetic predisposition, physical activity, diet and metabolism [1]. These two disorders are among the greatest health epidemics of the 21st century. In 2016 alone, nearly 2 billion adults were overweight and over 650 million adults were obese. The economic impact of this epidemic is overwhelming, with the annual cost of obesity estimated at USD 92 billion per year in USA, which is equivalent to 5% of total adult health expenditure. Other examples are the cost of £3.23 billion per year in UK or the annual cost of obesity in Canada range from CAD 1.27 billion to CAD 11.08 billion, nearly surpassing the economic costs of smoking, war, and terrorism [11]. With rates of obesity rapidly climbing, the management of obesity and its metabolic complications is at the forefront of modern research [12].

In the last decades, it has been recognized that the white adipose depots are metabolically active tissue, playing a role in the regulation of energy homeostasis and has significant pathological effects that result in many obesity-related diseases [1]. In fact, the inflammation of the adipose tissue is considered one of the causatives of the complications of obesity. This phenomenon happens because adipose tissue is infiltrated by bone-marrow derived macrophages that secrete adipokines and cytokines in the systemic circulation resulting in a chronic inflammatory state, which at the same time can lead to oxidative stress [13].

3.2. Oxidative stress

Oxidative stress has been defined as an imbalance between production of oxidants and antioxidant defenses that may result in damage to biological systems. It involves the chemistry of reactions of so-called reactive species derived from oxygen and nitrogen [14]. Superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen (1 O_2) are commonly defined reactive oxygen species (ROS), and they are generated as metabolic by-products by biological systems. Free radicals are necessary, as reactive ROS are involved in signal transduction to sustain life and defend bacteria, toxins and parasites [15], [16]. Some processes like apoptosis, protein

phosphorylation, activation of several transcriptional factors, differentiation, and immunity, are all dependent on a proper ROS production and presence inside cells that need to be kept at a low level. When ROS production increases, they start showing harmful effects on important cellular structures like lipids, proteins, and nucleic acids [16]. In addition, free radicals can cause mutations in certain genes to adapt to environmental change. Nevertheless, the existence of excessive oxygen free radicals also leads to damage of normal cells and tissues. Sun radiation, unhealthy eating habits and air pollution would force the body to produce more ROS, leading to DNA mutation, which is one of the reasons for aging and disease, and the damage of cell membrane, leading to loss of serum, anti-protease activity and cell mutation. Studies have shown that inflammation, aging and cancer, as well as certain diseases of the heart, lung, liver, skin and so on, are all closely related to the reduced ability of free radical scavenging [15]. In *Figure 2* it is shown a schematic representation of the sources of free radicals and their effects on the human body, as well as the antioxidant defense mechanism against these ROS.

ROS are mainly produced by mitochondria during both physiological and pathological conditions. Even though these organelles have an intrinsic ROS scavenging capacity, it is worth to note that this is not enough to address the cellular need to clear the amount of ROS produced by mitochondria. That is why cells deploy an antioxidant defensive system based mainly on enzymatic components, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), to protect themselves from ROS-induced cellular damage [16].

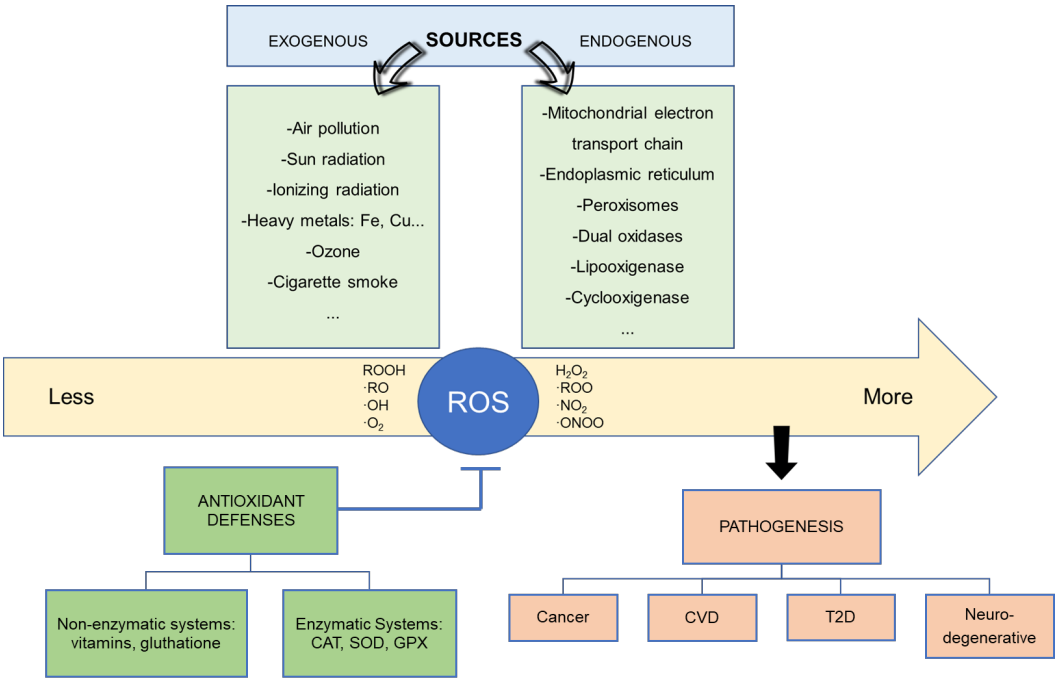

Figure 2. *Schematic representation of the sources of free radicals and their effects on the human body. CAT, catalase; CVD, cardiovascular disease; GPx, glutathione peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; T2D, type 2 diabetes.* *Adapted from* [17].

3.2.1. Obesity and oxidative stress

The liver is an important organ which has an essential role in metabolic homeostasis because it is responsible for the metabolism, storage, synthesis and redistribution of nutrients, carbohydrates, fats, and vitamins. The liver is also an important place for free radicals' formation [15]. That is the main reason why ROS play an important role in the development of obesity and its metabolic complications. These free radicals participate as regulatory factors of mitochondrial activity; they stimulate the differentiation of preadipocytes to mature adipocytes, they promote adipogenesis and lipogenesis, they modify the concentration of molecules taking part in inflammation, which is associated with a large number and size of adipocytes, and they play an important role as agents that regulate the energy balance in hypothalamic neurons that control appetite [13].

In cross-sectional studies, obese subjects have higher levels of oxidative stress biomarkers compared with their leaner counterparts [18]. In addition, it is shown that weight gain significantly increases the concentration of these biomarkers [19]. There are multiple sources for oxidative stress in relation to obesity. Some of them are related to increased adiposity and fat distribution, whereas others are the result of comorbidities or behavioral changes associated with being obese. Increased adipose tissue and, in particular, visceral adiposity are significantly correlated with systemic levels of oxidative stress biomarkers [1].

Obesity, as it is shown, is associated with several comorbidities, including hypertension, diabetes, IR and hyperlipidemia; each of these comorbidities alone can increase the oxidative stress burden [13]. However, most often these comorbidities occur simultaneously, as is the case of the MetS that is characterized by a cluster of cardiometabolic risk factors [20]. Compared with obese subjects without MetS, it is demonstrated that those with MetS have a greater degree of oxidative stress [1].

Maintaining a healthy lifestyle by being physically active and eating a balanced diet rich in antioxidants is associated with reduced oxidative stress [21]. Unfortunately, this protection is less effective among obese subjects, who are more sedentary, having lower serum vitamin levels and reduced intake of dietary antioxidants [1].

Over time, chronic oxidative stress in obesity has a cumulative effect that favors the development of end-stage liver disease [22]. This phenomenon has been mostly studied in the cardiovascular system and the liver in which chronic oxidative stress plays a critical role for the development of nonalcoholic hepatic steatosis and atherosclerosis [23], [24].

3.3. Seasonal variations

It is widely accepted that mammals are able to change their behavior, physiology and metabolism to anticipate changes among the seasons as climate and food availability. Accordingly, it has been observed that factors as reproduction, food intake, energy balance, and so on, vary during the year in some species to ensure their survival; that is why it is said that mammals are season- or photoperiod-sensitive [25].

Even though the seasonality has a limited impact because of the increased use of artificial lighting, air conditioning systems and heating, humans also display seasonal changes in different anthropometric, physiologic, metabolic and behavior parameters. An example of this is the increase in body fat in individuals who live in latitudes far from the equator during winter, where greater variations in temperature, climate and daylight hours are registered. This seasonal responsiveness is illustrated by higher fat mass accretion and fasting circulating levels of cholesterol, triglycerides, glucose, insulin, and leptin during winter and by increased energy expenditure and physical activity during summer. Taking this into account, the seasonal variations in these parameters seem to have a more negative impact on human health during the cold dark part of the year, since a clear negative correlation was found between cardiovascular disease mortality and day length [25], [26]. However, since the seasonal variation of these risk factors is influenced by changes in other exogenous factors different than day length, such as temperature and lifestyle, the relevance of seasonal day length variations on health is

far from being established. In this sense, the use of animal models, which can be maintained under constant temperature and social input, have emerged as a useful strategy to obtain more information, and better understand how exposure to different photoperiods impacts physiology and health [25], [27].

3.4. Polyphenols

Polyphenols constitute a large group of bioactive phytochemicals that include multiple sub-classes such as flavonoids, phenolic acids, lignans and stilbenes. *Figure 3* describes the classification of these phenolic compounds. One of the most studied groups of polyphenols is the flavonoids, which are structurally based on a 15-carbon skeleton of a chromane ring attached to a second aromatic ring. The flavonoids can be sub-divided into groups, including flavan-3-ols, flavones, flavonols, isoflavones, flavanones, and anthocyanins [28].

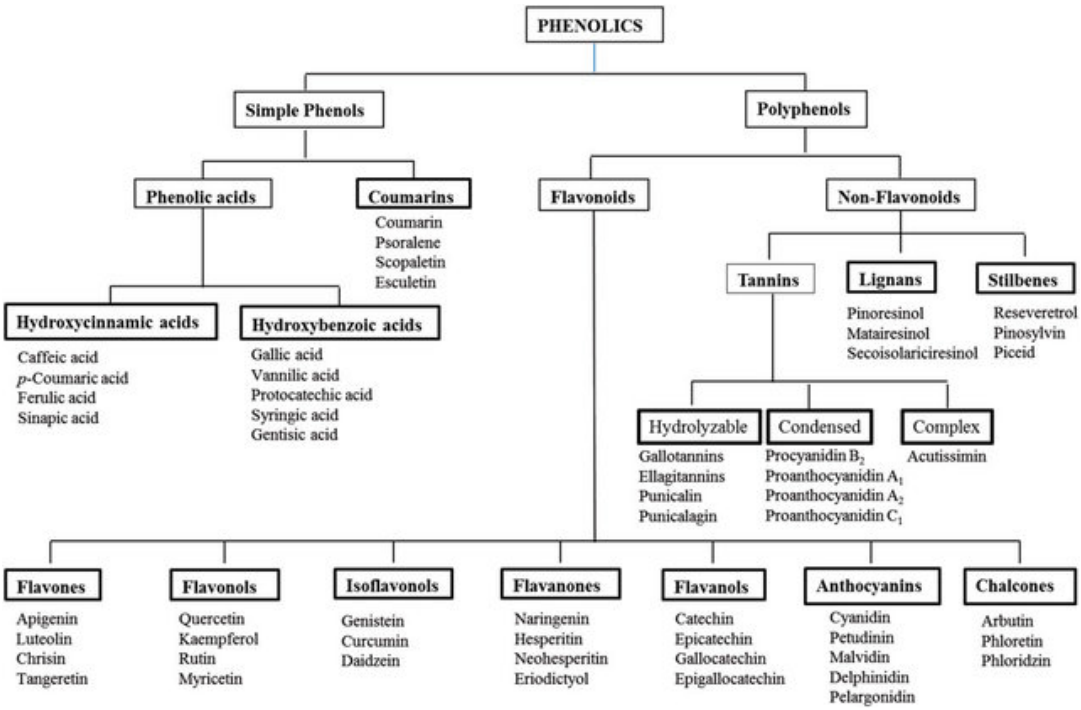

Figure 3. *Polyphenols classification. Obtained from* [28]

Firstly, it was believed that the primary mechanism of action of polyphenols was their direct antioxidant effects. However, it has been seen that these effects are no longer considered to be as relevant *in vivo*, as these compounds do not reach concentrations in most tissues that are high enough to have a significant direct effect in terms of scavenging free radicals [28]. Despite that, some other possible biochemical and molecular mechanisms have been identified, including pleiotropic effects within intra-

and inter-cellular signaling pathways, such as regulating nuclear transcription factors and fat metabolism. *In vitro* and *in vivo* tests have shown that polyphenols support anti-inflammatory functions and the human body's defense by inhibiting cytokine storm and modulating immune regulation; they also modulate cellular immunity, by inhibiting proinflammatory cytokines and acting as immunomodulators [29].

3.4.1. Proanthocyanidins

Proanthocyanidins (PACs) are polyphenols belonging to the class of non-flavonoids: the so-called condensed tannins. *Figure 4* shows the chemical structure of this phenolic compound. These phytochemicals are considered as offense and defense molecules because of their human health benefits, such as its antioxidant, anticancer, antidiabetic, neuroprotective, and antimicrobial capacity [30]. One of the most prominent PACs is grape seed proanthocyanidin extract (GSPE), which is a flavonoid polyphenolic compound extracted from grape seeds. It is composed of flavane-3-alcohol monomers, dimers, trimers, and tetramers, as well as oligomers of proanthocyanidins, +catechin, −epicatechin gallate, −epicatechin gallate and −epigallocatechin via C4–C6 or C4–C8 bond links. In the last years, GSPE has attracted a lot of interest due to its potential clinical value and significant health-promoting effects, including the ability to modify early cerebrovascular injury caused by hypertension, protection of the myocardium from injury, prevention and treatment of diabetes and its complications, alleviation of exercise fatigue and prevention of obesity and inflammatory reaction [31]. In addition, GSPE has potency as a powerful antioxidant for its ability to scavenge free oxygen radicals, with anti-cancer and anti-inflammatory effects, and it has proven to have protective effect against oxidative stress in the liver [32]**.**

By the study of the relationship between GSPE and obesity, it was found that GSPE has many activities, such as improving insulin resistance, lowering hepatic lipid droplets, plasma FFA, triacylglycerides (TAG) accumulation or lipogenesis and steatosis, improving the intestinal flora, regulating the metabolism and so on. So, this natural compound can effectively prevent or palliate obesity in many ways [31]**.**

Related to the oxidative stress, one study suggested that GSPE was effective in mitigating mitochondrial damage and significantly reducing the intracellular ROS and mitochondrial superoxide in the LPS-treated cells**.** This same study revealed that GSPE also reduced oxidative stress in Caco-2 cells by restoring mitochondrial membrane potential, reducing mitochondrial and intracellular ROS, and significantly increasing the expression of antioxidant enzymes as SOD1, SOD2, GSR and GPx1 [33].

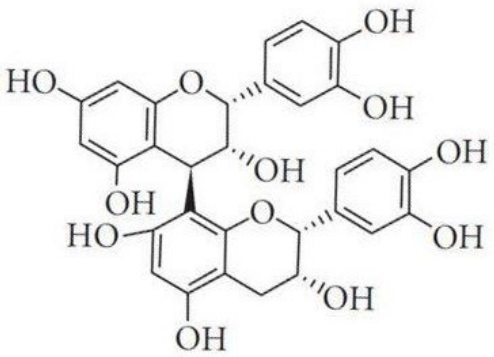

Figure 4. *Proanthocyanidin chemical structure. Obtained from* [30].

4. HYPOTHESIS AND OBJECTIVES

It has been demonstrated that photoperiod and diet are two factors that play an important role in metabolism and oxidative stress. In addition, a lot of research in the last decade is focusing on the study of the antioxidant and anti-inflammatory properties of polyphenols and the role they could play at the metabolic and oxidative stress level. Although this modulatory effect of polyphenols has not yet been studied in great depth, current data suggests that some subclasses of these phytochemicals, such as GSPE, might be able to ameliorate homeostatic dysregulations in crucial organs such as the liver.

HYPOTHESIS:

GSPE is capable of improve the metabolic homeostasis and oxidative stress dysregulation in the liver in obese rats, which could be modulated by different photoperiods.

The **OBJECTIVES** associated with this hypothesis are:

- Validate whether cafeteria diet (CAF) dysregulate the oxidative stress and metabolic homeostasis in the liver.
- Validate whether photoperiod changes affect the oxidative stress and metabolic homeostasis in the liver.
- Validate whether GSPE can ameliorate the cafeteria diet-induced dysregulation.
- Validate whether GSPE can ameliorate the affection induced by photoperiod changes.

5. METHODOLOGY AND MATERIALS

5.1. Animal treatment and experimental design

Fifty-four 12-week-old male Fisher 344 rats (Charles River Laboratories, Barcelona, Spain) were used in this experiment. Rats were housed in pairs at 22 ºC and 55% of humidity. A 4-day adaptation period was carried out where rats were fed with a standard diet (STD) ad libitum. The STD composition was 20% protein, 72% carbohydrates and 8% fat (Panlab, Barcelona, Spain). Rats were randomly divided into 2 groups depending on the diet, 18 rats were fed with STD and the other 36 rats with cafeteria diet (CAF). In addition, these rats were divided into 3 groups depending on the photoperiod: one group with a short photoperiod (6 light hours: 18 dark hours), another with a normal photoperiod (12 light hours: 12 dark hours) and the last one with a large photoperiod (18 light hours: 6 dark hours). In total, there were 6 groups of rats that were subjected to these conditions of diet and photoperiod during a 5-week pre-treatment. CAF consisted of biscuits with cheese and pâté, bacon, coiled puff pastry from Mallorca, carrots and sweetened milk (22% sucrose w/v). CAF composition was 14% protein, 76% carbohydrates and 35% fat. The treatment period started the 5th week and lasted for 4 weeks, and the rats continued with the diet they were fed during the pre- treatment period. At this time, all STD-fed rats were treated with condensed milk, vehicle (VH). CAF-feed rats were divided into two groups, 18 were treated with the VH, and the other 18, with 25 mg/kg GSPE (Les Dérivés Résiniques et Terpéniques, Dax, France) diluted 1/5 in condensed milk. So finally, we had 9 groups of rats (n=6) divided according to diet, photoperiod, and administration of GSPE or vehicle. GSPE was composed of catechin (58 μmol/g), dimeric procyanidins (250 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechin gallate (89 μmol/g), epigallocatechin gallate (1.40 μmol/g), hexameric procyanidins (0.38 μmol/g), pentameric procyanidins (0.73 μmol/g), tetrameric procyanidins (8.8 μmol/g) and trimeric procyanidins (1568 μmol/g). The treatment was orally administered daily using a syringe. During both the pre-treatment and treatment, body weight and food intake were weekly recorded. The rats were fasted for 3 hours and were sacrificed by decapitation. Blood was collected to obtain serum after a 15-min centrifugation (12,000× g and 4 ◦C) and stored at −80 ◦C. The liver was weighted, then collected and finally stored at −80 ◦C for further analysis. All animal care and experimental protocols with animals were approved by the Ethics Review Committee for Animal Experimentation of the *Universitat Rovira i Virgili* (reference number 9495, 18 September 2019) and were carried out in accordance with Directive 86/609EEC of the Council of the European Union and the procedure established by the *Departament d'Agricultura, Ramaderia i Pesca* of the *Generalitat de Catalunya*. The experimental design is depicted in *Figure 5*.

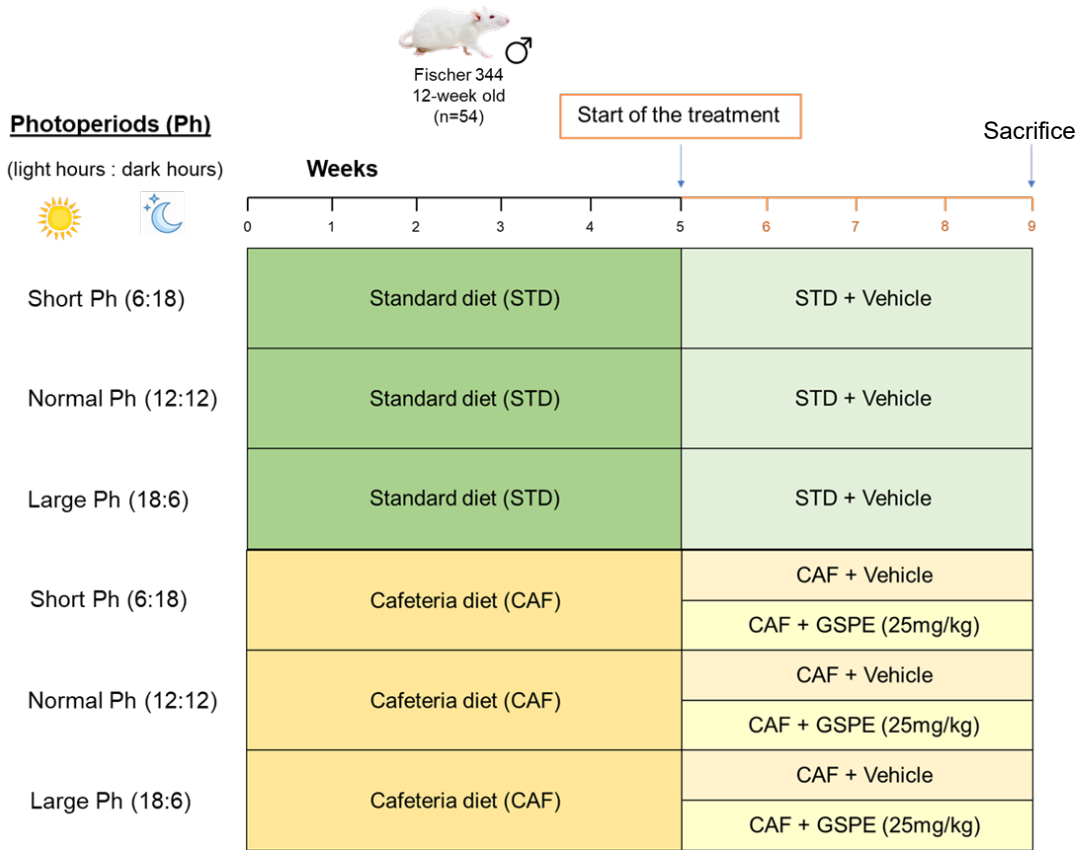

***Figure 5**. Experimental design workflow of the animal treatment.*

5.2. Biochemical liver parameters

The cholesterol, phospholipids and TAG were analyzed by colorimetric enzymatic assay kits (QCA, Barcelona, Spain) according to the manufacturer's instructions.

5.3. RNA extraction

A liver tissue portion (20 – 30 mg) was mixed with 1000 µl of Trizol® reagent (Thermo Fisher, Madrid, Spain) and homogenized 2-3 times by Tissue Lyser LT (Qiagen, Madrid, Spain). After a 10-minute centrifugation (12,000 g and 4 ºC), the pink phase of the homogenate was placed into a new eppendorf another centrifugation took place (12,000 g and 4 ºC) for 10 minutes. The liquid was placed into a new eppendorf and 250 µl of chloroform were added. Two phases were separated after a 15- minute centrifugation (12,000 g and 4 ºC). The aqueous phase (the upper one) was transferred into a new eppendorf and 500 µl of isopropanol were added. The samples were mixed and incubated at room temperature. The supernatant was discarded after a 10-minute centrifugation (12,000 g and 4 ºC). The pellet was cleaned twice with 500 µl of ethanol

70% and a 5-minute centrifugation (8,000 g and 4 ºC) took place. These cleaning steps were repeated one more time. The supernatant was discarded again, and the cleaned pellet was resuspended with 60 µl of nuclease-free water (Thermo Fisher, Madrid, Spain). The RNA concentration (ng/µl) and the purity were measured in Nanodrop ND-1000 spectrophotometer (Thermo Fisher, Madrid, Spain).

5.4. Gene expression analysis by real time RT-qPCR

The cDNA was obtained by a reverse transcription of the RNA extracted using a High-Capacity Complementary DNA Reverse Transcription Kit (Thermo Fisher, Madrid, Spain), according to manufacturer instructions in a Galaxy XP ClearLine Thermal Cycler (Dominique Dutscher, Brumach, France). The quantitative polymerase chain reactions (qPCRs) were performed in 96- well plates in a CFX96 Real-Time System (Bio-Rad, Madrid, Spain) using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Barcelona, Spain). The thermal cycle used in all qPCRs was 30 seconds at 90 ºC and 40 cycles of 15 seconds at 95 ºC and minute at 60 ºC. The liver genes SOD1, SOD2, Catalase, GPx1, GSR, MFN2 and FIS1 were normalized by the housekeeping gene peptidylprolyl Isomerase A (Ppia). The primers used for each gene were obtained from Biomers (Ulm, Germany) and can be found in *Table 1*. The relative expression of each gene was calculated using the $2^{-\Delta\Delta}$Ct method, as reported by Schmittgen and Livak [34].

Table 1. *Forward and reverse primers of SOD1, SOD2, Catalase, GPx1, GSR, MFN2, FIS1 and Ppia genes.*

Gene	Forward primer (5' → 3')	Reverse primer (3' → 5')
SOD1	GGTGGTCCACGAGAAACAAG	CAATCACACCACAAGCCAAG
SOD2	AAGGAGCAAGGTCGCTTACA	ACACATCAATCCCCAGCAGT
Catalase	GAATGGCTATGGCTCACACA	CAAGTTTTTGATGCCCTGGT
GPx1	TGCAATCAGTTCGGACATC	CACCTCGCACTTCTCAAACA
GSR	ATCAAGGAGAAGCGGGATG	GCGTAGCCGTGGATGACT
MFN2	GATGTCACCACGGAGCTGGA	AGAGACGTCACTCACTTTG
FIS1	GCACGCAGTTTGAATACGCC	CTGCTCCTCTTTGCTACCTTTGG
PPIA	TCAAACACAAATGGTTCCCAGT	ATTCCTGGACCCAAAACGCT

5.5. MDA analysis

A liver tissue portion (50 mg) was mixed with 0.154 M KCl buffer to make 10% homogenate. After a 15-minute centrifugation (3,000 rpm and 4 ºC), 500 µl of supernatant were transferred into a new 1.5 ml Eppendorf tube and 500 µl of 10% Trichloroacetic Acid (TCA) were added. Another 10-minute centrifugation (3,000 rpm and RT) took place and then 500 µl of supernatant were transferred to a 1.5 ml Eppendorf tube. Afterwards, the liquid was mixed with 500 µl of 0,67% Thiobarbituric Acid (TBA) and kept on Thermoblock (100 ºC) for 15 minutes. Finally, 200 µl of each sample in triplicates were pipetted in ELISA plate and the absorbance was measured at 531 nm.

5.6. Statistical analyses

Data were expressed as mean ± S.E.M. The body weight gain, Area Under Curve (AUC) of body weight gain, liver weight, liver biochemical related parameters, qPCR and MDA comparisons were performed by using two-way ANOVA with DMS post-hoc analysis. The significative differences were expressed + $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$, or # $p < 0.0001$ STD-VH versus CAF-VH; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, CAF-VH versus CAF-GSPE; $ $p < 0.05$, $$ $p < 0.01$ and $$$ $p < 0.001$ photoperiod effect. All statistical analyses were carried out using IBM SPSS Statistics 26 software and graphics were done by GraphPad Prism 8 software.

6. RESULTS

6.1. Body weight gain and biochemical liver parameters validate the obesogenic effect of cafeteria diet

In the 3 photoperiods it could be seen that there was an effect of diet on body weight gain since there were significant differences in all weeks. The CAF worked due to the difference in weight gain between CAF-rats and STD-rats *(Figure 6a, 6b* and *6c)*. Regarding GSPE, a significant effect was only seen at photoperiod L18 *(Figure 6a)*. It could be intuited that GSPE acted ameliorating the effect of the cafeteria diet, since CAF-rats treated gained less weight than CAF-rats without treatment. On the other hand, after analyzing the results at L12 *(Figure 6b)* and L6 *(Figure 6c)* photoperiods, no effect of GSPE was observed, as there was no significant difference between the body weight gain of CAF-GSPE rats and CAF-VH rats.

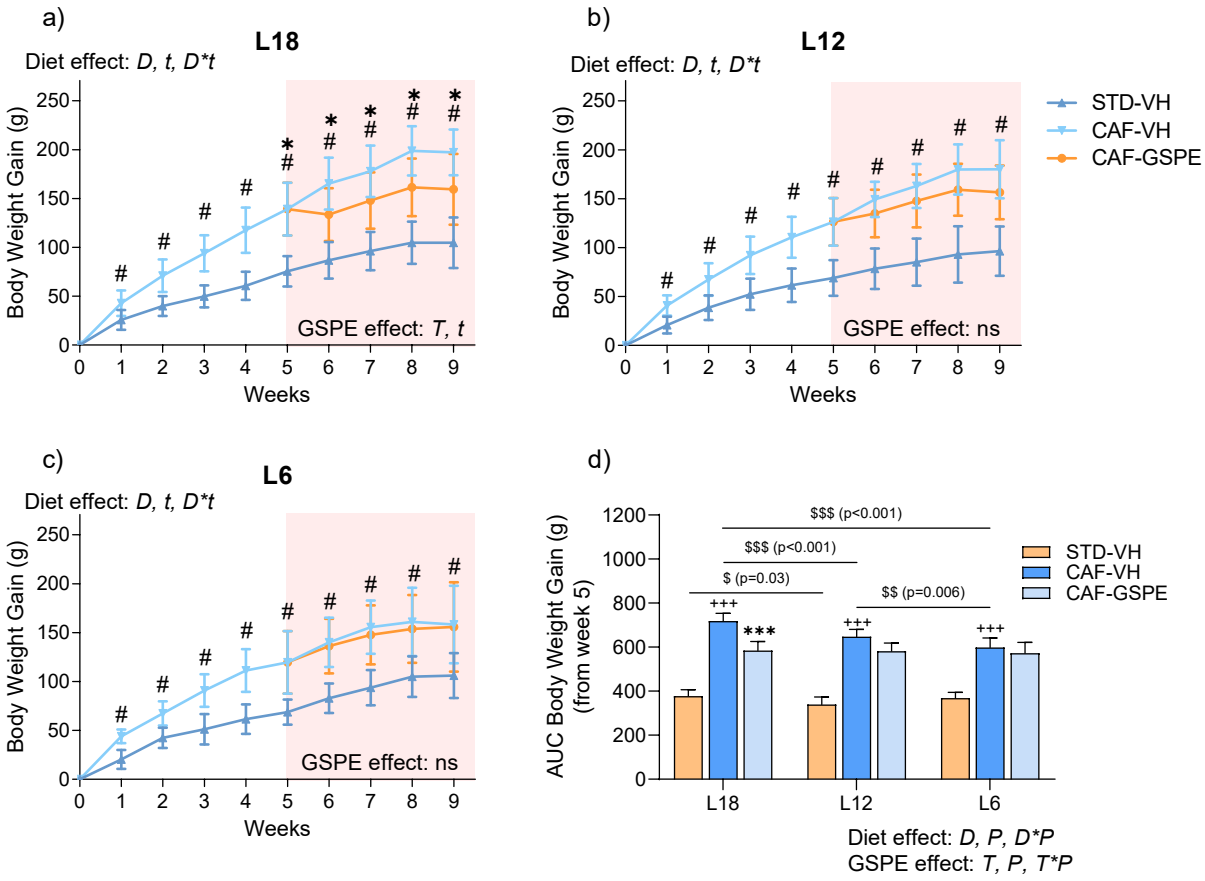

Figure 6: *Body weight gain during the nine weeks of experiment. a) Body weight gain in grams of L18 rats during the nine weeks of experiment. b) Body weight gain in grams of L12 rats during the nine weeks of experiment. c) Body weight gain in grams of L6 rats during the nine weeks of experiment. d) Area Under Curve (AUC) of Body weight gain during the four weeks of treatment with GSPE. Data are represented as mean ± Standard Deviation, SD (figures 6a, 6b and 6c) or mean ± Standard Error of Mean, SEM (Figure*

*6d). # means significant differences between STD-VH and CAF-VH groups (p ≤ 0.0001) using repeated measures ANOVA followed by Student's T-Test. * indicates significant differences between CAF-VH and CAF-GSPE (p < 0.05) using repeated measures ANOVA followed by Student's T-test for each week from the fifth week of the experiment. +++ means significant differences between STD-VH and CAF-VH (p < 0.001) using 2 way-ANOVA followed by a LSD post-hoc test. *** means significant differences between CAF-VH and CAF-GSPE (p < 0.001) using 2 way-ANOVA followed by a LSD post-hoc test. $ means significant differences (p < 0.05) between the same group; $$, p < 0.01; $$$, p < 0.001. D, Diet effect; T, Treatment effect; t, time effect; P, photoperiod effect; D*P, interaction between Diet and Photoperiod effect; T*P, interaction between Treatment and Photoperiod effect; ns, no significant differences.*

After making the two-way ANOVA, it was observed that for the 3 photoperiods *(Figure 6a, 6b* and *6c)* there was an effect by diet, by treatment, and also an effect by the interaction of the two factors different from the analysis of the factors separately.

In addition, the analysis of the AUC from the first week of treatment (week 5) was also performed. In *Figure 6d* it could be confirmed that there was an effect of diet in the 3 photoperiods on the weight gain of these rats, since there were significant differences between the group fed with STD diet and the group with CAF in each of the photoperiods. In case of GSPE, an effect at L18 can be observed because of the significant differences between CAF-GSPE rats and untreated CAF rats *(Figure 6d),* not being so in the other photoperiods.

6.2. Intake of obesogenic diet alters liver weight, TAG, and cholesterol levels in liver while GSPE effect is also observed

Firstly, significant differences were observed between STD-VH and CAF-VH rats with respect to liver weight *(Figure 7a)*, demonstrating a diet effect. On the other hand, although there are no differences of GSPE treatment in CAF rats within the same photoperiod, when compared by 2-way ANOVA, there is a significant effect produced by GSPE, which makes liver weight become lower in treated rats than in untreated rats.

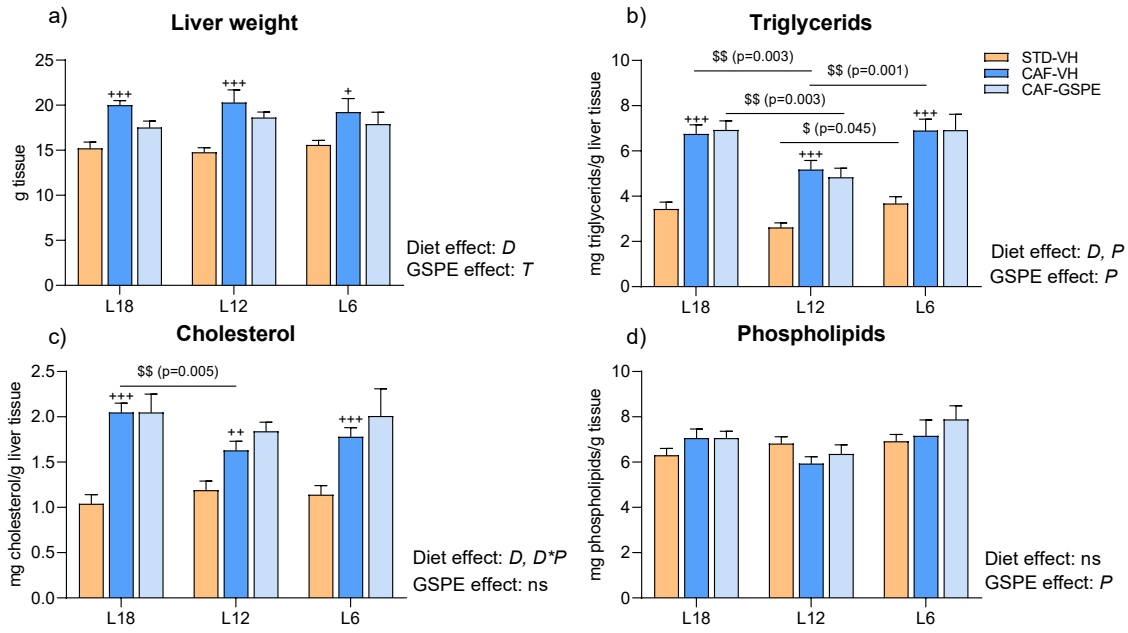

Figure 7: *Liver biochemical parameters. a) Liver weight in grams in L18, L12 and L6 photoperiods. b) Triglyceride levels in mg per grams of tissue in L18, L12 and L6 photoperiods. c) Cholesterols level in mg per grams of tissue in L18, L12 and L6 photoperiods. c) Phospholipid levels in mg per grams of tissue in L18, L12 and L6 photoperiods. Data are represented as mean ± Standard Error of Mean, SEM. + means significant differences between STD-VH and CAF-VH ($p < 0.05$) using 2 way-ANOVA followed by a LSD post-hoc test; ++, $p < 0.01$; +++, $p < 0.001$. *** means significant differences between CAF-VH and CAF-GSPE ($p < 0.001$) using 2 way-ANOVA followed by a LSD post-hoc test. $ means significant differences ($p < 0.05$) between the same group; $$, $p < 0.01$. D, Diet effect; T, Treatment effect; t, time effect; P, photoperiod effect; D*P, interaction between Diet and Photoperiod effect; T*P, interaction between Treatment and Photoperiod effect; ns, no significant differences.*

In the case of the triglyceride profile *(Figure 7b)*, a diet effect was also observed due to significant differences between STD-VH and CAF-VH rats. These results could be contrasted with those of body weight gain *(Figure 6d)* and liver weight *(Figure 7a)*, as an increase in weight of CAF rats could be partially due to an increase in their liver weight, and this increase in turn due to TAG accumulation. Apart from this, when STD-VH and CAF-VH groups are compared, a photoperiod effect was also observed, as significant differences were shown between L18 and L12 CAF rats and between L12 and L6 CAF rats. Taking this into account, a significant difference by photoperiod was also observed between GSPE-treated rats at L18 and L6. Altogether these results corroborated that the L12 photoperiod had a lower TAG profile than the other two photoperiods.

Regarding cholesterol levels *(Figure 7c)*, a diet effect could again be observed, due to significant differences between CAF-VH and STD-VH rats in all photoperiods. In addition, there was also a photoperiod effect at the diet level, as significant changes

were shown between L18 and L12 CAF-rats. After carrying out the two-way ANOVA, it was shown that there was an effect by the interaction of diet and photoperiod different from the analysis of the factors separately. However, an effect by GSPE was not visualized.

At the phospholipid level *(Figure 7d)*, there were no significant changes between groups despite a differential effect by photoperiod.

6.3. MDA analysis demonstrates neither a dietary disruption nor an ameliorating effect of the GSPE

In *Figure 8*, it could be observed that in L12 there was a significantly higher level of lipid peroxidation in CAF-rats compared to STD-rats, reinforcing the detrimental effect of CAF. A photoperiod effect could also be intuited by differences between L18 and L12 CAF rats, with L12 rats having higher lipid peroxidation. In addition, the analysis performed with two-way ANOVA showed that there was an effect by the interaction of photoperiod and diet.

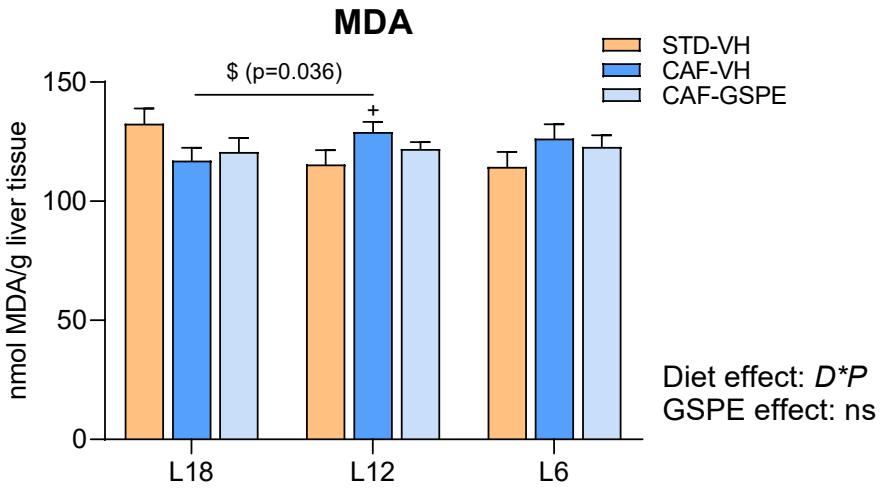

Figure 8. *Lipid peroxidation analysis. Data are represented as mean ± Standard Error of Mean, SEM. + means significant differences between STD-VH and CAF-VH ($p < 0.05$) using 2 way-ANOVA followed by a LSD post-hoc test. $ means significant differences ($p < 0.05$) between the same group. D, Diet effect; T, Treatment effect; t, time effect; P, photoperiod effect; D*P, interaction between Diet and Photoperiod effect; T*P, interaction between Treatment and Photoperiod effect; ns, no significant differences.*

With respect to GSPE, no significant differences were seen either between groups or between photoperiods.

6.4. Cafeteria diet and photoperiod changes have not been enough to cause significant oxidative stress

According to two-way ANOVA, there was an effect by diet and by photoperiod on SOD1 expression *(Figure 9a)*, as notable differences were seen between STD-VH rats and CAF-VH rats, especially at L12. In addition, an effect by photoperiod was also seen in the diet, as there were significant differences between L18 CAF-rats and both L12 and L6 CAF-rats.

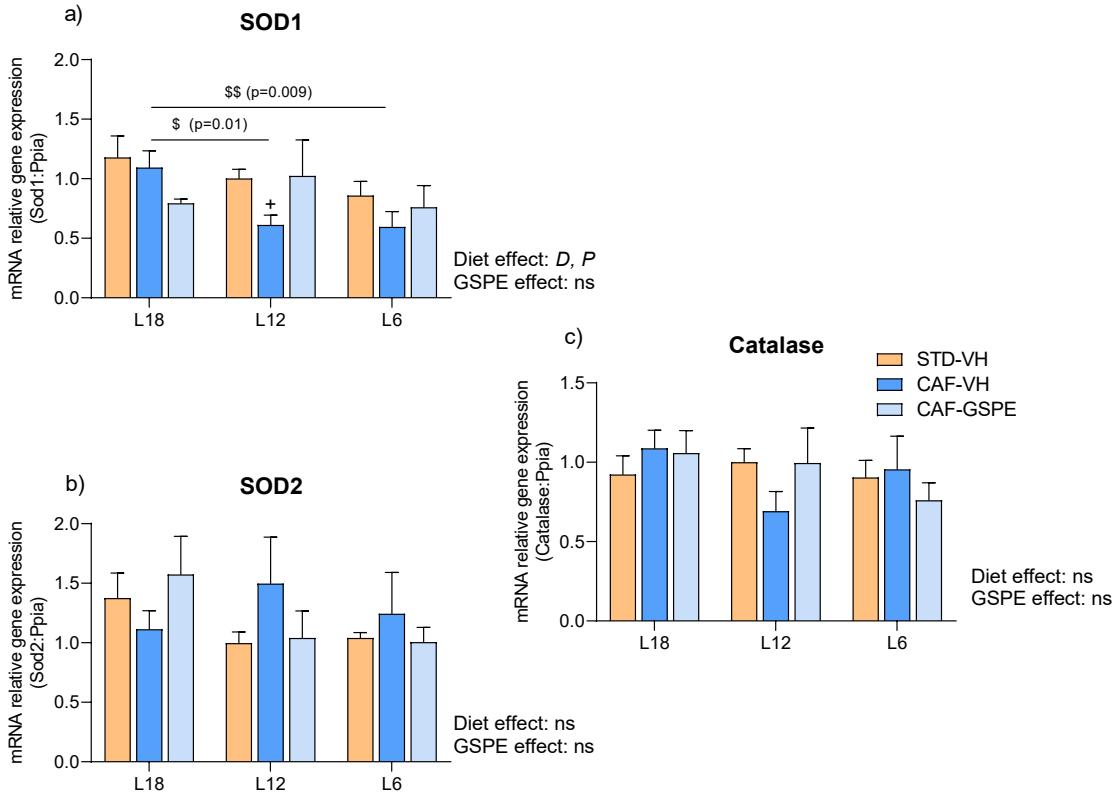

Figure 9: *Relative gene expression of antioxidant defense system genes in the liver. a) Relative gene expression of SOD1. b) Relative gene expression of SOD2. c) Relative gene expression of Catalase. Data are represented as mean ± Standard Error of Mean, SEM. + means significant differences between STD-VH and CAF-VH ($p < 0.05$) using 2 way-ANOVA followed by a LSD post-hoc test. $ means significant differences ($p < 0.05$) between the same group; $$, $p < 0.01$. D, Diet effect; T, Treatment effect; t, time effect; P, photoperiod effect; D*P, interaction between Diet and Photoperiod effect; T*P, interaction between Treatment and Photoperiod effect; ns, no significant differences.*

On the other hand, no significant effect was observed by GSPE, although the expression of the 3 genes *(Figure 9a, 9b* and *9c)* in treated CAF-rats was higher than untreated CAF-rats. This pattern is repeated in L12 and L6, but not in L18, where CAF-GSPE rats had a lower expression of the genes than CAF-VH rats.

Despite this fact, no significant differences in the expression of SOD1, SOD2 and Catalase genes were observed.

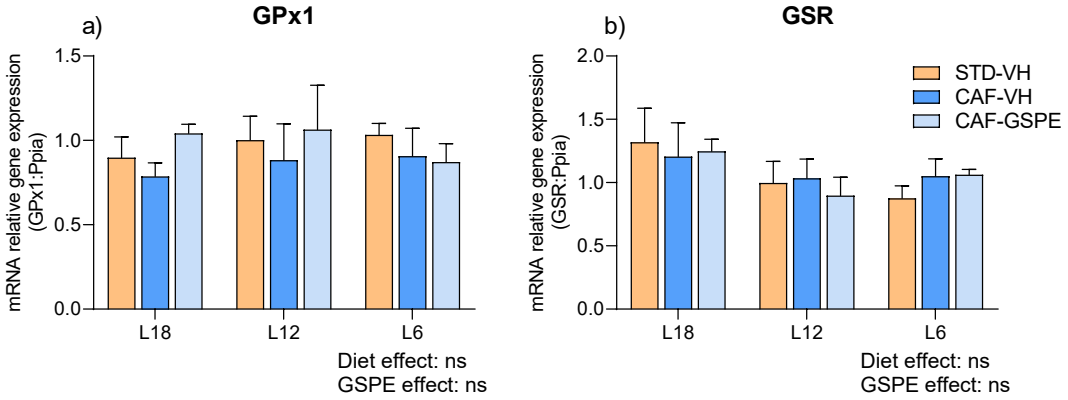

***Figure 10**: Genes related to the GSH cycle. a) Relative gene expression of GPx1. b) Relative gene expression of GSR. Data are represented as mean ± Standard Error of Mean, SEM. No statistical differences were found between groups.*

No significant changes were shown either between groups or between photoperiods in the expression of each gene, so no effect of diet or photoperiod could be intuited. Interestingly, although there were no significant changes between untreated and GSPE-treated rats, a pattern in the expression of the two genes was intuited: GSPE-treated rats tended to have a higher expression of the two genes *(Figure 10a* and *10b)* than untreated CAF rats, and even higher than STD rats at most photoperiods.

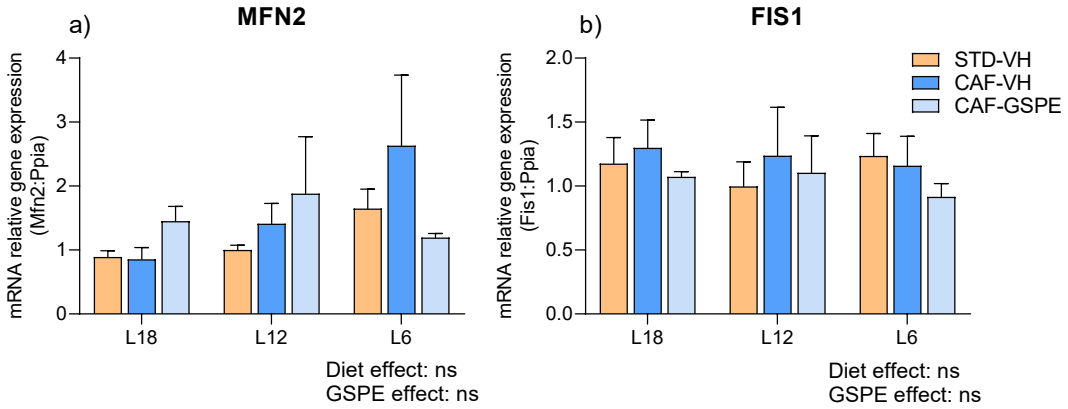

***Figure 11**: Mitochondrial dynamics genes - Fusion and fission. a) Relative gene expression of MFN2. b) Relative gene expression of FIS1. Data are represented as mean ± Standard Error of Mean, SEM. No statistical differences were found between groups.*

Although there were no significant changes that demonstrate an effect of diet or photoperiod on the expression of these two genes *(Figure 11a* and *11b)*, it seemed that in most photoperiods there was a higher expression of the genes in CAF-rats compared to STD-rats. On the other hand, it also seemed that CAF-GSPE rats had a higher expression than CAF-VH rats, although this was not true for all photoperiods. These patterns changed in both L6 of MFN2 *(Figure 11a)* and FIS1 *(Figure 11b)* expression.

7. DISCUSSION

It is undoubtedly that cafeteria diet is associated with the development of obesity and MetS, and specifically in the liver, with the development of oxidative stress and NAFLD [35]. In this study, a diet supplemented with grape polyphenols demonstrated the ability of proanthocyanidins to ameliorate the effects of a western diet (high in fats), lowering lean and fat mass, body weight, and hepatic steatosis. Additionally, CAF and metabolic diseases are linked with oxidative stress disruption, which polyphenols are also capable of modulating, as demonstrated by Pérez-Torres et al [36]. The authors consider that these phytochemicals have the potential for inducing weight loss and have been included in dietary strategies to abolish oxidative stress to prevent obesity.

CAF provides a highly relevant model in terms of examining the human diet in rodents, since the groups fed with this diet have an increase of body weight gain (*Figures 6a, 6b, 6c* and *6d*), liver weight (*Figure 7a*), and an increase in the liver of TAG and cholesterol levels (*Figures 7b* and *7c* respectively); this is consistent with the results of some studies of mice fed with CAF or/and high fat diet (HF) [37][38]. Rats fed with CAF exhibited voluntary hyperphagia and elevated fat intake which resulted in dramatic and rapid weight gain, as shown in *Figure 6d*.

In these studies, it is also remarked the role of CAF in the formation of NAFLD and, in most cases, in the progression of this disease to NASH, which is responsible for the inflammation of the liver and the development of these hepatic conditions. However, a NASH profile is not achieved with a Western-style diet until week 16 of the experiment, or at least in mice model. [39]. This diet is also associated with the appearance of inflammation of white and brown adipose tissue, as well as a MetS profile with insulin resistance and elevated blood glucose. After measuring lipid profile in the liver, no significant difference has been seen with respect to phospholipids, as shown in *Figure 7d*. This may be because CAF does not have a significant impact on the phospholipid level of the liver, as shown in a 2020 study published by Mašek et al [40]. However, in this study it is demonstrated that HF increases the level of hepatic phospholipids, so we can assume that no significant differences in phospholipid levels have been found because CAF and HF do not really have the same nutritional composition, which may have an influence at the metabolic level.

Another factor to study in this experiment is the effect of seasonal changes, since it is known that animals are season-sensitive and part of our behavior, metabolism, etc. can

change according to the seasons [25]. It is clear that diet has a strong effect on body weight gain, and it has been shown that there are also significant differences between photoperiods. This may be because the metabolic state of mammals also changes with respect to the seasons of the year [27], so that a differential effect by photoperiod can be observed both in body weight gain (*Figures 6a, 6b, 6c* and *6d*) and in TAG and cholesterol levels (*Figures 7a* and *7c*). However, no effect of seasonality was seen in oxidative stress generation, as no significant differences were seen in the expression of the different oxidative stress genes between any of the photoperiods (*Figures 9a, 9b, 9c, 10a, 10b, 11a* and *11b)*.

On the other hand, there is a clear effect of GSPE in increasing TAG decomposition reducing food intake and fat deposition, suggesting that consumption of GSPE can reduce obesity development and related metabolic pathways [41]. If we focus on body weight gain (*Figures 6a* and *6d*), a significant effect is only observed for GSPE at L18, ameliorating the effect of the CAF. This translates into GSPE-treated CAF rats having a lower body weight gain than untreated CAF rats, seeing one effect per treatment and photoperiod. In *Figure 7a*, an influence by GSPE treatment on liver weight is also appreciated, seeing an ameliorating effect of CAF diet. However, this does not happen in *Figure 7c* on cholesterol levels. In addition, in TAG and phospholipid levels (*Figures 7b* and *7d*), an effect by photoperiod can be seen with respect to GSPE. The variation in the effect of GSPE due to changes in photoperiod may be due to the fact that the bioavailability of polyphenols, including both metabolization and absorption, in the same fruit varies according to the seasons of the year and the time of day at which the polyphenols are administered [42]. In the case of grapes, from which GSPE is extracted, there is a different bioavailability and bioactivity of proactocyanidins depending on the season of the year and time of day, so it has been seen that there is a significant effect on L18 by GSPE with respect to body weight gain (*Figures 6a* and *6d*). On the other hand, significant differences are also seen for the effect of GSPE on TAG and phospholipid levels due to changes in photoperiod (*Figures 7b* and *7d*). In a study where organic and inorganic red grape extracts were analyzed, it could be shown that there were many factors that influenced the availability of polyphenols in the same fruit and, therefore, this then affected their bioactivity [43]. Another study published by Escobar-Martínez et al [44], showed that the mice treated during its resting phase with cherry out of season had a higher average concentration of total phenolic compounds compared to those treated during the active phase. This explains on the one hand, that fruit has different bioavailability of its polyphenols depending on whether it is in season or not,

and on the other hand, that the time of day at which these polyphenols are administered can also affect the metabolic level later on.

MDA (malondialdehyde) is one of oxidative stress biomarkers, since this is a product of lipid peroxidation used to measure oxidative stress in physiological conditions. The expected results were to find an increase of MDA in rats fed with CAF compared to STD rats, although what was obtained was that there were no significant differences between these two groups (*Figure 8*). So, the cause of increased free radicals formation and consequently of enhanced lipid peroxidation in obesity cannot be deduced from this study. This may be due to the time the animals have been subjected to the experiment, since, in a study published by Barroso et al [45], it is demonstrated that in feeding the animals with HF for 13 weeks, there is an increase of MDA in the liver and, therefore, of lipid peroxidation in these rats.

As it is said before, obesity and MetS are also related with oxidative stress because of the imbalance between the generation of free radicals and the antioxidant defense systems. Inflammation and the excessive generation of free radicals in cells induces the activation of defense mechanisms, such as antioxidant enzymes which protect the cells against oxidative stress. What was expected in this study was to find a decrease in the expression of oxidative stress genes in the liver, generated by CAF effect, as shown in articles published by or Zhang et al. in 2021 [46]. However, and as seen in our study in *Figures 9a, 9b, 9c, 10a* and *10b* no significant differences were found in the expression of oxidative stress SOD2, Catalase, GxP1 and GSR genes. Another gene that has been analyzed in this experiment is SOD1, the expression of which has been shown to be affected by diet and photoperiod, as we can see in *Figure 9a*. The effect of diet on SOD1 expression would make sense, as the environment of ROS and inflammation caused by CAF could cause it to decrease antioxidant defense by decreasing SOD1 expression, as demonstrated in a study published in 2019 by Wang et al [47].

In addition to the genes mentioned above, the expression of the mitochondrial fusion gene MFN2 (*Figure 11a*) and the mitochondrial fission gene FIS1 (*Figure 11b*) has also been studied. Fusion and fission processes are necessary to maintain healthy mitochondria, so it is very important to have controlled mitochondrial dynamics. On one side, some authors suggest overexpression of MFN2 causes mitochondrial dysfunction and cell death [48]. However, other authors demonstrate that overexpression of MFN2 improves liver fibrosis in hepatic stellate cells of mice [49]. On the other hand, several studies demonstrated that high-fat-diet-induced obesity mice showed overexpression of

FIS1, whereas inhibition or disruption of these genes led to an improvement in insulin resistance and mitochondrial dysfunction in skeletal muscle [50]. Because of these reports, what was expected from this study was that CAF would generate an increase in the expression of these two genes. However, no significant differences were found in this experiment, as it is shown in *Figure 11a* and *11b*. A 9-week experiment by Rodríguez et al. [51], showed overexpression of MFN2 and FIS1 genes caused by CAF. Nonetheless, our results may not be similar to this study because in our experiment the animals were not sacrificed at different times of the day, whereas in the aforementioned study they were. Therefore, we can intuit that this factor influences the higher or lower expression of these genes. Some studies reported the appearance of oxidative stress after 12 weeks [52] or 16 weeks [47] of diet containing 60% of energy intake provided by fat. Everything together leads us to think that the time over which the experiment was conducted was not long enough to cause significant oxidative stress in the liver of the rats studied, or that the time of slaughter of the animals is a factor to be taken into account when measuring this stress.

Moreover, we wanted to see if GSPE was able to ameliorate the oxidative stress caused by CAF or photoperiod changes. Since no significant oxidative stress has been found, the effect of GSPE in the treated rats compared to the untreated rats could not be seen either. There are several studies that showed some foods or substances able to ameliorate oxidative stress induced by CAF or HF. Some examples are lycopene [53], apple cider vinegar [54] and resveratrol [55]. In 2017, an experiment published by Gil-Cardoso et al. [56], showed that chronic supplementation with dietary proanthocyanidins (GSPE) protects from diet-induced intestinal alterations in obese rats [56]. All these studies reported that CAF or HF have worked and that there were some substances capable of lower oxidative stress. However, our results cannot corroborate what is said in the previous reports because no significant oxidative stress has been found, as it is shown in *Figures 9a, 9b, 9c, 10a, 10b, 11a* and *11b.*

After analyzing our results, has been seen that both the CAF and changes in the photoperiod have a differential effect at the metabolic level being ameliorated by GSPE. Thus, it is deduced that GSPE can be a bioactive compound with a certain potential in restoring metabolic homeostasis, as shown by the results on body weight gain (*Figures 6a* and *6b*), liver weight (*Figure 7a*) and TAG and cholesterol levels (*Figure 7b* and *7c* respectively).

On the other hand, CAF did not have the expected effect in terms of inflammation and oxidative stress, and therefore, the effect of GSPE in ameliorating this oxidative stress

by decreasing MDA and increasing the expression of antioxidant defense genes could not be demonstrated.

8. CONCLUSIONS

Firstly, regarding to the first 2 objectives, CAF was validated to be effective to dysregulate the metabolic homeostasis via increasing body weight gain, liver weight and lipid profile. Changes in photoperiod have also been shown to affect metabolism, resulting in differences in weight gain, TAG and cholesterol. Nevertheless, this diet or photoperiod changes were not enough to generate a significant oxidative stress.

Secondly, regarding to the last 2 objectives, the GSPE's ability to reverse the photoperiod or CAF- induced dysregulation was validated in some cases. It must be pointed that:

• GSPE was able to decrease body weight gain in L18.

• GSPE had an effect in L12 decreasing TAG level more than in the other two photoperiods.

• GSPE was capable of ameliorate CAF effect decreasing liver weight.

All in all, the main conclusion is that CAF and photoperiod changes generate dysregulation at the metabolic level, which can be restored by the GSPE. However, as no major impact of diet or seasonal variations at the level of oxidative stress has been seen, it cannot be confirmed that GSPE ameliorates this dysregulation.

9. REFERENCES

[1] F. Bonomini, L. F. Rodella and R. Rezzani, “Metabolic syndrome, aging and involvement of oxidative stress,” *Aging and Disease*, vol. 6, no. 2, pp. 109–120, 2015, doi: 10.14336/AD.2014.0305.

[2] D. H. Sherling, P. Perumareddi and C. H. Hennekens, “Metabolic Syndrome: Clinical and Policy Implications of the New Silent Killer,” *Journal of Cardiovascular Pharmacology and Therapeutics*, vol. 22, no. 4, pp. 365–367, Jul. 2017, doi: 10.1177/1074248416686187.

[3] A. Bovolini, J. Garcia, M. A. Andrade and J. A. Duarte, “Metabolic Syndrome Pathophysiology and Predisposing Factors,” *International Journal of Sports Medicine*, vol. 42, no. 3, pp. 199–214, Mar. 2021, doi: 10.1055/A-1263-0898/ID/R8170-0026.

[4] B. Balkau, M.Vernay, L. Mhamdi, M. Novak, D. Arondel, S. Vol, J. Tichet and E. Eschwege, “The incidence and persistence of the NCEP (National Cholesterol Education Program) metabolic syndrome. The French D.E.S.I.R. study,” *Diabetes and Metabolism*, vol. 29, no. 5, pp. 526–532, 2003, doi: 10.1016/S1262-3636(07)70067-8.

[5] E. E. Powell, V. W. S. Wong and M. Rinella, “Non-alcoholic fatty liver disease,” *The Lancet*, vol. 397, no. 10290, pp. 2212–2224, Jun. 2021, doi: 10.1016/S0140-6736(20)32511-3.

[6] Z. M. Younossi, “Non-alcoholic fatty liver disease - A global public health perspective,” *J Hepatol*, vol. 70, no. 3, pp. 531–544, Mar. 2019, doi: 10.1016/J.JHEP.2018.10.033.

[7] Z. Younossi, Q. M. Anstee, M. Marietti, T. Hardy, L. Henry, M. Eslam, J.George and E. Bugianesi, “Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention,” *Nat Rev Gastroenterol Hepatol*, vol. 15, no. 1, pp. 11–20, Jan. 2018, doi: 10.1038/NRGASTRO.2017.109.

[8] L. Xu, N. Nagata and T. Ota, “Impact of Glucoraphanin-Mediated Activation of Nrf2 on Non-Alcoholic Fatty Liver Disease with a Focus on Mitochondrial Dysfunction,” *International Journal of Molecular Sciences 2019, Vol. 20, Page 5920*, vol. 20, no. 23, p. 5920, Nov. 2019, doi: 10.3390/IJMS20235920.

[9] E. Muzurović, D. P. Mikhailidis and C. Mantzoros, “Non-alcoholic fatty liver disease, insulin resistance, metabolic syndrome and their association with vascular risk,” *Metabolism*, vol. 119, Jun. 2021, doi: 10.1016/J.METABOL.2021.154770.

[10] N. Stefan, H. U. Häring and K. Cusi, “Non-alcoholic fatty liver disease: causes, diagnosis, cardiometabolic consequences, and treatment strategies,” *Lancet Diabetes Endocrinol*, vol. 7, no. 4, pp. 313–324, Apr. 2019, doi: 10.1016/S2213-8587(18)30154-2.

[11] S. M. Aljunid, “Obesity, a Costly Epidemic,” *Laparoscopic Sleeve Gastrectomy*, pp. 13–22, 2021, doi: 10.1007/978-3-030-57373-7_2.

[12] Z. Zhang, V. Mocanu, C.Cai, J. Dang, L. Slater, E. C. Deehan, J. Walter and K. Madsen, “Impact of Fecal Microbiota Transplantation on Obesity and Metabolic Syndrome—A Systematic Review,” *Nutrients*, vol. 11, no. 10, p. 2291, Oct. 2019, doi: 10.3390/NU11102291.

[13] I. Pérez-Torres, V. Castrejón-Téllez, M. E. Soto, M. E. Rubio-Ruiz, L. Manzano-Pech and V. Guarner-Lans, “Oxidative Stress, Plant Natural Antioxidants, and Obesity,”

International Journal of Molecular Sciences, vol. 22, no. 4, pp. 1–26, Feb. 2021, doi: 10.3390/IJMS22041786.

[14] H. J. Forman and H. Zhang, “Targeting oxidative stress in disease: promise and limitations of antioxidant therapy,” *Nature Reviews. Drug Discovery*, vol. 20, no. 9, p. 689, Sep. 2021, doi: 10.1038/S41573-021-00233-1.

[15] R. Zhu, Y. Wang, L. Zhang, and Q. Guo, “Oxidative stress and liver disease,” *Hepatology Research*, vol. 42, no. 8, pp. 741–749, Aug. 2012, doi: 10.1111/J.1872-034X.2012.00996.X.

[16] G. Pizzino, N. Irrera, M. Cucinotta, G. Pallio, F. Mannino, V. Arcoraci, F. Squadrito, D. Altavilla and A. Bitto, “Oxidative Stress: Harms and Benefits for Human Health,” *Oxidative Medicine and Cellular Longevity*, vol. 2017, 2017. doi: 10.1155/2017/8416763.

[17] M. Sharifi-Rad, N. V. Anil Kumar, P. Zucca, E. M. Varoni, L. Dini, E. Panzarini, J. Rajkovic, P. V. Tsouh Fokou, E. Azzini, I. Peluso, A. Prakash Mishra, M. Nigam, Y. El Rayess, M. El Beyrouthy, L. Polito, M. Iriti, N. Martins, M. Martorell, A. O. Docea, W. N. Setzer, D. Calina, W. C. Cho and J. Sharifi-Rad22, “Lifestyle, Oxidative Stress, and Antioxidants: Back and Forth in the Pathophysiology of Chronic Diseases,” *Frontiers in Physiology*, vol. 11, Jul. 2020, doi: 10.3389/FPHYS.2020.00694.

[18] J. F. Keaney, M. G. Larson, R. S. Vasan, P. W. F. Wilson, I. Lipinska, D. Corey, J. M. Massaro, P. Sutherland, J. A. Vita and E. J. Benjamin, “Obesity and systemic oxidative stress: Clinical correlates of oxidative stress in the Framingham study,” *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 3, pp. 434–439, Mar. 2003, doi: 10.1161/01.ATV.0000058402.34138.11.

[19] H. K. Vincent, C. M. Bourguignon and A. G. Taylor, “Relationship of the Dietary Phytochemical Index to Weight Gain, Oxidative Stress and Inflammation in Overweight Young Adults,” *J Hum Nutr Diet*, vol. 23, no. 1, p. 20, Feb. 2010, doi: 10.1111/J.1365-277X.2009.00987.X.

[20] M. Litwin and Z. Kułaga, “Obesity, metabolic syndrome, and primary hypertension,” *Pediatr Nephrol*, vol. 36, no. 4, pp. 825–837, Apr. 2021, doi: 10.1007/S00467-020-04579-3.

[21] A. W. C. Man, H. Li and N. Xia, “Impact of Lifestyles (Diet and Exercise) on Vascular Health: Oxidative Stress and Endothelial Function,” *Oxidative Medicine and Cellular Longevity*, vol. 2020, 2020, doi: 10.1155/2020/1496462.

[22] S. Seen, “Chronic liver disease and oxidative stress – a narrative review,” *Expert Review of Gastroenterology & Hepatology*, vol. 15, no. 9, pp. 1021–1035, 2021, doi: 10.1080/17474124.2021.1949289.

[23] A. J. Kattoor, N. V. K. Pothineni, D. Palagiri and J. L. Mehta, “Oxidative Stress in Atherosclerosis,” *Curr Atheroscler Rep*, vol. 19, no. 11, Nov. 2017, doi: 10.1007/S11883-017-0678-6.

[24] J. Yang, M. Fernández-Galilea, L. Martínez-Fernández, P. González-Muniesa,1, A. Pérez-Chávez, J. A. Martínez and M. J. Moreno-Aliaga, “Oxidative Stress and Non-Alcoholic Fatty Liver Disease: Effects of Omega-3 Fatty Acid Supplementation,” *Nutrients*, vol. 11, no. 4, Apr. 2019, doi: 10.3390/NU11040872.

[25] R. Mariné-Casadó, C. Domenech-Coca, J. M. del Bas, C. Bladé, A. Caimari and L. Arola, “Cherry consumption out of season alters lipid and glucose homeostasis in normoweight

and cafeteria-fed obese Fischer 344 rats," *Journal of Nutritional Biochemistry*, vol. 63, pp. 72–86, Jan. 2019, doi: 10.1016/j.jnutbio.2018.09.013.

[26] J. P. Pell and S. M. Cobbe, "Seasonal variations in coronary heart disease," *QJM*, vol. 92, no. 12, pp. 689–696, 1999, doi: 10.1093/QJMED/92.12.689.

[27] R. Mariné-Casadó, C. Domenech-Coca, J. M. del Bas, C. Bladé, L. Arola and A. Caimari, "The exposure to different photoperiods strongly modulates the glucose and lipid metabolisms of normoweight fischer 344 rats," *Frontiers in Physiology*, vol. 9, p. 416, Apr. 2018, doi: 10.3389/fphys.2018.00416.

[28] C. G. Fraga, K. D. Croft, D. O. Kennedy and F. A. Tomás-Barberán, "The effects of polyphenols and other bioactives on human health," *Food & Function*, vol. 10, no. 2, pp. 514–528, Feb. 2019, doi: 10.1039/C8FO01997E.

[29] K. Chojnacka, D. Skrzypczak, G. Izydorczyk, A. Witek-Krowiak, K. Mikula and D. Szopa, "Antiviral Properties of Polyphenols from Plants," *Foods*, vol. 10, no. 10, p. 2277, Sep. 2021, doi: 10.3390/FOODS10102277.

[30] A. Rauf, M. Imran, T. Abu-Izneid, Iahtisham-Ul-Haq, S. Patel, X. Pan, S. Naz, Ana. S. Silva, F. Saeed and H. A. R. Suleriaj, "Proanthocyanidins: A comprehensive review," *Biomedicine & Pharmacotherapy*, vol. 116, p. 108999, Aug. 2019, doi: 10.1016/J.BIOPHA.2019.108999.

[31] M. Liu, P. Yun, Y. Hu, J. Yang, R. B. Khadka and X. Peng, "Effects of Grape Seed Proanthocyanidin Extract on Obesity," *Obesity Facts*, vol. 13, no. 2, p. 279, May 2020, doi: 10.1159/000502235.

[32] S. A. Rajput, L. Sun, N. Zhang,1 M. M. Khalil, Z. Ling, L. Chong, S. Wang, I. R. Rajput, D. M. Bloch, F. A. Khan, A. Shaukat and D. Qi, "Grape Seed Proanthocyanidin Extract Alleviates AflatoxinB1-Induced Immunotoxicity and Oxidative Stress via Modulation of NF-κB and Nrf2 Signaling Pathways in Broilers," *Toxins (Basel)*, vol. 11, no. 1, p. 23, Jan. 2019, doi: 10.3390/TOXINS11010023.

[33] R. Nallathambi, A. Poulev, J. B. Zuk and I. Raskin, "Proanthocyanidin-Rich Grape Seed Extract Reduces Inflammation and Oxidative Stress and Restores Tight Junction Barrier Function in Caco-2 Colon Cells," *Nutrients*, vol. 12, no. 6, Jun. 2020, doi: 10.3390/NU12061623.

[34] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001, doi: 10.1006/METH.2001.1262.

[35] E. Mezhibovsky, K. A. Knowles, Q. He, K. Sui, K. M. Tveter, R. M. Duran and D. E. Roopchand, "Grape Polyphenols Attenuate Diet-Induced Obesity and Hepatic Steatosis in Mice in Association With Reduced Butyrate and Increased Markers of Intestinal Carbohydrate Oxidation," *Frontiers in Nutrition*, vol. 8, p. 675267, Jun. 2021, doi: 10.3389/FNUT.2021.675267/FULL.

[36] I. Pérez-Torres, V. Castrejón-Téllez, M. E. Soto, M. E. Rubio-Ruiz, L. Manzano-Pech and V. Guarner-Lans, "Oxidative Stress, Plant Natural Antioxidants, and Obesity," *International Journal of Molecular Sciences*, vol. 22, no. 4, pp. 1–26, Feb. 2021, doi: 10.3390/IJMS22041786.

[37] N. Zeeni, C. Dagher-Hamalian, H. Dimassi and W. H. Faour, “Cafeteria diet-fed mice is a pertinent model of obesity-induced organ damage: a potential role of inflammation,” *Inflamm Res*, vol. 64, no. 7, pp. 501–512, Jul. 2015, doi: 10.1007/S00011-015-0831-Z.

[38] B. P. Sampey, A. M. Vanhoose, H. M. Winfield, A. J. Freemerman, M. J. Muehlbauer, P. T. Fueger, C. B. Newgard and L. Makowski, “Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet,” *Obesity (Silver Spring)*, vol. 19, no. 6, pp. 1109–1117, Jun. 2011, doi: 10.1038/OBY.2011.18.

[39] J. M. Eng and J. L. Estall, “Diet-Induced Models of Non-Alcoholic Fatty Liver Disease: Food for Thought on Sugar, Fat, and Cholesterol,” *Cells*, vol. 10, no. 7, Jul. 2021, doi: 10.3390/CELLS10071805.

[40] T. Mašek, J. Barišić, V. Micek and K. Starčević, “Cafeteria Diet and High-Fructose Rodent Models of NAFLD Differ in the Metabolism of Important PUFA and Palmitoleic Acid without Additional Influence of Sex,” *Nutrients*, vol. 12, no. 11, pp. 1–12, Nov. 2020, doi: 10.3390/NU12113339.

[41] M. Liu, P. Yun, Y. Hu, J. Yang, R. B. Khadka and X. Peng, “Effects of Grape Seed Proanthocyanidin Extract on Obesity,” *Obes Facts*, vol. 13, no. 2, pp. 279–291, May 2020, doi: 10.1159/000502235.

[42] A. Arola-Arnal, Á. Cruz-Carrión, C. Torres-Fuentes, J. Ávila-Román, G. Aragonès, M. Mulero, F. I. Bravo, B. Muguerza, L. Arola, M. Suárez, “Chrononutrition and Polyphenols: Roles and Diseases,” *Nutrients 2019, Vol. 11, Page 2602*, vol. 11, no. 11, p. 2602, Oct. 2019, doi: 10.3390/NU11112602.

[43] L. Iglesias-Carres, A. Mas-Capdevila, F. I. Bravo, G. Aragonès, A. Arola-Arnal and B. Muguerza, “A comparative study on the bioavailability of phenolic compounds from organic and nonorganic red grapes,” *Food Chemistry*, vol. 299, p. 125092, Nov. 2019, doi: 10.1016/J.FOODCHEM.2019.125092.

[44] I. Escobar-Martínez, V. Arreaza-Gil, B. Muguerza, A. Arola-Arnal, F. I. Bravo, C. Torres-Fuentes and M. Suárez, “Administration Time Significantly Affects Plasma Bioavailability of Grape Seed Proanthocyanidins Extract in Healthy and Obese Fischer 344 Rats,” *Molecular Nutrition and Food Research*, vol. 66, no. 3, Feb. 2022, doi: 10.1002/mnfr.202100552.

[45] M. V. Barroso, A. Graça-Reis, I. Cattani-Cavalieri, L. B. Gitirana, S. S. Valenca and M. Lanzetti, “Mate tea reduces high fat diet-induced liver and metabolic disorders in mice,” *Biomedicine & Pharmacotherapy*, vol. 109, pp. 1547–1555, Jan. 2019, doi: 10.1016/J.BIOPHA.2018.11.007.

[46] X. Zhang, J. Li, B. Yang, Q. Leng, J. Li, X. Wang, T. Chairak, J. Lu, O. J. Olatunji and J. Tang “Alleviation of Liver Dysfunction, Oxidative Stress, and Inflammation Underlines the Protective Effects of Polysaccharides from Cordyceps cicadae on High Sugar/High Fat Diet-Induced Metabolic Syndrome in Rats,” *Chemistry & Biodiversity*, vol. 18, no. 5, p. e2100065, May 2021, doi: 10.1002/CBDV.202100065.

[47] F. Wang, Y. Liu, J. Yuan, W. Yang and Z. Mo, “Compound C Protects Mice from HFD-Induced Obesity and Nonalcoholic Fatty Liver Disease,” *Int J Endocrinol*, vol. 2019, 2019, doi: 10.1155/2019/3206587.

[48] P. Huang, T. Yu and Y. Yoon, “Mitochondrial clustering induced by overexpression of the mitochondrial fusion protein Mfn2 causes mitochondrial dysfunction and cell death,” *European Journal of Cell Biology*, vol. 86, no. 6, pp. 289–302, Jun. 2007, doi: 10.1016/J.EJCB.2007.04.002.

[49] H. Zhu, Y. Shan, K. Ge, J. Lu, W. Kong and C. Jia, “Specific Overexpression of Mitofusin-2 in Hepatic Stellate Cells Ameliorates Liver Fibrosis in Mice Model,” *Hum Gene Ther*, vol. 31, no. 1–2, pp. 103–109, Jan. 2020, doi: 10.1089/HUM.2019.153.

[50] H. F. Jheng, P. J. Tsai, S. M. Guo, L. H. Kuo, C. S. Chang, I. J. Su, C. R. Chang and Y. S. Tsaia, “Mitochondrial fission contributes to mitochondrial dysfunction and insulin resistance in skeletal muscle,” *Mol Cell Biol*, vol. 32, no. 2, pp. 309–319, Jan. 2012, doi: 10.1128/MCB.05603-11.

[51] R. M. Rodríguez, A. J. Cortés-Espinar, J. R. Soliz-Rueda, C. Feillet-Coudray, F. Casas, M. Colom-Pellicer, G. Aragonès, J. Avila-Román, B. Muguerza, M. Mulero and M. J. Salvadó, “Time-of-Day Circadian Modulation of Grape-Seed Procyanidin Extract (GSPE) in Hepatic Mitochondrial Dynamics in Cafeteria-Diet-Induced Obese Rats,” *Nutrients*, vol. 14, no. 4, Feb. 2022, doi: 10.3390/NU14040774/S1.

[52] Y. Ji, Y. Gao, H. Chen, Y. Yin and W. Zhang, “Indole-3-Acetic Acid Alleviates Nonalcoholic Fatty Liver Disease in Mice via Attenuation of Hepatic Lipogenesis, and Oxidative and Inflammatory Stress,” *Nutrients*, vol. 11, no. 9, Sep. 2019, doi: 10.3390/NU11092062.

[53] T. Albrahim and M. A. Alonazi, “Lycopene corrects metabolic syndrome and liver injury induced by high fat diet in obese rats through antioxidant, anti-inflammatory, antifibrotic pathways,” *Biomedicine & Pharmacotherapy*, vol. 141, p. 111831, Sep. 2021, doi: 10.1016/J.BIOPHA.2021.111831.

[54] B. H. Halima, G. Sonia, K. Sarra, B. J. Houda, B. S. Fethi and A. Abdallah, “Apple Cider Vinegar Attenuates Oxidative Stress and Reduces the Risk of Obesity in High-Fat-Fed Male Wistar Rats,” *J Med Food*, vol. 21, no. 1, pp. 70–80, Jan. 2018, doi: 10.1089/JMF.2017.0039.

[55] A. Jimoh, Y. Tanko, A. Ahmed, A. Mohammed and J. O. Ayo, “Resveratrol prevents high-fat diet-induced obesity and oxidative stress in rabbits,” *Pathophysiology*, vol. 25, no. 4, pp. 359–364, Dec. 2018, doi: 10.1016/J.PATHOPHYS.2018.07.003.

[56] C. González-Quilen, K. Gil-Cardoso, I. Ginés, R. Beltrán-Debón, M. Pinent, A. Ardévol, X. Terra and M. T. Blay, “Chronic supplementation with dietary proanthocyanidins protects from diet-induced intestinal alterations in obese rats,” *Mol Nutr Food Res*, vol. 61, no. 8, Aug. 2017, doi: 10.1002/MNFR.201601039.

10. ACKNOWLEDGMENTS

I would like to express my most sincere gratitude to Dr. Miquel Mulero, for giving me the opportunity to do my internship in the nutrigenomics research group, for guiding me, helping me, making me see things more clearly and for believing in my work. He has taught me that "solving problems is easier if it is done horizontally, and not vertically".

I am very grateful to have met all the members of this group, for making my days more enjoyable and for the laughs in the office. In particular, I would like to thank Antonio Jesús Cortés, for being my partner during all these months and for being the best mentor anyone could have. He has taught me many things to become a good future researcher.

I also wanted to thank my friends, especially those at the university, because we have supported each other in this beautiful stage.

Finally, I would like to acknowledge the support and love of my mother, Pili; my father, Víctor; my sister Aina and my soulmate, Laura.