ROLE OF FLAVONOIDS IN THE MODULATION OF INTESTINAL ALTERATIONS ASSOCIATED WITH METABOLIC CHALLENGES: OBESITY AND AGING

Marta Sierra Cruz

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DOCTORAL THESIS

Tarragona 2022

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Role of flavonoids in the modulation of intestinal alterations associated with metabolic challenges: obesity and aging

Doctoral Thesis

Supervised by Dr. Raúl Beltrán Debón and Dr. Ximena Terra Barbadora

UNIVERSITAT ROVIRA i VIRGILI

Department of Biochemistry and Biotechnology

MoBioFood Research Group

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HAGO CONSTAR que el presente trabajo, titulado **"Role of flavonoids in the modulation of intestinal alterations associated with metabolic challenges: obesity and aging"**, que presenta **Marta Sierra Cruz** 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.

I STATE that the present study, entitled "**Role of flavonoids in the modulation of intestinal alterations associated with metabolic challenges: obesity and aging"**, presented by **Marta Sierra Cruz** 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.

Tarragona, 4 marzo 2022 / Tarragona, 4th March 2022

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A mi padre,

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"I was taught that the way of progress was neither swift nor easy"

Marie Curie

"Everything is theoretically impossible, until it is done"

Albert Einstein

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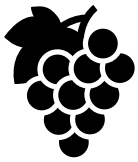

SUMMARIES

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SUMMARY

Obesity and aging have become issues of global concern due to their increasing prevalence in the population in recent decades. On one side, obesity is associated with a loss of intestinal barrier function as well as changes in the composition and functionality of the microbiota (dysbiosis). Together, this causes a greater transport of endotoxins into the bloodstream and, with it, a systemic inflammatory response that is directly related to the development of metabolic syndrome. Proanthocyanidins are bioactive compounds of natural origin present in foods of plant origin that have a protective effect against intestinal alterations associated with obesity induced by the consumption of high-fat/high-sugar diets. Specifically, proanthocyanidins reduce the translocation of endotoxins and act as an anti-inflammatory agent at the systemic level in young animal models. On the other side, it has been hypothesized that aging might derive in similar effects on the intestinal barrier functionality, but little is known about proanthocyanidin therapeutic effect during aging.

In that sense, the main objective of this thesis is to evaluate the role of proanthocyanidins in the modulation of metabolic and intestinal disruptions associated with obesity and aging. We found that a pharmacological dose (500 mg/kg of body weight) of a grape seed proanthocyanidin extract (GSPE) can reduce body weight gain by reducing food intake when administered preventively to aged rats fed a standard diet. This dose of GSPE also reduces body weight gain by acting on food intake, as well as fat accumulation in the liver when administered synchronically to aged rats receiving cafeteria diet. Furthermore, this thesis aims to identify the mechanisms by which GSPE modulates metabolic endotoxemia using a dual *in vitro/in vivo* model of dietary lipids-induced intestinal dysfunction. We found that GSPE reduces intestinal permeability by modulating the translocation of endotoxins through different routes as well as the intestinal microbiota.

In conclusion, the administration of GSPE exerts protective effects against metabolic changes associated with age and obesity. Doses and administration time in humans must be determined in future clinical studies.

RESUMEN

La obesidad y el envejecimiento han aumentado su prevalencia en la población en las últimas décadas. Por un lado, la obesidad se asocia a una pérdida de la función de barrera intestinal, así como a cambios en la composición y funcionalidad de la microbiota (disbiosis). Todo ello provoca un mayor transporte de endotoxinas al torrente sanguíneo y, con ello, una respuesta inflamatoria sistémica que está directamente relacionada con el desarrollo del síndrome metabólico. Las proantocianidinas son compuestos bioactivos naturales de origen vegetal que tienen un efecto protector frente a las alteraciones intestinales asociadas a la obesidad inducida por el consumo de dietas ricas en grasas y azúcares. En concreto, las proantocianidinas reducen la translocación de endotoxinas y actúan como agente antiinflamatorio a nivel sistémico en modelos animales jóvenes. Por otro lado, se ha planteado la hipótesis de que el envejecimiento podría tener efectos similares sobre la funcionalidad de la barrera intestinal, pero se sabe poco sobre el efecto terapéutico de las proantocianidinas durante el envejecimiento.

En este sentido, el objetivo principal de esta tesis es evaluar el papel de las proantocianidinas en la modulación de las alteraciones metabólicas e intestinales asociadas a la obesidad y al envejecimiento. Se vio que una dosis farmacológica (500 mg/kg de peso corporal) de un extracto de pepita de uva rico en proantocianidinas (GSPE) es capaz de reducir el peso corporal mediante la reducción de la ingesta cuando se administra de forma preventiva a ratas envejecidas alimentadas con una dieta estándar. Esta dosis de GSPE también reduce la ganancia de peso corporal actuando sobre la ingesta y la acumulación de grasa en el hígado cuando se administra sincrónicamente a ratas envejecidas alimentadas con dieta de cafetería. Además, esta tesis pretende identificar los mecanismos por los que el GSPE modula la endotoxemia metabólica utilizando un modelo dual *in vitro/in vivo* de disfunción intestinal inducida por lípidos dietéticos. El GSPE es capaz de reducir la permeabilidad intestinal, modulando la translocación de endotoxinas a través de diferentes vías así como la microbiota intestinal.

En conclusión, la administración de GSPE ejerce efectos protectores contra los cambios metabólicos asociados a la edad y la obesidad. Las dosis y el tiempo de administración en humanos deben determinarse en futuros estudios clínicos.

RESUM

En les darreres dècades, l'obesitat i l'envelliment s'han convertit en temes de preocupació mundial a causa de la creixent prevalença en la población. D'una banda, l'obesitat s'associa a una pèrdua de la funció de barrera intestinal, així com a canvis en la composició i funcionalitat de la microbiota (disbiosis). Tot això causa un major transport d'endotoxines a la sang, provocant una resposta inflamatòria a nivell sistèmic. Aquesta inflamació es relaciona directament amb el desenvolupament del síndrome metabòlic. Les proantocianidines són compostos bioactius naturals d'origen vegetal que tenen un efecte protector davant les alteracions intestinals associades a l'obesitat induïda pel consum de dietes riques en greixos i sucres. Concretament, les proantocianidines redueixen la translocació d'endotoxines i actuen com agents antiinflamatoris a nivell sistèmic en models animals joves. Tanmateix, s'ha plantejat la hipòtesi que l'envelliment podria producir efectes similars sobre la funcionalitat de la barrera intestinal, però encara es coneix poc sobre l'efecte terapèutic de les proantocianidines durant l'envelliment.

En aquest sentit, l'objectiu principal d'aquesta tesi és avaluar el paper d'un extracte de raïm ric en proantocianidines (GSPE) en la modulació de les alteracions metabòliques i intestinals associades a l'obesitat i a l'envelliment. Trobem que una dosi farmacològica de GSPE (500 mg/kg pes corporal) és capaç de reduir l'augment de pes corporal mitjançant la reducció de la ingesta quan s'administra de manera preventiva a rates envellides alimentades amb una dieta estàndard. Aquesta dosi de GSPE també redueix el guany de pes corporal actuant sobre la ingesta, així com l'acumulació de greix en el fetge, quan s'administra sincrònicament a rates envellides alimentades amb una dieta de cafeteria. A més, aquesta tesi pretén identificar els mecanismes pels quals el GSPE modula la endotoxemia metabòlica utilitzant un doble model *in vitro/in vivo* de disfunció intestinal induïda per lípids dietètics. Trobem que el GSPE és capaç de reduir la permeabilitat intestinal modulant la translocació d'endotoxines a través de diferents vies, així com la microbiota intestinal.

En conclusió, l'administració de GSPE exerceix efectes protectors contra els canvis metabòlics associats a l'edat i l'obesitat. Les dosis i el temps d'administració en humans han de determinar-se en futurs estudis clínics.

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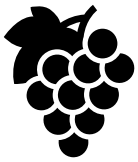

ABBREVIATIONS

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AMP	antimicrobial peptides
ApoB48	apolipoprotein B48
BAT	brown adipose tissue
BMI	body mass index
CAF	cafeteria diet
CD36	cluster of differentiation 36
CM	chylomicron
CNR1	cannabinoid receptor type 1
CVD	cardiovascular disease
DIO	diet-induced obesity
ECS	endocannabinoid system
F4D	fluorescein isothiocyanate- dextran 4 kDa
GALT	gut-associated lymphoid tissue
GFR	glomerular filtration rate
GI	gastrointestinal tract
GSPE	grape seed proanthocyanidin extract
HDL	high-density lipoprotein
HFD	high-fat diet
HOMA-IR	homeostatic model assessment of insulin resistance
HOMA-β	homeostasis model assessment of β-cell dysfunction

HPRT	hypoxanthine-guanine phosphoribosyl transferase
IAP	intestinal alkaline phosphatase
IESC	intestinal epithelial stem cell
IgA	immunoglobulin A
IL	interleukin
JAM-A	junctional adhesion molecule A
JNK	c-jun N-terminal kinase
LBP	LPS-binding protein
LM	lipid mixture
LPS	lipopolysaccharide
M cells	microfold cells
MLCK	myosin like chain kinase
MWAT	mesenteric white adipose tissue
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NEFAs	non-esterified fatty acids
NF-κB	nuclear factor κB
OVA	ovalbumin
OWAT	periovaric white adipose tissue
PACs	proanthocyanidins
PD	Parkinson's disease
PPIA	cyclophilin-E
ROS	reactive oxygen species

RPF	renal plasma flow
RWAT	retroperitoneal white adipose tissue
SCFA	short chain fatty acid
SOD	superoxide dismutase
SRB1	scavenger receptor class B type 1
T2D	type 2 diabetes
TAG	triglyceride
TEER	transepithelial electrical resistance
TGF-β	transforming growth factor β
TJ	tight-junction
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factor α
WAT	white adipose tissue
ZO	zoonulin

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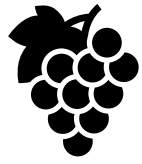

INTRODUCTION

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INTRODUCTION

1. Current metabolic challenges: obesity and aging

Obesity and aging are two major health issues of global concern. Social and environmental changes are relevant factors influencing changes in dietary and physical patterns, thus contributing to an increase of obesity prevalence among the world population. In fact, the worldwide prevalence of obesity has increased dramatically in the last decades, nearly tripled between 1975 and 2016, with 1.9 billion overweight adults and 650 million obese adults up to date according to Body Mass Index (BMI)[1]. The World Health Organization considers overweight individuals with a BMI from 25 to 29 kg/m^2 and obese when BMI is over 30 kg/m^2. However, this guide it may not correspond to the same degree of adiposity in different individuals [2]. Moreover, the elderly segment of the population is growing very fast, and it is estimated that by 2050, there will be two billion people over the age 60 [3]. Both obesity and aging process are characterized by an homeostasis disruption and associated to metabolic syndrome [3–5] which, at the same time, is a major risk factor for suffering from Type 2 Diabetes (T2D), cardiovascular diseases (CVDs), inflammatory diseases and neurodegenerative disorders, among others [5–8]. In the way of knowing deeper how to manage this health global concern, the first step is understanding metabolic alterations as well as the underlying mechanisms occurring in the development of getting obese and older.

1.1. Obesity and metabolic syndrome

The word *obesity* comes from the latin word *obesus*, which means *"having eating until fat"*. Obesity is considered a multifactorial complex metabolic disease caused by an imbalance between the energy intake and the energy expenditure, leading to tissue stress and dysfunction [4,9]. Obesity results from the interaction between genes, behavioural and environmental factors, mainly the consumption of unhealthy diets based on sugars and saturated fatty acids, the sedentary lifestyle, the poor physical activity and the socio-economic and cultural circumstances [2,10,11].

Obesity is associated to metabolic syndrome and insulin resistance [5,6,12]. Metabolic syndrome is not considered a disease but a clustering of individual risk

factors for disease [5] such as abnormal lipid profile, impaired glucose metabolism, hypertension and low-grade inflammation [4,5,8,12]. Nevertheless, conflicting data is found in the literature regarding metabolic risk and obesity because it has been recognized the presence of obese patients without associated metabolic complications [13]. Actually, 20-30% of obese people have no metabolic complications associated to obesity, so that characterized by high-insulin sensitivity, normal blood pressure, normal lipid profile and no signs of chronic inflammation [14]. Insulin resistance is defined as a tissue dysregulation in responding to insulin stimulation, especially in adipose tissue, liver and skeletal muscle due to their high metabolic demand for uptake and oxidation of glucose, glycogen synthesis and lipid oxidation processes, respectively [15,16]. Moreover, under an obesogenic and insulin resistance situation, adipocytes increase the release of free fatty acids and the synthesis of pro-inflammatory cytokines, mainly Tumour Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1) and Interleukin-6 (IL-6), promoting ectopic fat accumulation and low-grade inflammation [10,16–18]. Indeed, insulin resistance provokes a disruption in glucose uptake and fatty acid metabolism in liver, leading to accumulation of triglycerides (TAGs) which increases the risk of non-alcoholic fatty liver disease (NAFLD), also highly associated to obesity and metabolic syndrome [5,19]. In this sense, TNF-α secretion by adipose tissue feeds the vicious cycle of insulin resistance by interfering insulin signalling, thereby favouring steatosis and inflammation in liver, thus promoting pathogenesis of non-alcoholic steatohepatitis (NASH)[20]. Under this metabolic disruption, β-pancreatic cells try to compensate by secreting more insulin, so that leading to hyperinsulinemia [10,16,21,22]. All in all, obesity and metabolic syndrome are associated with a risk of suffering from other co-morbidities such as T2D, CVDs, hepatic steatosis, different types of cancer such as breast, colon and prostate and neurodegenerative disorders, among others (Figure 1) [4,9,23].

Additionally, obesity and metabolic syndrome are also considered risk factors in the mid-age for neurodegenerative disorders by favouring cognition decline in old age [6,24]. Chronic inflammation associated to obesity is involved in altering brain volume, promoting grey matter atrophy in different brain areas such as hippocampus or thalamus and reducing neural integrity. In that regard, literature suggests overnutrition might be the main responsible on obesity role in promoting brain damage, mainly neuroinflammation and oxidative stress, especially through

consumption of high-fat diets [25,26]. Interestingly, changes in blood-brain barrier permeability appear to develop after chronic consumption of diets containing high contents in fat and sucrose, whereas cognitive decline is observed earlier [14].

Taking all into account, it becomes clear the necessity of having reliable animal models for further research human obesity in order to understand the underlying mechanisms leading to metabolic syndrome, insulin resistance and other related co-morbidities and to find new therapeutic targets. In that regard, cafeteria diet is widely recognized as a more robust model for that purpose compared with high-fat diet [27].

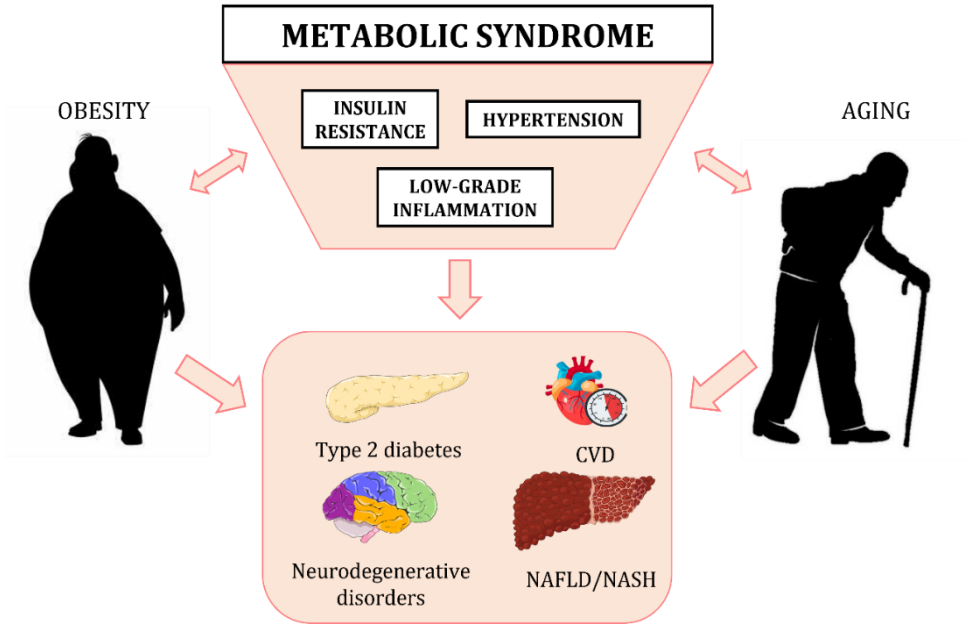

Figure 1. Schematic view of the relationship between metabolic syndrome features with obesity, aging and associated pathologies.

1.1.1. Cafeteria diet as a model of induced obesity

Laboratory animals play a crucial role in basic and clinical research. With the increasing prevalence of obesity among world population, both genetic and diet-induced animal models have been developed to further research obesity and associated co-morbidities, mainly metabolic syndrome. As reviewed by *Sampey et al.*, genetic models such as *ob/ob* mice, *db/db* mice and *Zucher fa/fa* obese rats represent a good contribution to science regarding the knowledge about genetic anomalies in

controlling energy homeostasis [27]. Nevertheless, since high-fat diet chronic consumption contributes directly to the development of metabolic diseases in humans [28], scientists have developed different animal models to imitate human dietary patterns and lifestyle. The two most prevalent models are Sprague-Dawley and Wistar rats fed with high fat and/or sugar diets, usually called diet-induced obesity (DIO) models, being the predominant ones the high-fat diet (HFD) and the cafeteria (CAF) diet [28], which in addition to high fat it contains high sugar.

HFDs are normally based on special pellets in which the amount, percentage and type of lipids, proteins, minerals and vitamins delivered to animals are previously determined, allowing experimenters to have control of each animal consumption. CAF-fed animals are offered *ad libitum*, and consist of fresh and highly palatable energetic food such as biscuits, sausages, bacon, carrots, muffins and milk with sugar in addition to standard chow diet [29–31]. Although HFDs are known to provoke an increase in body weight gain and adiposity, CAF diets better mimic actual human dietary behaviour and Western diet associated to obesity pandemics [27,32,33]. Moreover, CAF diet is a robust model of human metabolic syndrome compared to traditional HFD [27]. Specifically, as widely distributed in the literature, CAF diet provokes a rapid body weight gain, an increase in adiposity, low-grade inflammation and liver steatosis, thus promoting progression from NAFLD to NASH, in both young animals and adults [27,31,34]. Indeed, CAF diet also induces metabolic syndrome development in aged rats (21-months old), specifically provoking insulin resistance and hepatic macrosteatosis [35].

CAF diet also affects gut homeostasis at different levels causing appetite homeostasis disarrangement [36], intestinal damage, gut barrier dysfunction and subsequent systemic inflammation due to the entrance of microbiota-derived endotoxins into the bloodstream in a time-dependent manner [37–39]. Moreover, consumption of high-fat or high-fat/high-sugar diets has been demonstrated to be associated with cognitive decline, especially attention, memory, eating and food decisions and processing speed [14,40]. Interestingly, as well as in humans, sexual dimorphism in the development of metabolic diseases associated to obesity occurs in rodents, being males more susceptible to DIO than females [40]. Although little is known about the underlying mechanisms triggering metabolic disturbances caused by overnutrition,

oxidative damage derived from CAF diet consumption has been suggested to cause adipose tissue inflammation and systemic metabolic dysfunction in male Wistar rats, corroborating previous data in humans [41].

However, the principal disadvantage of CAF diet as a model of human disease is the poor standardization of ingredients and consumption period, depending not only on the animal choice but also on the own laboratory [29,32]. In that regard, recently, *Lalanza & Snoeren* have proposed a protocol for standardizing CAF diet consisting on 16 different products divided into four different menus [33], thus allowing a higher offer of flavours to animals and enhancing the effect of CAF diet in increasing body energy stores [42]. All in all, CAF diet is a good model to assess the influence of energetical dense Western diet not only in causing obesity but is also a relevant model for human metabolic and neurodegenerative complications derived from obesity. Further research needs to be done to standardize CAF diet and allow researchers comparing results.

1.2. Aging and metabolic syndrome

World population is getting old very fast. Lifestyle, economic, social and cultural opportunities are favouring an increase in life expectancy, especially in developed countries. In 2018, the World Health Organization estimated that there will be 1.4 billion old people in 2030 and about 2.1 billion in 2050, which represents a huge increase in just a few decades. Aging is considered as a disturbance of homeostasis characterized by morphological, functional and biological deterioration, thus increasing the risk for both metabolic and neurodegenerative disorders, such as T2D and Alzheimer's disease (AD), respectively [24,43]. Aging is accompanied by changes in body composition, principally fat accumulation in abdominal adipose tissue, liver and muscle and progressive loss of muscle mass, strength and quality, termed sarcopenia [3,43,44]. Furthermore, aging is also characterized by deep changes in sex hormones production, thus affecting insulin resistance phenotype due to differences in fat deposition and lipid profiles among men and women. Literature suggests that this can be an explanation for what life expectancy and prevalence of aging-associated diseases, such as metabolic syndrome and CVDs, vary among genders [43,45].

Since metabolic syndrome is associated to aging [3,44,46–48], insulin resistance and low-grade systemic inflammation are two major factors involved in promoting the development of aging-associated pathologies, especially T2D, CVDs, NAFLD and dementia (Figure 1). In fact, changes in dietary patterns triggering obesity, metabolic syndrome and diabetes determine longevity and cause alterations in the aging process [47]. With this scenario, together with the higher risk of neurodegenerative and metabolic disorders associated to aging, it is absolutely necessary to develop new tools to prevent or even cure diseases and guarantee a better quality of life for our elderly.

1.2.1. Immunosenescence and low-grade inflammation

Tissue inflammation is one of the most relevant immune system's mechanisms to alert about danger signals. Both humoral and cellular immune responses work together to repair cell damage and to fight against antigens, thus resulting in an increase of pro-inflammatory cytokines production, especially TNF-α, Interleukin-1β (IL-1β) and IL-6, followed by a controlled resolution leading to immune homeostasis, especially through lipid mediators which promote anti-inflammatory cytokines production, mainly Interleukin-10 (IL-10), Interleukin-37 (IL-37) and Transforming Growth Factor β (TGF-β) [49,50]. However, in old people, the immune system function declines, a process known as *immunosenescence*. *Immunosenescence* is characterized by changes in lymphocyte cell pool, stem cells alterations, thymus involution and consequent disruption of central tolerance and reduced immunity, therefore increasing predisposition to chronic inflammation and making them more sensitive and vulnerable to pathogens, cancer and autoimmune diseases [47,51,52].

In the elderly, the immune system loses the ability to control the balance between the pro-inflammatory response against the danger signal and the consequent resolution through an anti-inflammatory response, thus provoking a chronic low-grade inflammation state associated to aging called *inflammaging* [3,49]. Moreover, age-associated ectopic fat accumulation together with atrophy of adipose tissue contribute to hyperglycemia, insulin resistance and production of proinflammatory cytokines such as TNF-α and IL-6, thus contributing to *inflammaging* [53]. *Inflammaging* has been also linked to have a relevant role in aging development and related diseases such as dementia [6,44,48] and contributes to metabolic syndrome

and related co-morbidities [47,48], especially through the activation of inflammasome [49]. Inflammasome is a multicomplex protein structure involved in the activation of pro-inflammatory cascades, principally through Nuclear Factor κ-light-chain-enhancer of activated B cells (NF-κB) mediated-pathway [49,54,55]. Actually, metabolic syndrome and obesity have demonstrated to induce inflammasome activation by reducing sensitivity in adipose tissue [49], thus inducing a worse homeostasis maintaining during aging. Indeed, insulin resistance and aging contribute together to mitochondrial dysfunction, thus reducing ATP production and increasing oxidative stress [44,47] which, at the same time, feeds this process of chronic inflammation through triggering inflammasome [49,56].

Reactive oxygen species (ROS) provoke several cellular damages, hence increasing DNA damage, accumulation of misfolded proteins and activation of inflammasome. Under homeostatic conditions, senescent cells have receptors for cytotoxic cells, especially natural killer cells, in charge of senescent-cells clearance. However, in the elderly, senescent cells are able to escape from immune system, so that promoting their accumulation and subsequent inflammation [49,56]. Moreover, both reduced telomeres length and telomerase activity have demonstrated to promote *inflammaging*, especially through accumulation of senescent-T-cells which produce pro-inflammatory cytokines in response to oxidative damage. In fact, telomerase activity might also regulate immune response through interaction with NF-kB pathway [51,54].

2. Intestinal dysfunction associated to obesity and aging

Human intestinal mucosa, with approximately 300 m^2, is the largest surface in the body [57,58]. Besides its important role in water, ions and nutrients absorption, intestine plays a major immunological function by acting as both physical and biochemical barrier to avoid the entrance of potential harmful microbes, toxins and antigens [58,59]. Gut microbiota inhabits in the human gastrointestinal tract, especially in the large intestine, and is composed by millions of heterogenous populations of bacteria, archaea, eukaryotes and viruses which are essential in maintaining gut health [58,60]. Gut microbiota remains essential in the immunological tolerance, defence against pathogens, maintaining of intestinal homeostasis and regulation of metabolic processes. However, intestinal integrity and

gut microbiota can be disrupted by several factors such as dietary patterns, drug abuse, physical activity or aging [57,59,61]. This intestinal homeostasis impairment provokes the entrance of microbiota-derived molecules into the bloodstream, therefore activating immune system and provoking chronic inflammation which, at last, participates in the pathogenesis of non-intestinal disorders such as NAFLD, diabetes, obesity or AD [57,62,63]. All in all, deeper research is needed to identify the underlying mechanisms occurring during obesity and aging which cause intestinal damage and subsequent intestinal dysfunction and increase permeability to non-desirable molecules such as bacterial endotoxins, especially lipopolysaccharide (LPS), thus leading to endotoxemia and associated pathologies.

2.1. The intestinal barrier: a multilayer system

The intestinal barrier is a complex system formed by physical, biochemical and immunological layers which allow the separation of apical and basolateral sides (Figure 2). The very first line of intestinal defence is the mucus layer, composed by highly glycosylated proteins, which not only lubricates the progression of luminal content through the gut but also acts as physical barrier capturing gut microbiota, antigens, toxins and enzymes potentially harmful for intestinal epithelium [64]. Antimicrobial peptides (AMPs), principally defensins and cathelicidins, remain essential to display an immunoregulatory activity against pathogens, activating both innate and adaptive immune responses and protecting the underlying intestinal epithelium from damage [59,65,66]. Intestinal epithelium consists on a single layer of intestinal epithelial cells: (1) enterocytes, in charge of nutrients, ions, vitamins and water uptake; (2) enteroendocrine cells which secrete hormones involved in hunger and energy balance such as leptin [66,67]; (3) goblet cells, specialized in mucus production but also chemokines and cytokines important for gut immunity [68]; (4) Paneth cells, specialized in secretion of AMPs and other proteins with immunomodulatory properties, thus helping to maintain intestinal homeostasis [69]; and (5) microfold (M) cells, which protect gut-associated lymphoid tissue (GALT), especially Peyer's patches of the small intestine, and act as antigen-presenting cells to the immune cells in lamina propria: macrophages, dendritic cells, mast cells, immunoglobulin A (IgA) secreting plasmatic cells and T cells [70,71].

Furthermore, the gut, especially the large intestine, is a huge reservoir of commensal bacteria which participate not only in the digestion function but are also fundamental to maintain gut health through modulation of GALT. In fact, correct functioning of both gut and gut microbiota depends on this symbiotic relationship, having a double bidirectional communication. Hence, intestinal barrier can regulate gut microbiota diversity and, at the same time, gut microbiota has an influence on barrier integrity by modulation of immune system through different bacterial components such as LPS, peptidoglycan or flagellin and also by production of short-chain fatty acids (SCFAs) derived from degradation of non-digestible dietary fiber [58,72–74]. With this complex intestinal scenario, it is evident that maintaining the correct balance of the intestinal barrier, including the gut microbiota, is essential to avoid intestinal disruptions, mainly an increase in permeability which, ultimately, leads to intestinal inflammation and associated diseases.

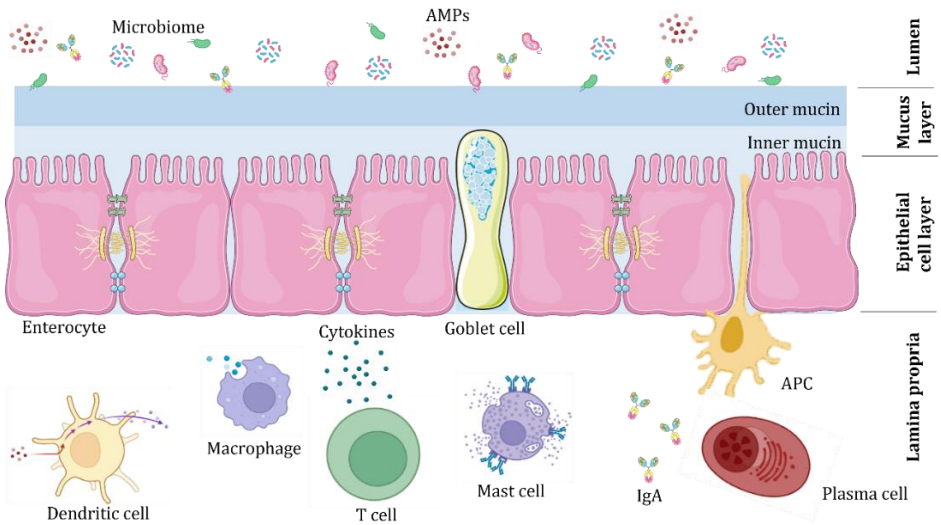

Figure 2. The multilayer intestinal system. Intestinal barrier is composed by four different layers extending from lumen to *lamina propria*. Gut microbiota, AMPs, IgA and microbiota-derived compounds such as LPS are located in the lumen. Mucin layer avoids contact between gut microbiota and epithelial cells. Epithelial cell layer is composed mainly by enterocytes but also other secretory cell types such as goblet and Paneth cells, in charge of mucus and AMPs production. Different immune cell types are in *the lamina propria*, protecting the body from antigens and bacteria to cause systemic inflammation. *Abbreviations: AMPs, antimicrobial peptides; APC, antigen presenting cells; IgA, immunoglobulin A.* Adapted from *Gosh et al.* (2020).

2.2. Intestinal permeability

The intestinal barrier must ensure the absorption of nutrients but, at the same time, avoid the translocation of macromolecules, including microbial components and pro-inflammatory agents. Maintaining intestinal homeostasis between external environment and internal milieu remains essential for human health [61,75,76]. For that reason, intestinal epithelial cells which form the physical barrier are connected to each other by complex dynamic protein structures called tight-junctions (TJs) that allow a selective pass of molecules. The structure of TJs consist of: (1) transmembrane proteins like claudin family, which form the paracellular pore and are associated to other proteins such as occludins and Junctional adhesion molecule A (JAM-A); and (2) cytoplasmatic junctional complex proteins, such as zoonulin (ZO), with specific domains to interact with both cytoskeleton and intracellular signalling molecules, especially Myosin like chain kinase (MLCK). MLCK regulates assembly and disassembly of TJs by phosphorylation and dephosphorylation, thus modulating paracellular flux [63,77–79].

Different insults such as diet, drugs, toxins, stress and pathogens have an effect on intestinal permeability, thus leading to translocation of luminal content to *lamina propria* and subsequent intestinal inflammatory response, which is associated with metabolic alterations such as obesity, metabolic syndrome, insulin resistance and aging [75,80,81]. Diet has been widely studied as a contributing factor for intestinal homeostasis alteration. Several studies demonstrated that high-fat-high-sugar diets induce intestinal dysfunction, mainly by increasing permeability and intestinal inflammation and influencing gut microbiota composition [82–85]. Both HFD and CAF diet provoke hyperpermeability of the intestinal barrier through reduction of TJs structure in animal models [63], thus permitting the leakage of bacterial endotoxins, mainly LPS into circulation [86]. HFD also provokes changes in gut microbiota population proportions, leading to an increase in gut permeability and translocation of bacterial endotoxins to circulation [87]. CAF diet also induces gut barrier dysfunction at different levels: increase of oxidative stress, dysbiosis and increased intestinal permeability, thus provoking the entrance of bacterial endotoxins and subsequent metabolic endotoxemia, both contributing to systemic inflammation [37–39,88]. Moreover, long-chain fatty acids from dietary fats also induce immune system

activation, thus provoking an increase in inflammatory mediators such as TNF-α, IL-6 and IL-1β which, at the same time, downregulate TJs protein expression and upregulate MLCK [57,63]. As reviewed recently by *Merra et al.*, Mediterranean diet, in contrast to Western dietary patterns, could have beneficial effects on gut microbiota composition and function, thus contributing to the reduction of cardiovascular and metabolic diseases onset [89].

Aging has been associated with different pathologies affecting the gastrointestinal tract. In fact, the prevalence of gut-related disorders is considerable in the elderly population [76,90]. Due to the fact the intestinal tract is a hostile environment, epithelium requires a continuous process of renewal thanks to the presence of intestinal epithelial stem cells (IESCs) located in the intestinal crypts. It has been reported that changes in JNK signalling pathway in IESCs may be responsible of the intestinal homeostasis disruption during aging [91]. However, literature regarding intestinal permeability in the elderly is controversial. Some studies in rodents and non-human primates reported that intestinal permeability is increased with age but some others showed no variations in TJs protein expression and pro-inflammatory cytokines secretion in aged individuals [76,92]. Moreover, the thickness of the mucus layer in duodenum seems to remain unchanged in aged healthy people[93] but is decreased in colon in a transgenic mice model of accelerating aging [94]. Some AMPs are downregulated with age, such as α-defensin, and some others are upregulated, such as angiogenin-4 [95]. More consensus has been found in the literature about pro-inflammatory cytokines levels in the gut, which tend to increase with age [57,63,92]. Gut microbiota also experiments profound changes in composition and functioning in the elderly, not only affecting the age but also other factors such as nutrition, genetics and geographical/social patterns [96]. Especially relevant is the increase of IL-6 expression in aged people in modulating expression of TJs proteins in gut, thus leading to an increase in gut permeability [57,97]. Hence, these changes in gut homeostasis during aging have very relevant consequences, mainly a higher risk of microbial infections and gut inflammation due to the poor response against pathogens. Interestingly, disruption of epithelial intestinal barrier and associated microbiota in aged people has been correlated with neurodegeneration typical from Parkinson's disease (PD), AD or multiple sclerosis [57,96,98].

All in all, when gut-gut microbiota balance is compromised at different levels in a context of obesity, aging and metabolic syndrome, LPS can enter the circulation and provoke systemic inflammation, a process called as endotoxemia.

2.2.1. Metabolic endotoxemia: intestinal and organism consequences

Different gut microbiota populations live in the luminal side of intestine, therefore being the major source of LPS of the whole body [60]. LPS is the major glycolytic component of the outer membrane of Gram-negative bacteria. It is an amphipathic molecule composed by a hydrophobic lipid A with endotoxin activity attached to a hydrophilic polysaccharide chain composed by a core oligosaccharide and the distal O-antigen polysaccharide [99–101]. Endogenous LPS is normally released after bacteria cell division or death and its bioactivity depends on the molecular shape of the lipid A [99]. Bacterial endotoxin plays a critical role in the maintaining a balanced immune system, mainly through stimulation of immune cells maturation in the GALT [102].

Under normal conditions, bacterial endotoxin is not allowed to be absorbed in the gut, mainly by potent mechanisms limiting its access such as the mucus layer or the inhibitory effects of IgA and AMPs in the apical side of the intestine. Interestingly, enterocytes also secrete intestinal alkaline phosphatase (IAP), an enzyme capable of detoxifying LPS and preventing it to pass through the intestinal layer. It has been reported that small quantities of LPS can cross intestinal barrier in healthy individuals, thus suggesting an hormetic effect on gut health, especially remaining essential in maintaining gut homeostasis through activation of intestinal immune system [60,103]. However, in a context of metabolic syndrome, the high intake of dietary lipids provokes an increase in gut permeability and alterations in microbiota composition (dysbiosis) which leads to an increase of endotoxin translocation into the circulation and inducing inflammation [104–106]. Inflammation occurs due to the interaction of endotoxin-lipid A domain with Toll-like receptor 4 (TLR4), present in the cell membrane of macrophages and other immune cells in the *lamina propria*, where triggers a cascade of pro-inflammatory cytokines production via NFκB pathway activation [101,107,108]. Indeed, LPS can be transported to peripheral tissues within the blood as a free molecule but also associated to lipoproteins, mainly high-density lipoprotein (HDL) during fasting and chylomicrons (CMs) during post-

prandial state, thus inducing at the same time chronic systemic low-grade inflammation [82,109].

The presence of LPS in the bloodstream due to the consumption of a high-fat/high-sucrose diet was named by *Cani et al. (2007)* as metabolic endotoxemia. They demonstrated for the first time that plasma concentration of LPS is enough to induced metabolic disruptions induced by HFD consumption. Besides, enough scientific evidence in animal models have demonstrated that consumption of HFD or CAF diet positively correlated with higher gut permeability and higher quantities of circulating LPS from the gut lumen, so that acting as inductors of metabolic endotoxemia. Saturated fats have been associated with changes in gut microbiota populations: increased levels of *Firmicutes* (Gram-positive) and decreased levels of *Bacteroidetes* (Gram-negative) in animal models but controversial results exist in humans [82,102,110,111]. HFDs also induce an increase in bile acid production which lead to intestinal permeability and facilitates LPS translocation [82].

Dietary fatty acids, as well as LPS, are able to activate TLR4, thus inducing the pro-inflammatory profile and causing gut damage. Moreover, TLR4 is also expressed in adipocytes and hepatocytes [112], so that when LPS crosses the intestinal barrier and reaches the circulation can trigger an inflammatory response in both adipose tissue and liver. In adipose tissue, endotoxin induces infiltration of macrophages which, together with adipocytes, contribute to local inflammation by producing pro-inflammatory cytokines and chemokines. Moreover, as a result of high dietary intake of fat and local inflammation, adipose tissue develops hypertrophy, thus causing energy balance derangements such as insulin resistance and lipolysis. Consequently, hepatic fatty acids ectopic accumulation together with the entrance of LPS via the portal vein into the liver, normally associated to lipoproteins, results in local hepatic inflammation and insulin resistance [99,111,113]. Moreover, as reviewed by *Damns-Machado et al.* (2017)[107], LPS translocation has been proven to cause liver inflammation and liver steatosis in experimental models of obesity as well as in obese humans.

All in all, as LPS translocation has important consequences at both tissue and systemic level, metabolic endotoxemia might be an important factor in promoting obesity and aging-related pathologies, not only intestinal ones such as colitis ulcerosa or Crohn

disease but also metabolic diseases such as insulin resistance or NAFLD, neurodegenerative pathologies such as AD and PD as well as cardiovascular diseases [60,106,107,114,115]. Specifically, during neurodegeneration, it has been described that endotoxin promotes accumulation of protein aggregates and activation of microglia cells that induce neuroinflammation [116]. At that point, knowing the specific mechanisms by which LPS is translocated in the gut will allow us to better understanding of metabolic endotoxemia and the multiple connections between gut and the rest of the organism, especially gut-brain axis, deeply involved in obesity, metabolic syndrome and aging processes.

2.2.2. LPS translocation across the intestinal barrier

Although the precise pathway by which LPS is translocated across the intestinal barrier remains controversial, different mechanisms have been proposed: (1) paracellular transport by passive diffusion linked to a higher gut permeability associated to TJs proteins complexes alterations; (2) transcellular pathway through cell-associated antigen passage such as microfold cells or goblet cells; (3) receptor-mediated endocytosis via clathrin- or caveolin-dependent transcytosis in enterocytes and colonocytes; or (4) chylomicron-associated pathway during postprandial period (Figure 3). Paracellular transport of LPS has been demonstrated in a wide of in *vitro* studies with intestinal cells lines and *in vivo* studies with animal models [101,117]. In fact, LPS itself induced pro-inflammatory cytokines production in gut which finally leads to an increase in gut permeability and a higher rate of LPS translocation via paracellular pathway [101,118]. Besides, intestinal M cells express ANXA5, a protein with specifically interacts with the lipid A domain of LPS and permits its endocytosis and subsequent transport to lamina propria, where GALT-associated cells start the inflammatory cascade [119].

CMs are lipoproteins produced in small intestine after a meal, thus increasing its concentration during post-prandial state [60]. Due to its lipid nature, CMs have a high affinity for LPS-lipid A domain, being also involved in its translocation across the intestinal barrier and being involved in endotoxin detoxification and preventing inflammation [120,121]. CMs are synthesized in endoplasmic reticulum of enterocytes, where apolipoprotein B48 (ApoB48) is associated to other lipids such as cholesterol and phospholipids, forming pre-CMs. These immature CMs are then

transported in vesicles to Golgi apparatus, where LPS is attached. Finally, mature CMs are secreted to lymphatic system [108,122] and reach the liver for LPS clearance and CMs recycling [82]. However, under a chronic intake of dietary fat, as in the case of Western diet, HFD or CAF diet, the excessive CM formation provokes a prolonged hepatic exposure to LPS which leads to aggravate metabolic endotoxemia [82]. Although LPS can bind to CMs by itself, LPS-binding protein (LBP) facilitates this association, thus improving endotoxin detoxification [121]. Both quantity and quality of dietary fat have been suggested to be crucial in modulating postprandial lipemia and LPS absorption in both cell-culture, animal models and human subjects [117,123–129].

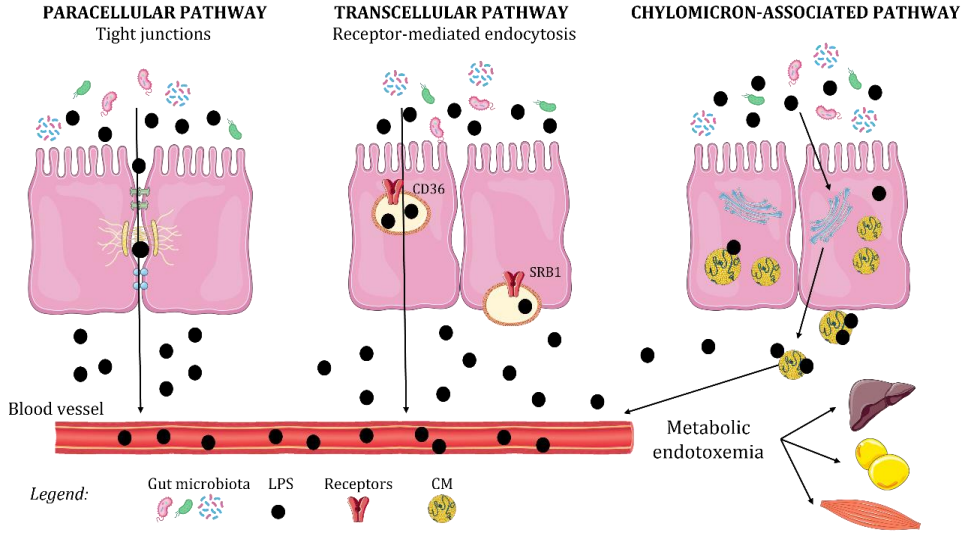

Figure 3. Schematic view of the proposed mechanisms for LPS intestinal translocation. *Abbreviations: CD36, Cluster of Differentiation 36; CM, chylomicron; GSPE, Grape seed proanthocyanidin extract; LPS, lipopolysaccharide; SRB1, Scavenger Receptor Class B Type 1.*

Cluster of Differentiation 36 (CD36) and Scavenger Receptor Class B type 1 (SR-B1) are both fatty acid translocases proteins located in the apical side of enterocytes. They act as lipid sensors for the incorporation of fatty acids, cholesterol and triglycerides needed for CMs synthesis during post-prandial state as well as scavenger receptors for LPS, thus being involved in CMs-associated LPS transport [108,130–132]. CD36 and SR-B1 are also expressed in other cell types such as adipocytes, macrophages,

hepatocytes and Kupffer cells, so that playing an important role in the development of metabolic endotoxemia and subsequent inflammation in peripheral tissues, especially adipose tissue and liver, so that promoting metabolic syndrome [108,132–134].

Giving the importance of metabolic endotoxemia in disturbing systemic health homeostasis status and its potent link with the development of a huge variety of pathologies, intestinal LPS transport could be a promising good therapeutical target. Up to date, hypocaloric diets, prebiotics and probiotics have been proposed as useful dietary interventions to reduce endotoxemia. However, dietary flavonoids could be a very powerful alternative in both preventing and treating metabolic endotoxemia.

3. Bioactive compounds

Dietary patterns are different among cultures, hence having a direct both beneficial and detrimental effects on human health [135]. It is widely recognized that consumption of a well-balanced diet rich in fruits, vegetables, legumes, fish and poor in saturated fat displays a protective role in the onset of different metabolic and neurodegenerative diseases as well as cancer. In the last decades, the current public concern about having a healthy lifestyle together with globalization, have pointed out the necessity for searching new food sources with positive effects on human health. In that regard, bioactive compounds have received increasing attention in the field of functional food.

Bioactive compounds are natural chemical compounds present in small quantities in food, vegetables and grains [136]. Curiously, they are responsible of different organoleptic characteristics of fruits and vegetables such as colour or flavour but are also important in the response of plants against stress. Bioactive compounds have been recognized to have protective effects on health like anticancer, antioxidative, anti-inflammatory and anti-obesity properties through the modulation of metabolic and immunological processes [135–138].

Bioactive compounds derived from plants are classified into three major classes depending on their chemical structure and function: terpenes and terpenoids, alkaloids, and phenolic compounds [139]. The exhibition of their beneficial effects strongly depends on the bioavailability, that is, the capacity of food components to be

accessible, absorbed, transformed and finally modulate biological processes in the organism [140].

Phenolic compounds, commonly named as polyphenols, deserve special attention because of its role in protection against biotic and abiotic stress in plants such ultraviolet radiation, pathogens and herbivores but also in pigmentation and reproduction [138,141,142]. Polyphenols contribute to bitter taste of different foods such as grapes, red wine, dark chocolate, tea or coffee and their classification depends on the number of phenol rings and the attached hydroxyl groups substituents within the molecule: phenolic acids, stilbenes, coumarins, tannins, quinones, lignans, curcuminoids and flavonoids [136,142].

3.1. Flavonoids: description and classification

Flavonoids are the most abundant phenolic compounds in the human diet, especially in fruits, vegetables, and some beverages [143,144]. Structurally, they are comprised of two aromatic rings and, depending on the third heterocyclic ring, they can be categorized in several major subclasses such as flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones and isoflavones (Figure 4) [145–147].

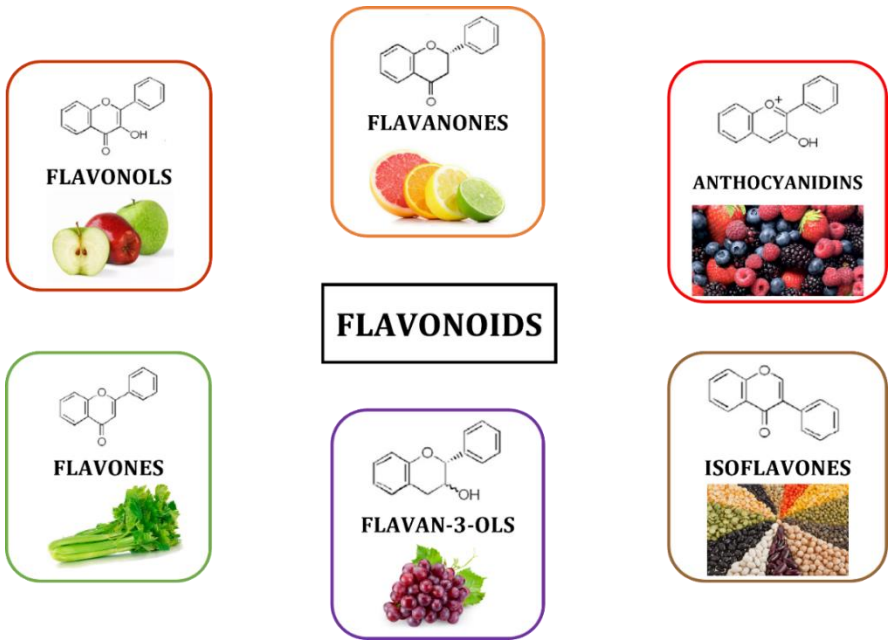

Figure 4. Flavonoids classification

Due to their wide variety, flavonoids have been assessed as potential natural compounds for multiple biological targets involved in the onset and development of obesity, aging, and associated pathologies. Mainly, they have been attributed to exert as anti-diabetic, anti-inflammatory, antioxidant, anti-carcinogenic and other biological properties [142,144,145].

The most complex type of flavonoid are proanthocyanidins (PACs), also known as condensed tannins. PACs are dimers, trimers, oligomers and polymers of elementary flavan-3-ols subunits, mainly epicatechin, catechin, gallocatechin, and epigallocatechin (Figure 5) [148,149]. The degree of polymerization can range between 3 and 11 subunits [150] and are classified into class-A and class-B PACs attending to the interflavanic linkages, distribution and biological activities (Figure 5) [148]. PACs are responsible for red, blue, and purple colours of fruits and flowers, such as grapes, strawberries, cinnamon, cocoa or tea [151].

They exert a wide range of bioactivities due to their complex hydroxylation patterns, structure and other chemical modifications such as methylations and glucuronidation [147,152]. Dimeric and oligomeric PACs can be absorbed in small intestine by passive diffusion through TJs-mediated paracellular transport, whereas polymeric PACs are normally partially degraded in small phenolic compounds by intestinal microbiota in colon (Figure 5) [142,152]. PACs are subjected to phase-II metabolism in gut and liver [147,152] and, once in circulation, they performed different potential health benefits by metabolism modulation through both biochemical and epigenetic mechanisms in a context of obesity [152] but it must be already determined during aging process [146]. As well as other phenolic compounds, PACs have been found to function as anti-oxidant, cardioprotective, neuroprotective, immunomodulatory, anticancer, antidiabetic and anti-obesity agents [150,153,154].

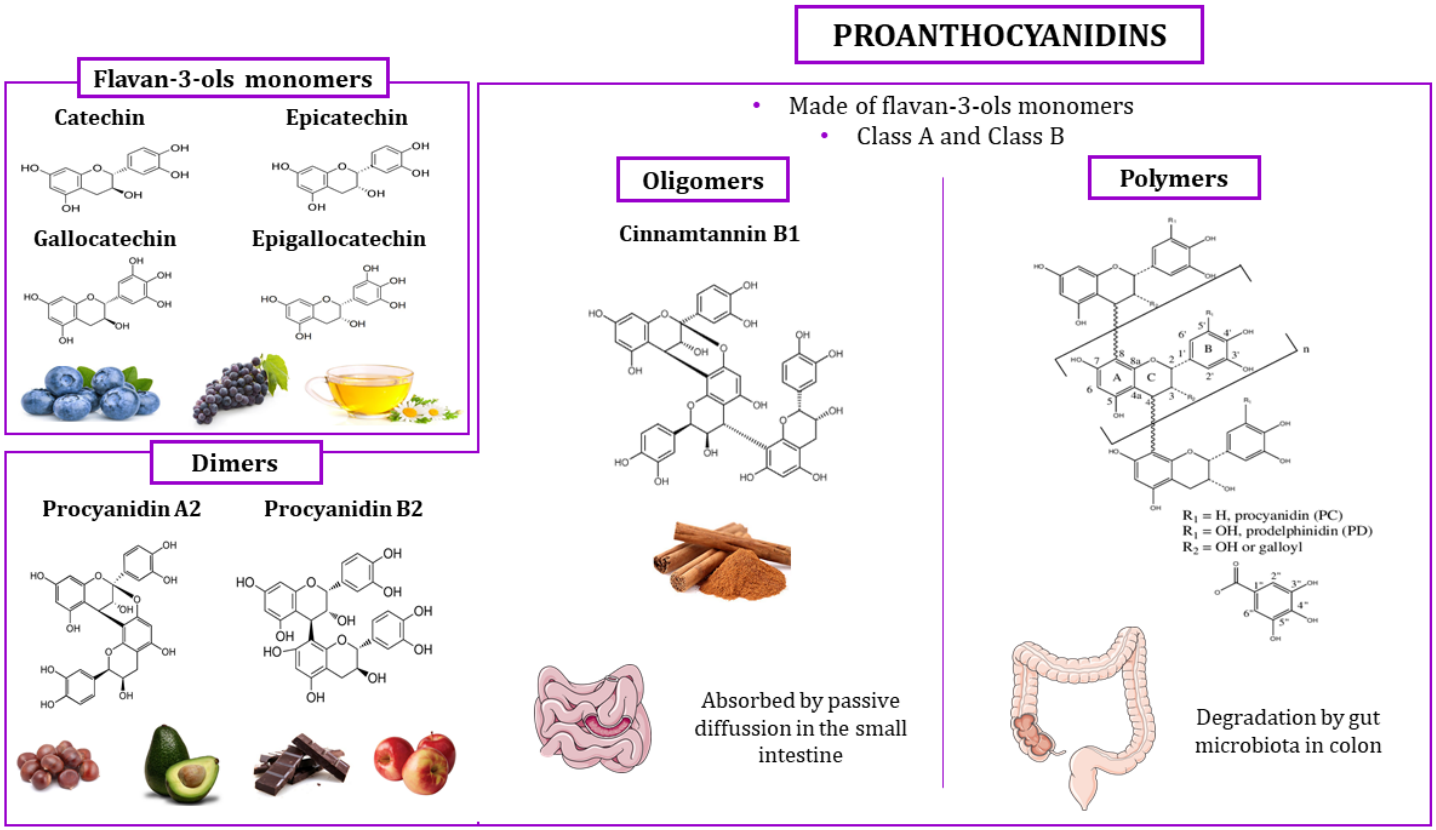

Figure 5. Proanthocyanidins classification, natural sources and metabolism.

3.2. Proanthocyanidins and metabolic syndrome

PACs are the most abundant type of flavonoids in occidental human diets [152]. In recent years, proanthocyanidins have been gaining prominence as a nutraceutical in the treatment and prevention of obesity and metabolic syndrome through metabolism modulation [155]. However, molecular mechanisms of action of PACs are not fully understood nowadays. It is known that PACs are able to affect lipid metabolism by inhibiting lipases and amylases, causing a reduction in fat depots and, consequently, body weight in both animal and human studies [151,156,157]. Moreover, PACs have been found to decrease TAGs and cholesterol levels in plasma, which are also associated to obesity, T2D and CVDs development [37,158]. Epigenetic modifications at DNA, histone and microRNA level have been pointed out as other PACs-associated anti-obesity mechanism [151,152,159]. Regarding the protective role at intestinal level, PACs diminish inflammation by interaction with gut microbiota and modulating NF-κB signalling pathway [151,160].

Particularly, grape-seed proanthocyanidin extract (GSPE) has been widely attributed as anti-obesity agent, especially when administered preventively or simultaneously to the obesogenic challenge. GSPE induces a reduction in food intake, adiposity and body weight gain in healthy [161] and obese young rats [157,162,163]. GSPE is also effective in reducing food intake, body weight and tumour development in healthy aged rats [164], as well as body weight gain, adiposity and hepatic steatosis in obese aged rats [35]. So that, GSPE would be an interesting candidate as dietary supplement in the prevention and treatment of metabolic syndrome-associated disruptions.

All in all, PACs constitute a good therapeutic alternative to traditional dietary and physical interventions in the treatment of obesity and metabolic syndrome due to their numerous positive effects on health. However, new studies are necessary to standardize optimal doses and time of administration, as well as trying to elucidate the underlying specific mechanisms of action.

3.3. Polyphenols in the modulation of endotoxemia and intestinal barrier integrity in obesity and aging

Up to date, little is known about the role of proanthocyanidins in the modulation of metabolic endotoxemia, especially the underlying mechanisms of their bioactivity in

obese and aged individuals. For that reason, we decided to focus in this section on the more general group of bioactive compounds, the polyphenols.

Resveratrol is one of the polyphenols that has been shown to have the most positive effects in reducing endotoxemia. In cells, resveratrol is able to reverse the pro-inflammatory profile of LPS-stimulated macrophages in a dose-dependent manner [165]. Both quercetin and curcumin improve intestinal integrity by modulation of TJs organization in *in vitro* models for intestinal barrier function [166,167]. In animal studies, resveratrol showed protective effects against endotoxemia induced with LPS administered intraperitoneally, both at the adrenal and cerebral levels, by reducing oxidative stress levels [168,169] . Moreover, quercetin was effective in limiting intestinal inflammation in LPS-treated mice organoids [170].

Chen et al. demonstrated that resveratrol is also able of modulating metabolic endotoxemia by decreasing plasma levels of LPS, systemic inflammation, hepatic steatosis, and intestinal permeability in mouse and rat models of HFD-induced obesity. The authors suggested that a possible mechanism of action in ameliorating metabolic endotoxemia was by increasing gene expression of TJs, thus improving intestinal barrier integrity as well as modulating the endocannabinoid system in the colon [171,172]. Activities of PACs strongly depends on the degree of polymerizations: oligomers are more effective in reducing body weight gain and development of insulin resistance whereas polymeric PACs are better in maintaining gut barrier function and protecting from intestinal inflammation [173]. In this regard, several protective properties have been attributed to GSPE in a context of intestinal dysfunction and derived metabolic endotoxemia and both intestinal and systemic inflammation at different dietary and pharmacological doses as well as points of administration [37,88,174].

Regarding clinical trials, *Wong et al.* demonstrated that a grape polyphenol extract was efficient in reducing endotoxemia not only *in vitro* but also in people with overweight and obesity but without influencing systemic inflammation [175]. Moreover, a green tea extract rich in catechins was able to restore intestinal integrity and decrease endotoxin absorption in people with metabolic syndrome compared with healthy controls [176]. GSPE administered to human colon tissues from patients with colorectal carcinoma also exerted beneficial effects by preventing epithelial

barrier disruption in a dose-dependent manner [81]. Therefore, more research is needed to develop proper experimental models adequate to explore the potential of GSPE and other natural extracts to modulate barrier integrity. These models will also be useful in the study of polyphenols as nutraceuticals in humans.

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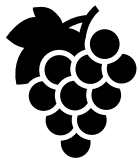
UNIVERSITAT ROVIRA I VIRGILI
ROLE OF FLAVONOIDS IN THE MODULATION OF INTESTINAL ALTERATIONS ASSOCIATED WITH METABOLIC CHALLENGES: OBESITY AND AGING
Marta Sierra Cruz



HYPOTHESIS & OBJECTIVES

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HYPOTHESIS & OBJECTIVES

Worldwide incidence of obesity and aging have dramatically increased in the last decades. A direct contribution of obesity to the aging process can be established, so they have become a focus of attention for researchers to find effective anti-obesity treatments to improve elderly life quality. Consumption of high-fat/high-sugar diets provokes microbiota dysbiosis as well as intestinal damage, leading to metabolic endotoxemia. The subsequent inflammatory state is a very relevant factor in the onset and development of both obesity and aging-related pathologies.

Proanthocyanidins, natural compounds present in some foods from plant origin, have a protective effect against obesity-associated metabolic and intestinal impairment caused by a nutritional challenge as high-fat/high-sugar diets. In fact, in previous studies, we have demonstrated that a pharmacological dose (500 mg/kg body weight) of grape seed proanthocyanidin extract (GSPE) exerts protective effects against cafeteria diet-induced metabolic disruption and intestinal damage in young rats, although the underlying mechanisms are unknown yet.

Based on these results, we hypothesize that GSPE could prevent or delay the loss of metabolic flexibility associated with obesogenic diet and aging via interacting with the gastrointestinal tract, thus reducing metabolic endotoxemia and systemic inflammation.

In order to demonstrate our hypothesis, we stablished the following objectives:

Objective 1. To characterize the long-term effectiveness of the grape seed proanthocyanidin extract (GSPE) on metabolic disruptions associated to aging.

A dose of 500 mg/kg b.w. of GSPE is known to reduce food intake, adiposity and body weight gain in both healthy young and obese rats fed with cafeteria diet. Preventive GSPE supplementation effects were maintained for several weeks after the treatment in young rats. Hence, we aimed to determine whether GSPE could also modulate food intake, body weight gain and metabolic alterations in aged obese rats when administered preventively, probably exerting a long-term anti-aging effect.

Objective 2. To evaluate the effect of GSPE on the modulation of metabolic alterations and systemic inflammation associated to obesity and aging.

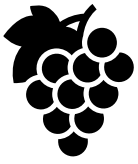
GSPE has been showed to have beneficial effects against metabolic syndrome in young and adult animal models for obesity. Pharmacological doses of GSPE reverted lipid and glucose metabolism-alterations as well as systemic inflammation caused by cafeteria diet consumption in young rats. So, we aimed to evaluate the potential effects of GSPE on maintaining metabolic homeostasis in aged rats facing an obesogenic challenge.

Objective 3. To identify the mechanisms through which GSPE modulates endotoxin intestinal translocation and subsequent metabolic endotoxemia.

GSPE can reduce intestinal permeability and subsequent endotoxemia, however its mechanisms of action are not fully understood. We aimed to characterize LPS intestinal translocation pathways and whether GSPE could modulate metabolic endotoxemia in both a Caco-2 cell *in vitro* model and *in vivo* model of cafeteria diet-induced obesity.

Objective 4. To analyse the protective effects of GSPE on intestinal alterations associated to aging and obesity.

Both dietary and pharmacological doses of GSPE haven been demonstrated to improve obesity-associated intestinal damage and decrease intestinal inflammation. GSPE is able to modulate metabolic endotoxemia by reducing endotoxin translocation via paracellular pathway. Hence, we aimed to evaluate cafeteria diet-associated intestinal damage in aged rats.



RESULTS

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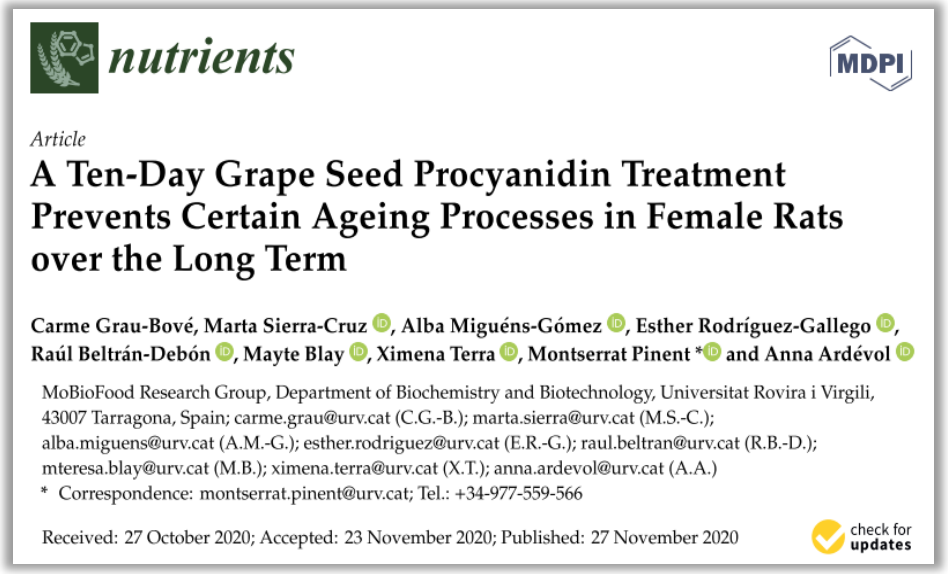
MANUSCRIPT 1



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A Ten-Day Grape Seed Procyanidin Treatment Prevents Certain Ageing Processes in Female Rats over the Long Term

Carme Grau-Bové, Marta Sierra-Cruz, Alba Miguéns-Gómez, Esther Rodríguez-Gallego, Raúl Beltrán-Debón, Mayte Blay, Ximena Terra, Montserrat Pinent * and Anna Ardévol

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A ten-day grape seed procyanidin treatment prevents certain ageing processes in female rats over the long term

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Abstract: Adaptive homeostasis declines with age and this leads to, among other things, the appearance of chronic age-related pathologies such as cancer, neurodegeneration, osteoporosis, sarcopenia, cardiovascular disease and diabetes. Grape seed-derived procyanidins (GSPE) have been shown to be effective against several of these pathologies, mainly in young animal models. Here we test their effectiveness in aged animals: 21-month-old female rats were treated with 500 mg GSPE/kg of body weight for ten days. Afterwards they were kept on a chow diet for eleven weeks. Food intake, body weight, metabolic plasma parameters and tumor incidence were measured. The GSPE administered to aged rats had an effect on food intake during the treatment and after eleven weeks continued to have an effect on visceral adiposity. It prevented pancreas dysfunction induced by ageing and maintained a higher glucagon/insulin ratio together with a lower decrease in ketonemia. It was very effective in preventing age-related tumor development. All in all, this study supports the positive effect of GSPE on preventing some age-related pathologies.

Keywords: ageing; procyanidins; food intake; adiposity; glucagon/insulin; tumor

1. Introduction

Adaptive homeostasis is a highly conserved process whereby cells, tissues and whole organisms transiently activate various signalling pathways in response to short-term mild internal or external perturbations, thereby resulting in transient changes in gene expression and stress resistance. There is a great deal of evidence that suggests that adaptative homeostasis declines with age. In fact, ageing is associated with a twofold detrimental impact on adaptive homeostasis [1]. Firstly, aged organisms lose their ability to rapidly modulate the adaptive homeostatic response and secondly, the compensatory basal increase in stress-responsive enzymes further compresses the maximal range of responses thus diminishing cellular ability to efficiently mitigate damage. All of this loss of adaptation leads to, among other things, chronic age-related pathologies such as cancer, neurodegeneration, osteoporosis, sarcopenia, cardiovascular disease and diabetes [2].

Metabolic derangement is one of the "seven pillars" considered among the basic mechanisms associated with age-related pathologies [3]. As glucose metabolism plays a key role in the energy management of the whole organism, its dysregulation with ageing affects several metabolic aspects [4]. Age changes in hepatic glucose output and peripheral insulin sensitivity seems to be more closely related to changes in body composition than to the ageing process itself [5]. Indeed, the intra-abdominal or visceral fat pad shows the highest association risk for diabetes mellitus, hypertension, atherosclerosis, dyslipidemia, cancers and mortality compared with peripheral obesity [6]. There is also controversy regarding the role of age in the ability of β-cells to function [5] and also its effect on β-cells although a few authors point to a higher hepatic sensitivity to plasma glucagon in older subjects [7].

Polyphenols are a broad spectrum of structures widely found in fruits and vegetables and derived products and also in beverages such as chocolate, green tea, coffee and wine [8]. They have been shown to protect against most age-related pathologies (cancer [9]; hypertension [10–12]; sarcopenia [13,14], neurodegeneration [15–20], osteoporosis [21,22] and cataract formation [23]). One group of these polyphenols is grape seed-derived procyanidins (GSPE), which include several flavanols, procyanidins and some phenolic acids [24]. They have been widely studied as antiobesogenic and antidiabetic agents [8,25,26], as being protective against

atherogenic indexes [27] and renal failure [28] and as anti-cancer agents [29]. However, their effectiveness on ageing processes in the metabolism has not been proved.

We have previously shown that some GSPE doses act as satiating agents in young healthy rats [30], limiting their body weight increase and adiposity [25] among other properties effective against metabolic syndrome [31,32]. Indeed, we have shown that their effects on body weight and adiposity [33] continue over the long term once the GSPE administration has finished [34]. Considering these beneficial effects of GSPE and the evidence that dietary restriction has been proven to extend lifespan [35], we hypothesize that the doses of GSPE with satiating properties may be beneficial in counteracting the ageing-induced loss of buffering in the body. We therefore aim to demonstrate this hypothesis after a 10-day GSPE oral treatment in aged rats and to observe its long term anti-ageing outcome, focusing our study on body weight and metabolism.

2. Materials and Methods

2.1. Proanthocyanidin Extract

The grape seed extract rich in proanthocyanidins (GSPE) came from Les Dérivés Résiniques et Terpéniques (Dax, France). According to the manufacturer, the GSPE used in this study (lot 207100) had a total proanthocyanidin content of 76.9% consisting of a mixture of monomers of flavan-3-ols (23.1%), dimers (21.7%), trimers (21.6%), tetramers (22.2%) and pentamers (11.4%).

2.2. Animal Model

In this study, 34 female Wistar rats were used, 10 of which were two months old (weighing 210–220 g) and 24 of which were 21 months old (weighing 300–350 g). The rats were obtained from Envigo (Barcelona, Spain). They were housed individually at a room temperature of 23 ◦ C with a standard 12 h light-dark cycle, ventilation and ad libitum access to a standard chow diet (2014 Teklad Global 14% protein rodent maintenance diet, Envigo, Barcelona, Spain) and tap water.

2.3. Experimental Design

The experiment was divided into two parts (Figure 1). The first consisted of 10 days of treatment with GPSE by oral gavage and an evaluation of its immediate effects on food intake and body weight. The second consisted of an assessment of the long term effects of this 10-day GSPE treatment on the metabolism. All procedures were approved by the Experimental Animal Ethics Committee of the Generalitat de Catalunya, Spain (Department of Territory and Sustainability, General Directorate for Environmental and Natural Policy, project authorization code: 10183).

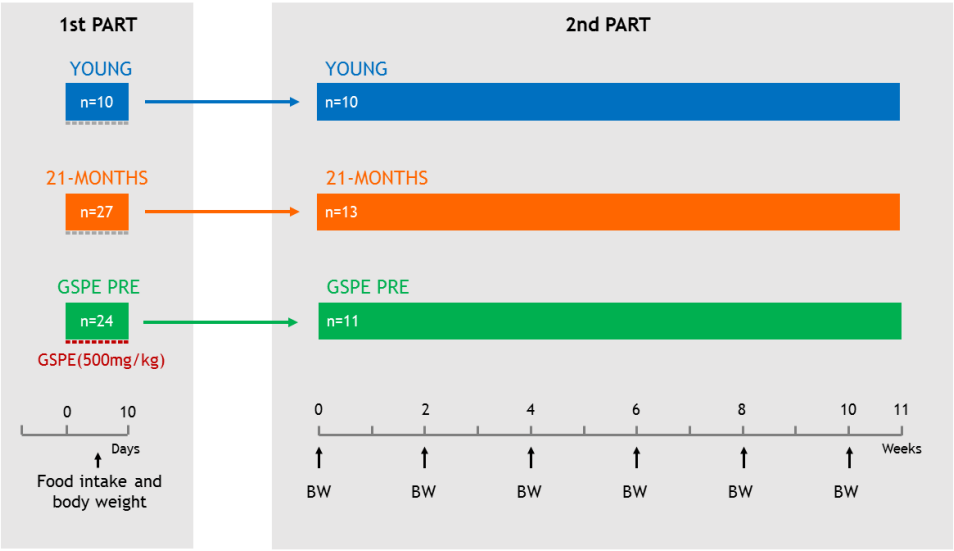

Figure 1. Schematic diagram of the experimental design. The experiment was divided into two parts. In the first part, the group named GSPE PRE animals were gavaged daily with a dose of 500 mg/kg of grape seed-derived procyanidins (GSPE) for 10 days while YOUNG and 21-MONTHS animals were gavaged with a vehicle. Food intake was recorded daily and body weight (BW) was measured after the 10 days of treatment. In the second part, all rats were maintained equally for 75 days and body weight was recorded every two weeks.

For the first part of the study, after a week of adaptation to the environment and another week of adaptation to oral gavage, the rats were weighed and divided into three experimental groups as follows: (1) YOUNG, which consisted of 10 two-month-old rats; (2) 21-MONTHS, which consisted of 27 twenty-one-month-old rats; (3) GSPE PRE, which consisted of 24 twenty-one-month-old rats.

For 10 days, all of the animals were fasted from 15:00 h. The GSPE was dissolved in tap water and orally gavaged to the GSPE PRE animals at a dose of 500 mg GSPE/kg of body weight at 18:00 h, one hour before the dark onset. Animals in the YOUNG and 21-MONTHS groups received an equivalent volume of tap water at the same time points. The chow diet was administered at the dark onset (19:00 h). The chow intake was measured after 20 h, the next day at 15:00 h, when the animals were fasted again. At the beginning and end of the 10-day treatment, the rats were weighed.

After the treatment, 13 animals from the 21-MONTHS group and 11 animals from the GSPE PRE group together with all of the YOUNG animals (n = 10) entered the second part of the study, which consisted of maintaining them for 75 more days with a chow diet and body weight records every two weeks.

2.4. Blood and Tissue Collection

At the end of the study, the animals were fasted for 12 h and euthanized by decapitation. The blood was collected using heparin (Deltalab, Barcelona, Spain) as anticoagulant. Plasma was obtained by centrifugation (1500 g, 15 min, 4ºC) and stored at −80ºC until analysis. The different white adipose tissue depots (retroperitoneal (rWAT), mesenteric (mWAT) and periovaric (oWAT)) and the brown adipose tissue (BAT), liver, kidneys, spleen, stomach, caecum and femur were rapidly removed, weighed, snap-frozen in liquid nitrogen and stored at −80ºC. When identified, tumorous tissues were excised and weighed.

2.5. Biochemical Variables

Commercial colorimetric enzymatic kits were used to measure levels of glucose, triacylglycerol, cholesterol, urea, creatinine (QCA, Tarragona, Spain), non-esterified fatty acids (NEFAs) (Wako, Neuss, Germany) and β-hydroxybutyrate (Ben Biochemical Enterprise, Milano, Italy) in the plasma samples in accordance with the manufacturers' instructions. Commercial ELISA kits were used to quantify plasma levels of insulin (Millipore, Madrid, Spain) and glucagon (Mercodia, Uppsala, Sweden).

2.6. Statistical Analysis

At the end of the study, statistical analysis was performed with all collected data using one-way ANOVA with Dunnet's post-hoc test taking the 21-MONTHS group as control. A chi-squared test was used to assess the association between tumor incidence and the variables of interest (age, treatment) in XLSTAT 2020.1 (Addinsoft, Spain) statistical software. The statistical significance for both tests was set at $p < 0.05$.

3. Results

3.1. GSPE Reduces Food Intake and Body Weight in the Short-Term in Aged Rats

Our first goal was to find out whether a dose of GSPE with satiating properties in young animals [25] was also effective in aged rats. The daily food intake was equivalent between the YOUNG and 21-MONTHS groups (Figure 2a). In agreement, the accumulated food intake for the 10 days was not different between the two groups (Figure 2b). The GSPE treatment over 10 days reduced the 20 h food intake almost daily in comparison with the 21-MONTHS rats (Figure 2a). In this case, there was a statistically significant effect on the accumulated food intake for the 10-day GSPE treatment group versus the 21-MONTHS group, as shown in Figure 2b. This reduction in the energy entering the organism over these days brought about a slightly lower body weight after the end of the treatment in the GSPE PRE rats compared with the 21-MONTHS rats (Figure 2c). As expected, the young rats showed a lower body weight than the 21-MONTHS rats (Figure 2c).

3.2. The GSPE Effect on Body Weight and Adiposity Continued for Several Weeks after Administration

Once the GSPE treatment was finished, the rats were kept for eleven weeks to evaluate the long term effects of GSPE on the 21-MONTHS rats [33]. Figure 3a shows the percentage of body weight increase during this period. The highest percentage increase was found in the young rats because they were undergoing a growth period in their lives. The 21-MONTHS rats showed a significantly lower weight increase. Initially the GSPE-treated rats showed a body weight increase similar to that of the 21-MONTHS rats (Figure 3a). However, in the tenth and eleventh weeks they

significantly increased their weight to reach a final body weight close to the 21-MONTHS group (Figure 3b).

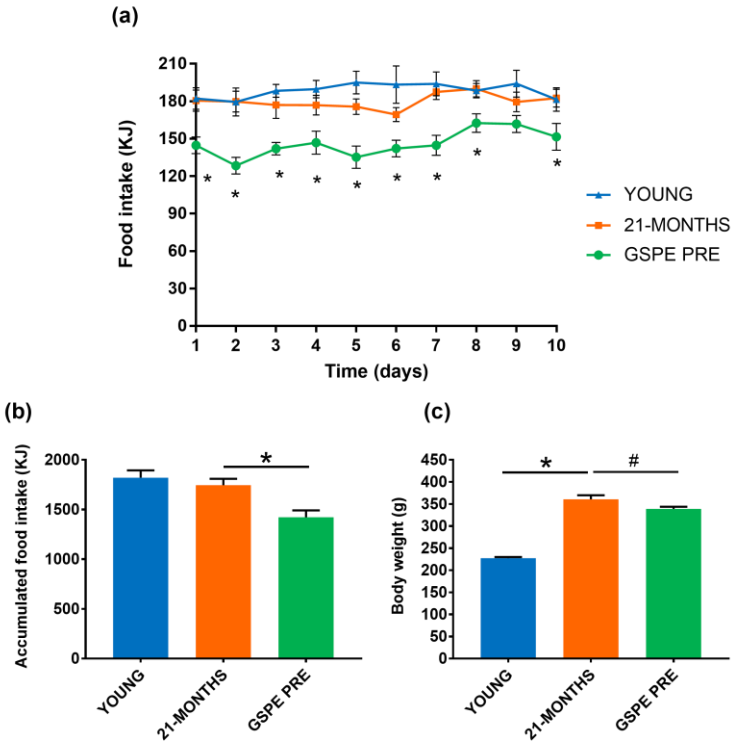

Figure 2. Effect of the 10-day treatment on food intake and body weight. (a) Daily food intake, where blue, orange and green represent YOUNG, 21-MONTHS and GSPE PRE groups, respectively. **(b)** Accumulated food intake over the 10 days of treatment. **(c)** Body weight at day 10. Values are means ± SEM. * p-value < 0.05, # p-value < 0.1 compared with 21-MONTHS rats.

Table 1 shows the weight of the tissues at the end of the experiment. As expected, due to their relative difference in size, all of the 21-MONTHS animals presented bigger organs than the young animals. When we compared the GSPE-treated rats with the untreated 21-MONTHS group, despite the similar body weight, the percentage of visceral adiposity was significantly lower in the GSPE PRE rats (Table 1). There were no differences in most of the other tissues except for the liver, which was also smaller in the GSPE-treated rats. The kidney showed a trend towards a lower size too.

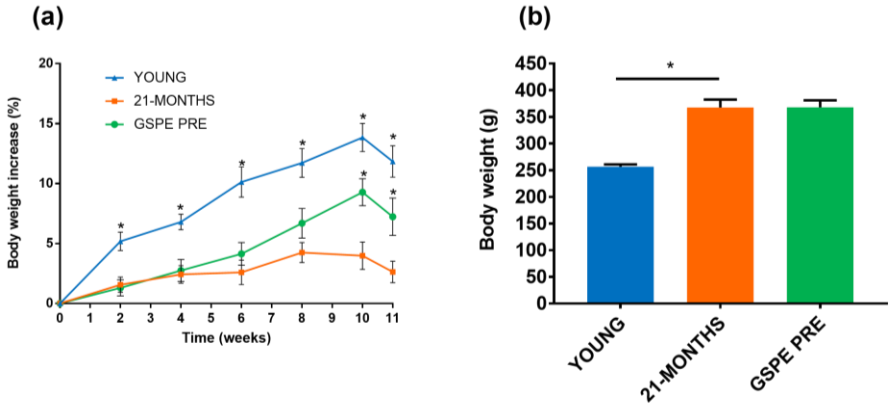

Figure 3. Body weight changes after GSPE pre-treatment. (a) Percentage of body weight increase from day 10 of the experiment. Body weight was measured once every two weeks throughout the whole experiment. Blue, orange and green represent YOUNG, 21-MONTHS and GSPE PRE groups, respectively. **(b)** Body weight at the end of the experiment. Values are means ± SEM. * $p < 0.05$ compared with 21-MONTHS rats.

3.3. Aged GSPE Pre-Treated Rats Showed a Higher Fasting Glucagon/Insulin Ratio Eleven Weeks after the Treatment

Looking at the biochemical parameters in the plasma of fasted YOUNG and 21-MONTHS rats, glucose, non-esterified fatty acids (NEFA), urea and creatinine levels were unaffected by ageing (Table 2). A GSPE pre-treatment (GSPE PRE) showed a trend towards increasing urea. Regarding endocrine pancreas hormones, plasma insulin and glucagon were greatly increased by the ageing process (Table 2) and the GSPE pre-treatment limited the increase in the insulinemia. To gain a better picture of the metabolic status of these animals, we worked on some ratios that provided us with more information. Figure 4a shows that the GSPE pre-treatment did not avoid the increase in insulin resistance brought about by the ageing processes as indicated by the index of insulin resistance HOMA-IR. Conversely, the GSPE pre-treated group (GSPE PRE) showed a normalized pancreatic response as indicated by the lower HOMA-β of this group versus the 21-MONTHS group (Figure 4b). When we compared the glucagon/insulin ratio in the plasma of fasted animals, we found no statistically different results due to the ageing process but we did find that the GSPE pre-treated group clearly showed a higher ratio (Figure 4c). To complete the picture, Figure 4d shows that the 21-MONTHS animals produced a limited amount of β-

hydroxybutyrate derived from NEFA. Finally, there were no changes in renal functionality due to ageing or GSPE pre-treatment (GSPE PRE) as defined by the urea/creatinine ratio (Figure 4e).

Table 1. Morphometric characteristics at the end of the experiment (week 11)

Variable	YOUNG	21-MONTHS	GSPE PRE
n	10	13	11
Body weight (g)	256.6 ± 4.3 *	367.4 ± 15.0	366.8 ± 14.2
mWAT (g)	3.7 ± 0.2 *	13.1 ± 1.2	10.5 ± 1.3
oWAT (g)	6.8 ± 0.2 *	16.6 ± 1.5	15.5 ± 1.5
rWAT (g)	4.0 ± 0.3 *	11.1 ± 1.1	10.6 ± 1.1
Total visceral WAT (g)	14.6 ± 0.2 *	39.5 ± 3.4	34.8 ± 3.3
BAT (g)	0.4 ± 0.0 *	0.7 ± 0.1	0.7 ± 0.1
% visceral adiposity	5.4 ± 0.2 *	11.3 ± 0.6	9.5 ± 0.7 *
Liver (g)	6.2 ± 0.2 *	8.7 ± 0.4	7.7 ± 0.2 *
Spleen (g)	0.5 ± 0.0 *	0.8 ± 0.0	0.8 ± 0.0
Kidney (g)	0.8 ± 0.0 *	1.0 ± 0.0	0.9 ± 0.0 #

mWAT: mesenteric white adipose tissue; oWAT: periovaric white adipose tissue; rWAT: retroperitoneal white adipose tissue; BAT: brown adipose tissue; total visceral WAT: sum of all white adipose tissues. Values are means ± SEM. * $p < 0.05$ compared with 21-MONTHS rats. Trends: # $p < 0.1$ compared with 21-MONTHS rats.

3.4. GSPE Limits the Development of Tumors in 21-MONTHS Rats

One of the characteristics of ageing is an increase in the presence of tumors [36]. When the rats were dissected, all of the tumors found were counted, weighed and classified (Supplementary Table S1). Figure 5 shows that we found no tumors in the YOUNG rats but the incidence of spontaneous tumors in the 21-MONTHS rats was 46.2%. The GSPE pre-treatment limited their presence. The chi-squared test, comparing both aged groups, showed a significant reduction of the present of tumors with the GSPE pre-treatment of 9.1% (Fisher's exact test, $p < 0.078$).

Table 2. Plasma biochemical characteristics at the end of the experiment (week 11).

Variable	YOUNG	21-MONTHS	GSPE PRE
Plasma			
Glucose (mM)	7.3 ± 0.3	7.0 ± 0.3	8.1 ± 0.6
TAG (mM)	0.4 ± 0.1 #	0.6 ± 0.1	0.5 ± 0.1
NEFA (mM)	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
Cholesterol (mM)	2.6 ± 0.1 *	4.5 ± 0.4	4.2 ± 0.4
β-Hydroxybutyrate (mM)	0.7 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
Urea (mM)	4.2 ± 0.2	3.8 ± 0.2	4.3 ± 0.1 #
Creatinine (μM)	7.1 ± 0.3	7.7 ± 0.6	7.3 ± 0.7
Insulin (pM)	182.7 ± 1.0 *	322.2 ± 36.4	233.7 ± 13.4 *
Glucagon (pM)	7.2 ± 1.3 *	18.2 ± 2.5	18.8 ± 1.9

Values are means ± SEM. * $p < 0.05$ compared with 21-MONTHS rats. Trends: # $p < 0.1$ compared with 21-MONTHS rats. TAG: triglycerides; NEFA: non-esterified fatty acids.

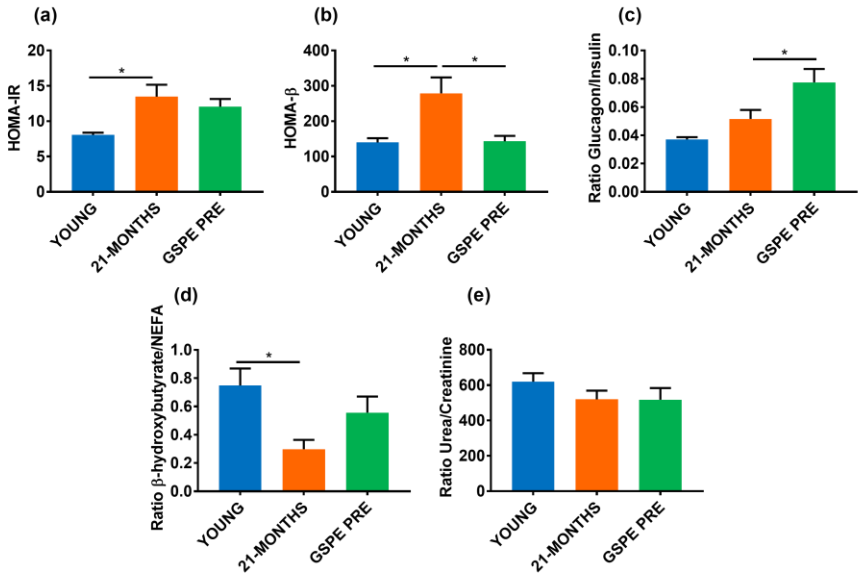

Figure 4. Biochemical characteristics of the groups analyzed at 13 weeks. (a) Insulin resistance HOMA-IR index. **(b)** Insulin resistance HOMA-β index. **(c)** Glucagon/insulin ratio. **(d)** β-hydroxybutyrate/non- esterified fatty acids (NEFA) ratio. **(e)** Urea/creatinine ratio. Values are means ± SEM. * $p < 0.05$ compared with 21-MONTHS rats.

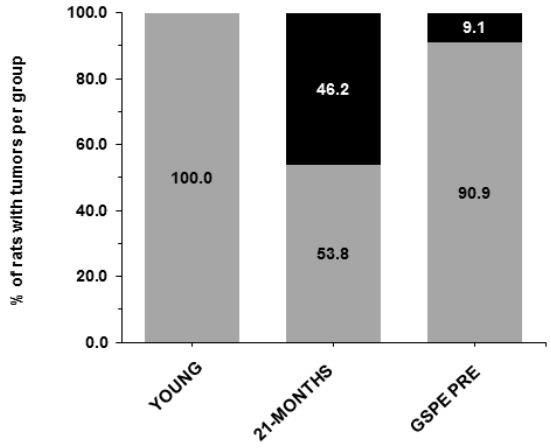

Figure 5. Percentage of tumors per group. Values are the percentage of rats without tumors (grey) and with tumors (black) per group studied.

4. Discussion

Ageing is a physiological process characterized by metabolic changes that lead to obesity, insulin resistance and dyslipidemia, which are risk factors for ageing-associated pathologies such as diabetes and cardiovascular disease [5,36]. GSPE has the ability to prevent several of these metabolic disruptions in young rats [33,34,37]. Here we have shown that GSPE maintains its ability in the 21-MONTHS animals. In addition, this GSPE treatment showed a trend to prevent the development of tumorigenic tissue growths, also closely associated with the ageing processes.

In previous studies, we defined a dose of 500 mg/kg GSPE as responsible for reducing food intake and body weight in young rats [25]. Here we have found a similar effect on food intake in aged animals over the 10 days that the treatment lasted. In this case, the food intake reduction of around 20% had only a slight effect on body weight immediately after the treatment because these animals had already reached adulthood and their body weight remained constant. In young rats, studies on a caloric restriction of 20% have shown reductions in body weight increase of around 40% in fifteen days [25,38]. In 21-month-old animals, the same caloric restriction takes several weeks to obtain a 30% decrease in body weight [35]. We found that in young rats the effects of GSPE were maintained for several weeks after the treatment had finished [33]. We have now shown that in aged rats, the GSPE effect limiting body weight increase was also maintained for eight more weeks after the end of the treatment. This was probably due to the ability of proanthocyanidins to limit adipose accrual several weeks after the end of the treatment [34]. Indeed, we also found that GSPE was able to maintain a lower percentage of visceral adiposity 11 weeks after the treatment. The effects of this GSPE dose as a preventive works in a similar way to caloric restriction interventions that reverse ageing-associated visceral fat increase and have an important impact on decreasing insulin resistance [39]. Our results eleven weeks after the end of the treatment with GSPE showed a preventive effect on the increase in the HOMA-β index as also found in some other models of caloric restriction [40]. However, it did not show any clear protective effect on the insulin resistance index (HOMA-IR). This lack of effect of GSPE on insulin resistance could be the explanation for the dyslipidemic profile found in the GSPE pre-treated group at the end of the study. There is a definite cause/effect associated with insulin resistance

on muscle and adipose tissue [41] and dyslipidemia [42]. Dyslipidemia, together with a decreased ketosis, has also been observed in recent work with 24-month-old male Wistar rats [43,44] and is in line with what we saw in our 21-month-old female rats. There have been some controversial results regarding caloric restriction effects on ketone bodies. A lower ability with ageing to synthetize ketone bodies in the intestine has been reported [45] as has a lower consumption of ketone bodies in the kidney [46]. In both examples, these reductions were reverted by caloric intake restriction. We previously found a higher expression of HMGCS2 in the liver of young rats five weeks after the last GSPE dose [34] suggesting a greater ability in the GSPE-treated rats to produce ketone bodies. In the present study, we did not have a statistically different effect on plasma β-hydroxybutyrate but this could be due to the length of the study.

In a fasting situation when blood insulin is low, glucagon levels are high and therefore fat oxidation and ketosis are increased and hepatic lipogenesis is activated [47]. Glucagonemia was also increased in our 21-MONTHS rats as found by Fernández et al. [44], although there is no wide consensus on glucagonemia and ageing [7]. Here, GSPE brought about a situation different than the 20% caloric restriction of Fernández et al. [44]. This higher glucagonemia in GSPE pre-treated rats clearly produced a higher glucagon/insulin ratio despite the weeks without treatment. This could explain the trend found in the limited increase in visceral adiposity in these animals. A higher presence of glucagon versus insulin during a fasting situation favors higher fat oxidation [48], which together with a higher liver sensitivity to glucagon on ageing [7] would produce a higher hepatic gluconeogenesis. We did not find a statistical change on glycemia in these GSPE pre-treated rats sacrificed in a fasting situation, but we cannot discard that it happened at some point earlier than the several weeks post-treatment when we were measuring it.

One of the significant organ systems that declines with ageing is the kidney. Changes in renal structure (reduction in mass) and function (glomerular filtration rate (GFR)) accompany advancing age [49]. Here, we found no effect of age on kidney size nor did we find any on urea, creatinine or their ratio. However, we did observe a GSPE trend after several weeks towards a smaller kidney size and an increase in urea versus the 21-MONTHS group but without any significant change in the urea/creatinine ratio,

suggesting the maintenance of the glomerular filtration rate. A similar dose of 500 mg/kg GSPE had beneficial effects on reducing induced acute renal injury and chronic kidney fibrosis in young mice [50] and diabetic-associated renal injury in young rats [28]. Our results therefore suggest no clear preventive effects on kidney functionality.

All in all, this GSPE pre-treatment produced a long term effect close to a caloric restriction state in the rats. Its preventive effect against tumorigenesis was also observed. Caloric restriction prevents tumorigenesis by decreasing the metabolic rate and oxidative damage [51]. Here, we found spontaneous tumors on 46.2% of the 25-month-old rats at the end of the study, a value that was lower than the reported number of age-associated tumors on female Wistar rats [52]. With the sample size used in this study, the GSPE treatment showed a trend towards a lowered incidence of tumors on GSPE PRE rats to 9.1%. A bigger sample size would be necessary to fully demonstrate this effect. Related to the possible reasons involving caloric restriction, the GSPE tumor suppressing effect may be explained by its modulation of antiproliferative and proapoptotic genes [53] such as the tumor suppressing factor p53 [54] and NF-κβ [55] observed in different cancerous cell lines, their anti-inflammatory properties [56] and antioxidant properties [57].

5. Conclusions

We can conclude that GSPE showed interesting properties on 21-MONTHS rats. It acted to limit food intake resulting in a decreased body weight after treatment. Eleven weeks after the treatment, GSPE maintained its effects on limiting visceral adipose tissue growth, prevented the increase in the HOMA-β index and maintained a higher glucagon/insulin ratio together with a reduced incidence of age-associated spontaneous tumors. A few of these effects might be related to their caloric restriction mimetic effect.

Author Contributions: Conceptualization, A.A., M.P., X.T. and M.B.; Methodology and Investigation, C.G.-B., M.S.-C. and A.M.-G.; Data Curation, E.R.-G. and R.B.-D.; Writing—Original Draft Preparation, C.G.-B. and M.S.-C.; Writing—Review & Editing, E.R.-G., A.A. and M.P.; Visualization, R.B.-D., X.T. and M.B.; Supervision, M.B. and R.B.-D.; Project Administration, M.P. and E.R.-G.; Funding Acquisition, A.A. and X.T. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Supplementary material

Supplementary table 1. Number, weight and classification of tumour per group.

Group	Total animals (n)	Animals with tumour (n and %)	Weight (g)	Type of tumor (n and %)				
				Ovaries and Fallopian tubes	Intestine	Subcutaneous	Hypophysis	Pancreas
Young	10	0 (0.0%)	0.0 ± 0.0	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
21-MONTHS	13	6 (46.2%)	2.1 ± 1.4	2 (15.4%)	1 (7.7%)	2 (15.4%)	0 (0.0%)	1 (7.7%)
GSPE PRE	11	1 (9.1%)	0.01 ± 0.001	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (9.1%)	0 (0.0%)

Number of animals with tumours are shown in total units and percentage in parenthesis. Weight of tumours per group is shown as mean ± SEM. The tumours found in these rats were located in ovaries or fallopian tubes, subcutaneous, hypophysis and pancreas. The number of animals with each of these types of tumour are shown in total units and percentage in parenthesis.

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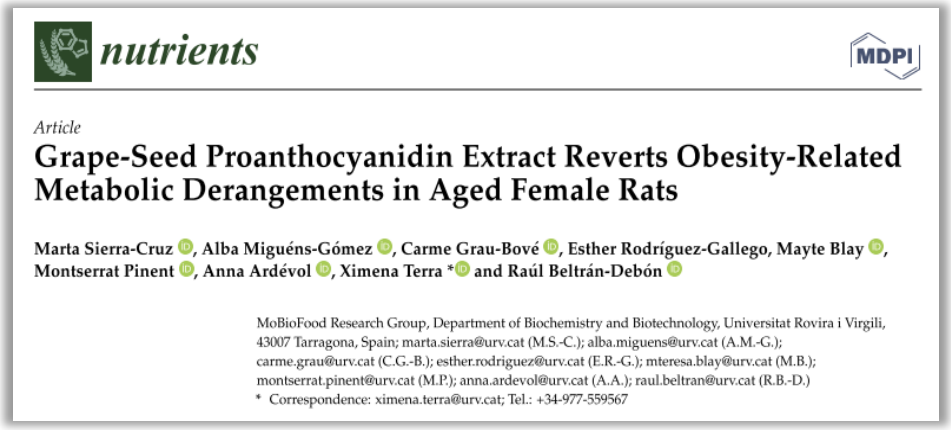

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Grape-Seed Proanthocyanidin Extract Reverts Obesity-Related Metabolic Derangements in Aged Female Rats

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Grape-seed proanthocyanidin extract reverts obesity-related metabolic derangements in aged female rats

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Abstract: Obesity and ageing are current issues of global concern. Adaptive homeostasis is compromised in the elderly, who are more likely to suffer age-related health issues, such as obesity, metabolic syndrome, and cardiovascular disease. The current worldwide prevalence of obesity and higher life expectancy call for new strategies for treating metabolic disorders. Grape-seed proanthocyanidin extract (GSPE) is reported to be effective in ameliorating these pathologies, especially in young animal models. In this study, we aimed to test the effectiveness of GSPE in modulating obesity-related pathologies in aged rats fed an obesogenic diet. To do so, 21-month-old rats were fed a high-fat/high-sucrose diet (cafeteria diet) for 11 weeks. Two time points for GSPE administration (500 mg/kg body weight), i.e., a 10-day preventive GSPE treatment prior to cafeteria diet intervention and a simultaneous GSPE treatment with the cafeteria diet, were assayed. Body weight, metabolic parameters, liver steatosis, and systemic inflammation were analysed. GSPE administered simultaneously with the cafeteria diet was effective in reducing body weight, total adiposity, and liver steatosis. However, the preventive treatment was effective in reducing only mesenteric adiposity in these obese, aged rats. Our results confirm that the simultaneous administration of GSPE improves metabolic disruptions caused by the cafeteria diet also in aged rats.

Keywords: obesity; ageing; metabolic syndrome; proanthocyanidins; adiposity; liver steatosis

1. Introduction

The prevalence of obesity has been increasing worldwide over the last 30 years [1]. Obesity is associated with low-grade inflammation and metabolic syndrome, which is characterized by alterations in glucose, fatty acids, and amino acid metabolism and leads both to a decrease in insulin sensitivity and a decline in one's ability to adjust to energy availability [2].

The prevalence of obesity is increasing steadily among the aged population [3] at a time when the number and proportion of older people are growing worldwide. By 2050, there will be roughly two billion people over the age of 60 [4]. Ageing is associated with the progressive loss of physiological functions [5] as well as metabolic alterations, such as increases in (1) abdominal white adipose tissue, (2) fat deposition in skeletal muscle and the liver, and (3) the expression of pro-inflammatory cytokines, all of which lead to a decrease in insulin sensitivity [5]. Together, obesity and ageing contribute to the development of associated diseases, mainly type-2 diabetes, cardiovascular diseases, and several types of cancer.

Since obesity contributes directly to the ageing process [6], effective anti-obesity treatments are needed to improve the quality of life of the elderly population. Traditional strategies have been based on physical exercise and dietary interventions. Studies on caloric restriction conducted in animal models have shown that this type of intervention increases life expectancy [7]. More specifically, caloric restriction can extend the life expectancy of mice and rats by 50% compared to control animals fed ad libitum [8,9]. However, weight loss in old, obese adults can also lead to a high loss of skeletal muscle or bone mass, which can be detrimental [4]. There is, therefore, an urgent need for innovation in therapeutic interventions aimed at treating obesity and ageing-related processes, mainly to improve quality of life and increase life expectancy. One interesting strategy that targets the elderly obese in this context is based on food bioactive compounds.

Food bioactive compounds are components (found in small quantities) of plants and lipid-rich foods [10]. An important example of these compounds are polyphenols. A group of polyphenols with potential beneficial effects on human health are proanthocyanidins (PACs) [11]. These are oligomers and polymers of monomeric

flavan-3-ols mainly found in everyday food and beverages such as grapes, cocoa, chocolate, red wine, and green tea [11–13]. The literature shows controversial results regarding the effect of PACs on adiposity and body weight. While some studies have shown that PACs may lead to a decrease in body weight by up-regulating energy expenditure-related genes, others have reported no such effectiveness [12,14,15]. Standardized methods, doses, and times of administration are therefore required. Apart from body weight modulation, several beneficial effects of PACs have been demonstrated in young experimental animals, including (1) a decrease in fatty acid synthesis and fat uptake and an increase in energy expenditure in skeletal muscle and the liver [13,15]; (2) a modulation of the neuropeptides involved in food intake and satiety [13]; (3) an inhibition of digestive enzymes, mainly amylase and lipase, which leads to a reduction in lipid and glucose absorption from the gut [12,13]; (4) an antioxidant beneficial effect on inflammatory processes through reduction in the activity of antioxidant enzymes, such as catalase or superoxide dismutase (SOD) [12]; and (5) hypolipidemic and hypotriglyceridemic effects that lead to an improvement in lipid metabolism and the attenuation of hepatic steatosis in mice fed a high-fat diet [16] and rabbits fed a high-fat, high-cholesterol diet [17].

These studies have then demonstrated that grape-seed proanthocyanidin extract (GSPE) shows potential beneficial effects against obesity and metabolic-related pathologies in young and adult animal models [18–20]. However, little is known about the potential benefits of GSPE in improving metabolic alterations in the elderly. Therefore, with this background and given the relevance of the increasing prevalence of the aged-obese population, it becomes essential to take actions to promote healthy ageing where PACs might have a role. We have previously evaluated the effects of a pharmacological dose of GSPE on young rats under an obesogenic challenge and found that GSPE reverted several features of the metabolic syndrome [21]. As far as we know, this is the first study to evaluate the effects of a PAC extract against both obesity and ageing. In this study we analyse the potential beneficial effects of an oral administration of a grape-seed proanthocyanidin extract on body weight gain and homeostatic buffering loss linked to obesity and ageing when administered either as a preventive or simultaneous treatment to aged rats fed an obesogenic diet.

2. Materials and Methods

2.1. Proanthocyanidin Extract

The grape-seed extract rich in proanthocyanidins (GSPE) was provided by Les Dérivés Résiniques et Terpéniques (Dax, France). According to the manufacturer, the GSPE used in this study (batch number: 207100) contains a total procyanidin content of 76.9% and consists of a mixture of monomers (23.1%), dimers (21.7%), trimers (21.6%), tetramers (22.2%), and pentamers (11.4%) of flavan-3-ols.

2.2. Animal model

A total of 42 aged Wistar female rats (21 months old), each weighing 300–350 g, were acquired from Charles River Laboratories (Barcelona, Spain). After one week of adaptation, the rats were individually housed in the animal quarters at 22ºC with a 12-h light/12-h dark cycle and fed a standard chow diet (Teklad 2014 Envigo, Barcelona, Spain) ad libitum and tap water. Laboratory rats start reproductive senescence at approximately 20 months old [22]. According to the age correlation with humans at this period of life, our model of study could be useful to understand alterations linked to obesity and ageing in 60-year-old humans. The rats were then randomly divided into four experimental groups (n = 14) and fed a standard chow diet ad libitum. The control group (STD) received only the standard chow diet throughout the experiment. In addition to the standard chow diet, the other groups received a cafeteria diet as a model of a high-fat/high-sucrose diet (CAF groups). The cafeteria diet consisted of bacon, sausages, paté and biscuits, carrots, muffins, and sugared milk, which induces voluntary hyperphagia [23]. This diet was offered freshly ad libitum every day for 75 days. The energy contents of the meals offered to the animals are shown in Table 1.

Two of the cafeteria-fed groups were supplemented with GSPE. An oral dose of 500mg/kg BW (body weight) was administered (1) as a preventive treatment for 10 days prior to the cafeteria diet intervention (CAF PRE) and (2) simultaneously with the cafeteria diet for 5 days once per month (CAF MONTHLY). The GSPE was dissolved in tap water and administered as an oral gavage to the animals at 6 pm, three hours after all available food had been removed. Fresh food was given to the animals one hour after they received the GSPE dose. The animals that did not receive

supplementation with GSPE received water as a vehicle. The experimental design is illustrated in Figure 1.

Table 1. Composition of the cafeteria diet offered

Component offered	kJ/g	% Carbohydrate (g)	% Protein (g)	% Lipid (g)	% Fiber (g)
Bacon	14.43	1.0	14.9	31.7	0.0
Sausages	8.36	8.0	14.0	18.0	0.0
Paté	6.57	0.7	8.5	11.0	0.0
Biscuits	18.4	22.0	6.6	10.3	2.0
Muffins	18.8	30.0	4.1	23.1	1.7
Carrot	1.66	0.7	0.1	0.0	2.6
Milk	2.74	4.7	3.1	3.8	0.0
Sugar	16.73	100.0	0.0	0.0	0.0
STD chow diet	12.13	48.0	14.3	4.0	4.1

STD, standard chow provided to the control group

2.3. Blood and Tissue Collection

At the end of the study, the animals were euthanized by decapitation after they had fasted for 12 h. The blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Plasma was obtained by centrifugation (1500× g, 15 min, 4ºC) and stored at −80ºC until analysis. White adipose tissue depots (retroperitoneal (rWAT), mesenteric (mWAT), and periovaric (oWAT)), brown adipose tissue (BAT), and the liver, kidneys, and spleen were rapidly removed, weighed, snap-frozen in liquid nitrogen, and stored at −80ºC.

2.4. Morphometric and Biochemical Variables

Body weight was monitored twice a month. Commercial colorimetric enzyme kits were used to measure the concentrations of plasma glucose, triacylglycerol (TAG), cholesterol, urea, creatinine (QCA, Tarragona, Spain), non-esterified fatty acids (NEFAs, Wako, Neuss, Germany), and β-hydroxybutyrate (Ben Biochemical Enterprise, Milan, Italy). Commercial ELISA kits were used to quantify plasma levels of insulin (Millipore, Madrid, Spain), glucagon (Mercodia, Uppsala, Sweden), tumour necrosis factor-α (TNF-α), and interleukin-6 (IL-6) (Thermo Scientific, Spain). Homeostatic model assessment of insulin resistance (HOMA-IR) and homeostasis

model assessment of β-cell dysfunction (HOMA-β) were calculated using glucose and insulin fasting values.

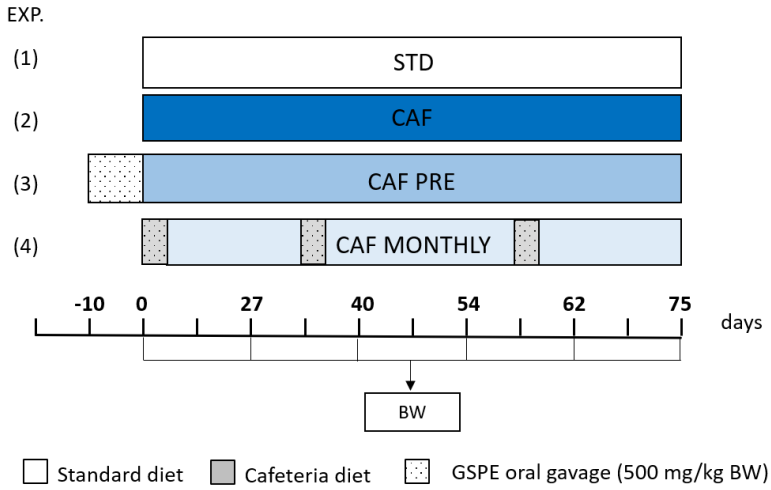

Figure 1. Schematic diagram of the experimental design. All groups were adapted to the environment and to oral gavage during one week before experiment started. Body weight was measured every two weeks. (1) STD: rats receiving standard diet during the whole experiment; (2) CAF: rats receiving standard diet before cafeteria diet intervention; (3) CAF PRE: rats receiving GSPE preventive treatment for 10 days before the cafeteria diet intervention started; (4) CAF MONTHLY: rats receiving a 5-day GSPE treatment simultaneously with the cafeteria diet once per month. Abbreviations: CAF, cafeteria diet; GSPE, grape seed proanthocyanidin extract; BW, Body weight.

Liver homogenization was performed in a tissue lyser (50 s, 2 cycles at maximum potency) using a 0.1% Triton X-100 phosphate-buffered solution after 4ºC centrifugation. The supernatant collected was used for further triacylglycerol measurement.

2.5. Analysis of Liver Steatosis

Liver samples were added to a 4% formaldehyde solution for 24 h and transferred to a 70% ethanol solution until paraffin inclusion. Tissue sections 4 μm thick were cut from paraffin blocks and placed on glass slides. Haematoxylin and eosin (H&E) staining was performed using standard procedures. These sections were analysed under a light microscope to detect changes in tissue architecture. Samples were

scored 0–3 according to the percentage of hepatocytes affected by fatty infiltration (0: <5%; 1: 6–33%; 2: 34–66%; 3: 67–100% of the surface). The histological diagnosis of liver steatosis was based on the criteria described by Brunt et al. [24]. Scoring was done blindly by a specialist.

2.6. Statistical Analysis

Body weight and morphometric variables are represented as the mean ± standard error of the mean (SEM). Kruskal–Wallis and Mann–Whitney non-parametric statistical tests were assessed. Analyses were performed with XLSTAT 2020.1 (Addinsoft, Spain). p-values < 0.05 were considered statistically significant.

3. Results

3.1. GSPE Prevents Body Weight Increase in Obesogenic Conditions When Administered Simultaneously with the Cafeteria Diet

To evaluate the effects of GSPE in aged rats after an obesogenic challenge, such as the cafeteria diet, we first tested its effectiveness on body weight gain. Figure 2 shows that ingesting a cafeteria diet significantly increased body weight gain from day 15 to the end of the experiment (day 75). CAF MONTHLY rats, which received GSPE once a month simultaneously with the cafeteria diet, showed a significant decrease in body weight gain from day 35 to the end of the experiment and reached an 8.4% greater reduction than rats in the CAF group (Figure 2). The body weight gain of CAF PRE rats was also lower than that of rats in the CAF group throughout the experiment. However, this difference did not reach statistical significance.

We have previously reported that GSPE acutely reduces food intake [25]. We measured food intake on the days that included GSPE or vehicle treatment. Cafeteria diet consumption significantly increased food intake compared to chow diet (485 vs. 198 kJ/day, $p < 0.0001$). GSPE significantly reduced food intake when administered with cafeteria diet (CAF vs. CAF MONTHLY; 485 vs. 343 kJ/day, $p < 0.0001$). In the 10-day pre-treatment with GSPE, the animals slightly reduced their food intake (CAF vs. CAF PRE groups before cafeteria diet onset; 175 vs. 141 kJ/day, $p < 0.0001$).

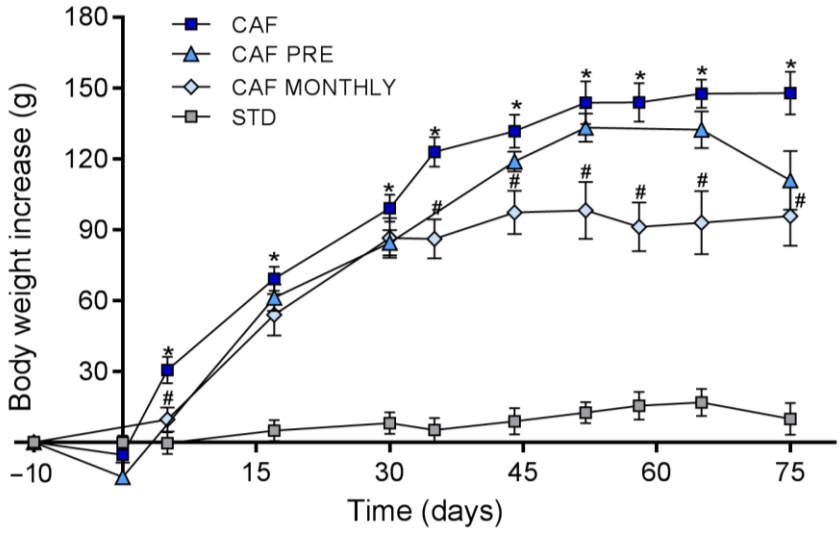

Figure 2. Body weight gain during the experiment. STD: lean rats fed a standard diet; CAF: rats fed a cafeteria diet; CAF PRE: rats receiving preventive treatment of GSPE during 10 days before cafeteria diet intervention; CAF MONTHLY: rats receiving GSPE treatment during 5 days once per month simultaneously fed with cafeteria diet. Values are means ± SEM. * p-value < 0.05 compared to STD rats. # p-value < 0.05 compared to CAF rats.

3.2. Preventive and Simultaneous GSPE Treatments Reduced Mesenteric Adiposity

The effect of GSPE treatments on several morphometric variables is described in Table 2. As expected, the obesogenic diet increased body weight and expanded white adipose tissue depots in CAF rats when compared to STD rats. Brown adipose tissue weight also increased in CAF rats compared to STD rats. Taken together, the cafeteria diet increased the adiposity index of CAF rats compared to control rats. Moreover, the obesogenic diet induced weight gain in the liver, spleen, and kidneys.

Both preventive and simultaneous GSPE treatments reduced mesenteric adipose tissue weight. Moreover, CAF MONTHLY rats experienced a significant reduction in visceral adiposity compared to CAF rats.

No differences were observed in the weight of the liver, spleen, or kidneys between rats in the GSPE-treated groups and CAF rats.

Table 2. Morphometric variables of the groups studied

Variable	STD	CAF	CAF PRE	CAF MONTHLY
n	13	12	12	8
Morphometric measurements				
Initial body weight (g)	364.0 ± 14.2	366.4 ± 11.8	355.7 ± 9.6	352.36 ± 2.5
Final body weight (g)	367.4 ± 15.0	516.0 ± 20.1*	470.2 ± 17.2	472.3 ± 18.9
mWAT (g)	13.1 ± 1.2	28.1 ± 2.0*	21.0 ± 1.5#	18.8 ± 1.4#
oWAT (g)	16.6 ± 1.5	31.7 ± 1.2*	31.8 ± 2.3	27.5 ± 2.3
rWAT (g)	11.1 ± 1.1	22.2 ± 1.2*	20.3 ± 1.2	19.3 ± 1.2
Total visceral WAT (g)	39.5 ± 3.4	80.7 ± 4.9*	72.1 ± 4.2	65.8 ± 2.4$
BAT (g)	0.7 ± 0.1	1.3 ± 0.1*	1.1 ± 0.1	1.5 ± 0.4
% visceral adiposity	11.3 ± 0.6	16.3 ± 0.5*	15.4 ± 0.6	14.0 ± 0.5#
Liver (g)	8.7 ± 0.4	12.2 ± 0.8*	11.2 ± 0.6	10.9 ± 0.3
Spleen (g)	0.8 ± 0.0	1.0 ± 0.0*	0.9 ± 0.1	0.9 ± 0.0
Kidney (g)	1.0 ± 0.0	1.2 ± 0.1*	1.2 ± 0.0	1.1 ± 0.0

STD: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; CAF PRE: rats receiving a GSPE preventive treatment 10 days before the cafeteria intervention; CAF MONTHLY: rats receiving a GSPE treatment during 5 days synchronized with the cafeteria diet; mWAT: mesenteric white adipose tissue; oWAT: periovaric white adipose tissue; rWAT: retroperitoneal white adipose tissue; WAT: white adipose tissue; BAT: brown adipose tissue. Values are means ± SEM. *p < 0.05 compared to STD group. #p < 0.05 compared to CAF group; Trends: $0.05 < p-value < 0.1 compared to CAF rats.

3.3. GSPE Effects on the Glucidic and Lipidic Profile of Obese, Aged Rats

Since obesity and ageing are linked to metabolic dysfunctions, such as dyslipidaemia and insulin resistance, we also analysed the metabolic state of the animals after they were subjected to the obesogenic challenge.

Glucidic profile was altered due to the cafeteria diet. Glucose and insulin levels increased significantly in the CAF group compared to the STD group (Figure 3A, B, respectively).

To explore this profile in greater detail, we also determined the HOMA-IR and HOMA-β ratios. HOMA-IR measures insulin-resistance in Wistar rats [26] while HOMA-βmeasures β-cell function both on the basis of fasting glucose and insulin levels. In this study, HOMA- IR was significantly higher in the CAF group than in the STD group (Figure 3D), but no differences were observed in HOMA-β values (Figure 3E). Since insulin and glucagon hormones have opposite functions, we also calculated the glucagon/insulin ratio in order to better understand the balance between catabolism and anabolism. Although the cafeteria diet did not lead to a significant reduction in glucagon levels in plasma, the glucagon/insulin ratio was lower in the CAF group than in the control group (Figure 3F). Taken together, aged rats fed an obesogenic diet presented higher insulin resistance than aged rats fed a standard diet. However, GSPE was unable to ameliorate this condition (Figure 3).

With regard to lipid metabolism, CAF rats had higher TAG levels (Figure 4A), but no differences were observed in cholesterol, β-hydroxybutyrate, or NEFA parameters (Figure 4B–D, respectively). We also determined the β-hydroxybutyrate/NEFA ratio in order to evaluate the ketogenesis rate. Only the cafeteria diet tended to increase this rate (Figure 4E). Neither preventive nor simultaneous GSPE supplementation had any effect on lipid metabolism-related parameters (Figure 4).

3.4. Renal Function in Aged Rats Fed the Cafeteria Diet Is Not Modified by the Obesogenic Diet or GSPE Treatments

The kidneys are one of the organs that are most sensitive to age-related changes, especially those that affect renal plasma flow (RPF) and the glomerular filtration rate (GFR) [27]. Moreover, obesity is widely reported to have biological consequences on kidney function in certain experimental animals [28]. Since neither the cafeteria diet nor GSPE supplementation affected urea or creatinine levels, no effects on kidney function were observed (Figure 5A, B, respectively).

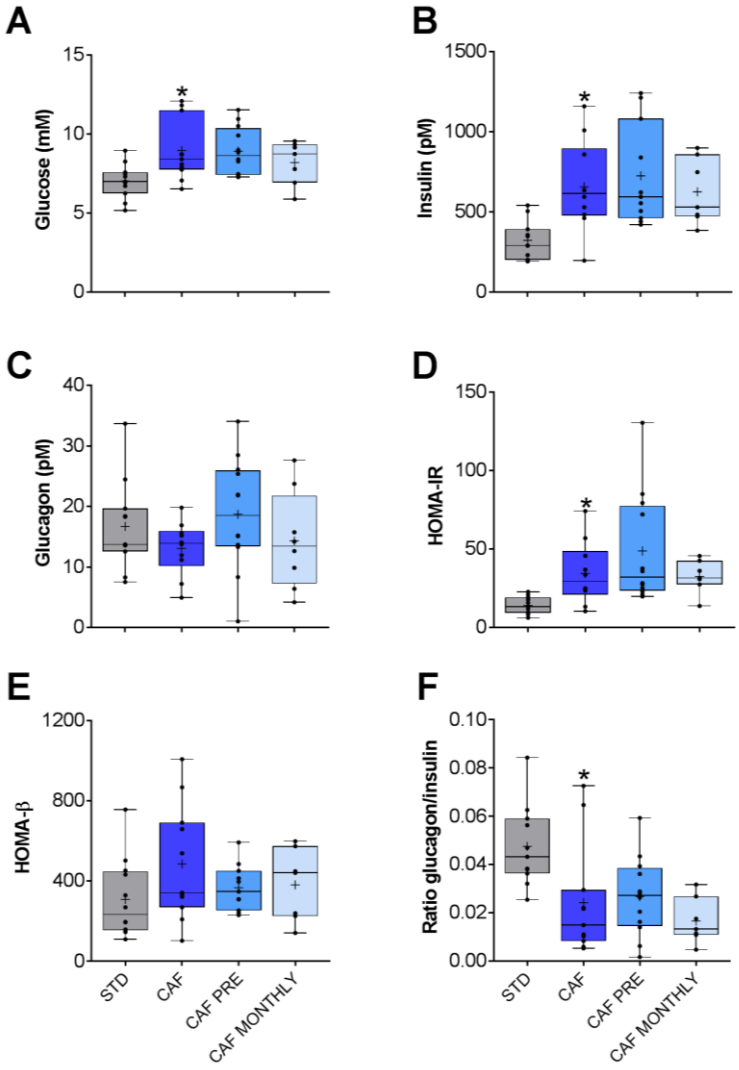

Figure 3. Glucose metabolism-related biochemical parameters: (A) Glucose. **(B)** Insulin. **(C)** Glucagon. **(D)** HOMA-IR. **(E)** HOMA-β. **(F)** Glucagon/insulin ratio. STD: lean rats fed a standard diet; CAF: rats fed a cafeteria diet; CAF PRE: rats receiving preventive treatment of GSPE during 10 days before cafeteria diet intervention; CAF MONTHLY: rats receiving GSPE treatment during 5 days once per month simultaneously fed with cafeteria diet. Values are represented as boxplots showing the median and the IQR. Means are represented as +. * p-value < 0.05 compared to STD rats.

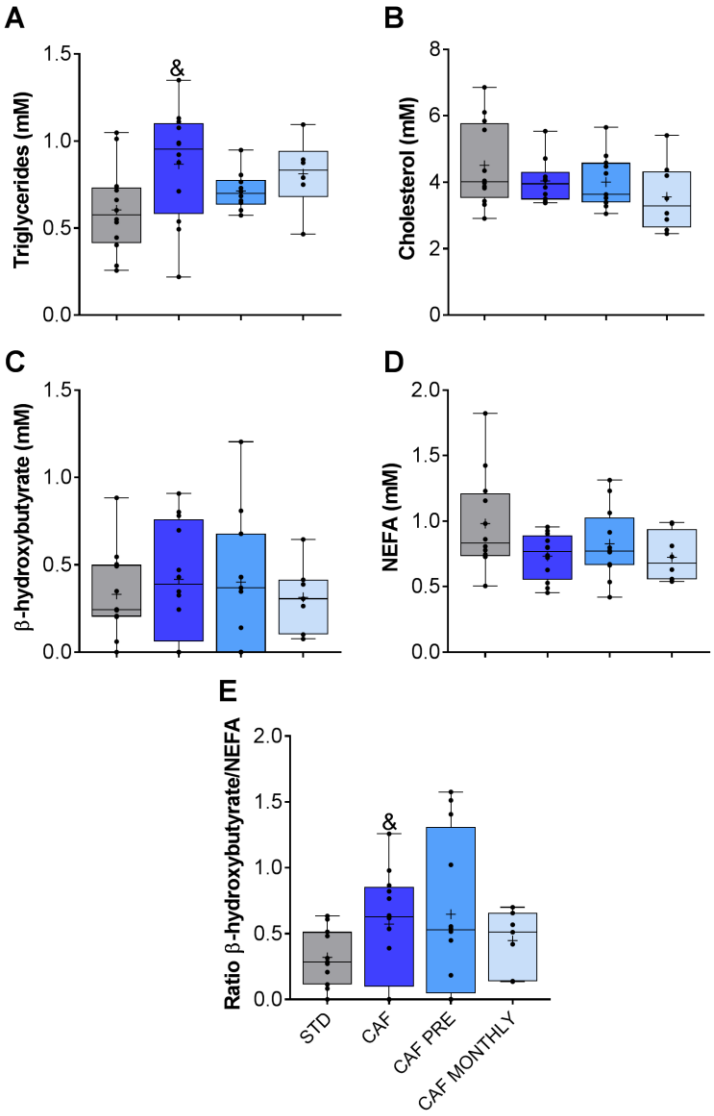

Figure 4. Lipid metabolism-related biochemical parameters. (A) Triglyceride levels. **(B)** Cholesterol levels. **(C)** β-hydroxybutyrate levels. **(D)** NEFA levels. **(E)** β-hydroxybutyrate/NEFA ratio. STD: lean rats fed a standard diet; CAF: rats fed a cafeteria diet; CAF PRE: rats receiving preventive treatment of GSPE during 10 days before cafeteria diet intervention; CAF MONTHLY: rats receiving GSPE treatment during 5 days once per month simultaneously fed with cafeteria diet; NEFA, non- esterified fatty acids). Values are represented as boxplots showing the median and the IQR. Means are represented as +. Trends: $^{\&}0.05 < \text{p-value} < 0.1$ compared to STD rats.

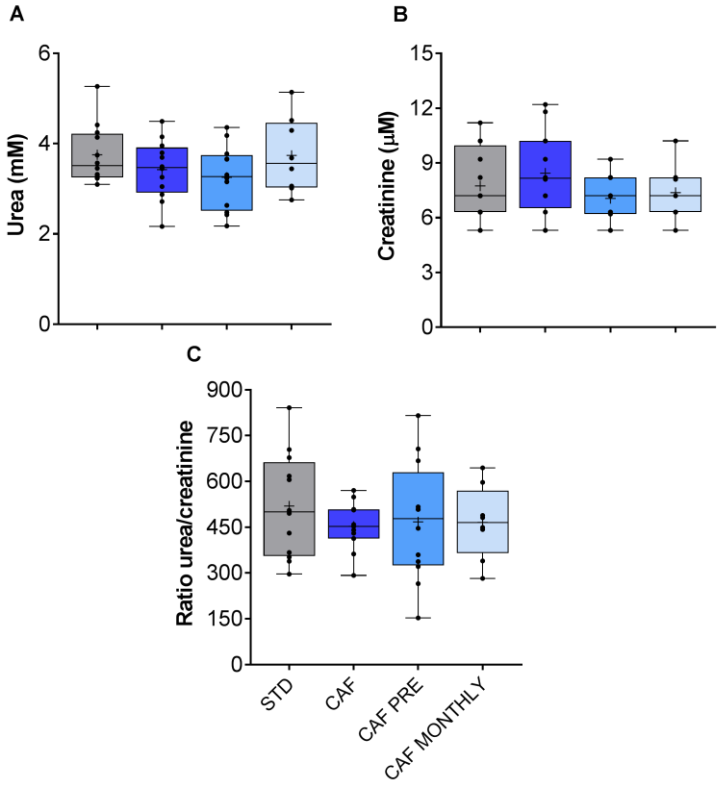

Figure 5. Renal metabolism-related biochemical parameters. (A) Urea levels. **(B)** Creatinine levels. **(C)** Urea/creatinine ratio. STD: lean rats fed a standard diet; CAF: rats fed a cafeteria diet; CAF PRE: rats receiving preventive treatment of GSPE during 10 days before cafeteria diet intervention; CAF MONTHLY: rats receiving GSPE treatment during 5 days once per month simultaneously fed with cafeteria diet. Values are represented as boxplots showing the median and the IQR. Means are represented as +.

3.5. GSPE Reduces Liver Lipid Content in Obese, Aged Rats

An important characteristic of the metabolic syndrome associated with obesity is ectopic fat accumulation in the liver, which triggers hepatic steatosis. In our experiment, rats in the CAF group showed greater TAG accumulation in the liver than rats in the STD group (Figure 6A). Although preventive treatment with GSPE had no effect on diminishing TAG accumulation, the CAF MONTHLY group tended to reduce TAG levels compared to the CAF group (Figure 6A). To corroborate these data, we analysed fat accumulation to evaluate the presence of hepatic steatosis (Figure 6B). However, only macrovesicular steatosis was observed. As expected, the CAF group

had a greater accumulation of lipid droplets in hepatocytes than the STD group (Figure 6C). Interestingly, although GSPE preventive treatment had no strong effect on reducing macrovesicular steatosis, when administered simultaneously, GSPE reduced diet-induced fat accumulation in the liver (Figure 6B, C).

3.6. Effect of GSPE on Systemic Inflammation in Aged Rats Fed an Obesogenic Diet

Finally, we also measured TNF-α in plasma as a biomarker of systemic inflammation. TNF-α levels were undetectable in all samples analysed (data not shown). To further evaluate systemic inflammation in this model, we measured IL-6 levels in plasma. In agreement with TNF-α results, IL-6 levels were generally very low in aged rats. The CAF diet did not induce systemic inflammation in comparison with STD rats, and GSPE supplementation did not change inflammatory status (Figure 7).

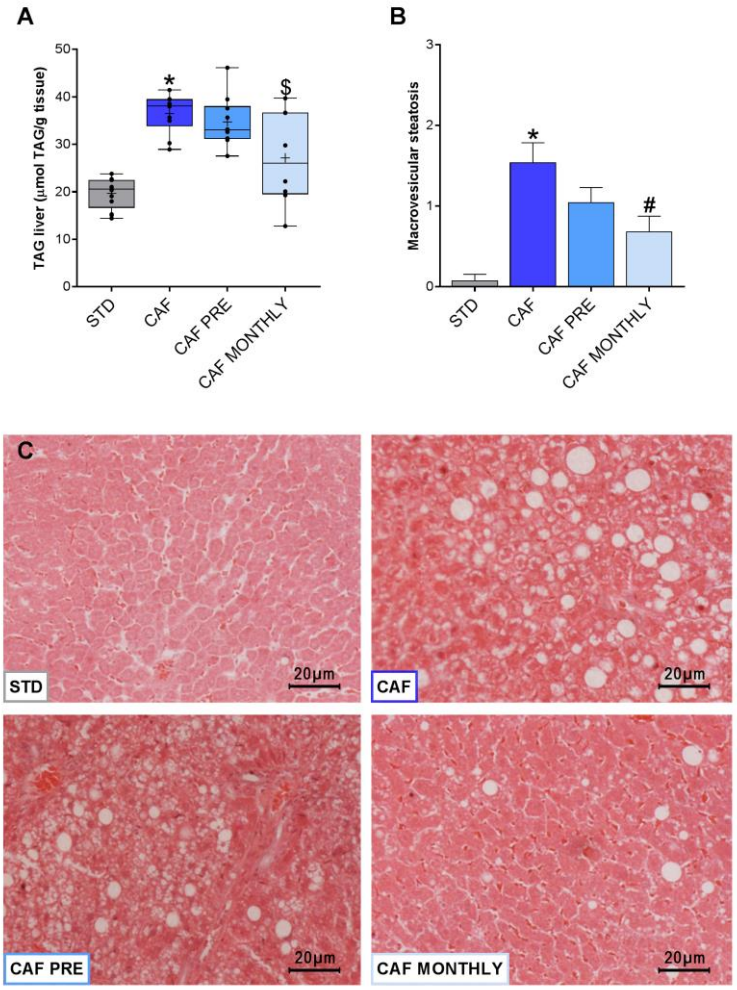

Figure 6. Effects of GSPE treatment on liver steatosis. (A) Simultaneous treatment with GSPE reduces liver lipid accumulation; **(B)** Macrovesicular steatosis reduction after simultaneous supplementation with GSPE; **(C)** Hematoxilin-eosin staining of representative histological sections of liver from STD, CAF, CAF PRE, and CAF MONTHLY rats. Scale bar = 10 µm in all pictures. STD: lean rats fed a standard diet; CAF: rats fed a cafeteria diet; CAF PRE: rats receiving preventive treatment of GSPE during 10 days before cafeteria diet intervention; CAF MONTHLY: rats receiving GSPE treatment during 5 days once per month simultaneously fed with cafeteria diet. Levels of triglycerides are represented as boxplots showing the median and the IQR. Means are represented as +. Macrovesicular steatosis score values are represented as means ± SEM. * p-value < 0.05 compared to STD rats. # p-value < 0.05 compared to CAF rats. Trends: $0.05 < p-value < 0.1 compared to CAF rats.

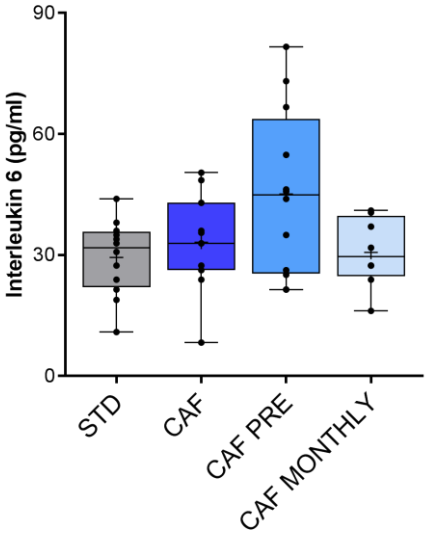

Figure 7. Interleukin-6 levels as biomarker of systemic inflammation. STD: lean rats fed a standard diet; CAF: rats fed a cafeteria diet; CAF PRE: rats receiving preventive treatment of GSPE during 10 days before cafeteria diet intervention; CAF MONTHLY: rats receiving GSPE treatment during 5 days once per month simultaneously fed with cafeteria diet. Values are represented as boxplots showing the median and the IQR. Means are represented as +.

4. Discussion

The prevalence of obesity is increasing in parallel with the worldwide growth in the aged population. This scenario makes it absolutely necessary to find new strategies aimed at mitigating the harmful effects of ultra-processed foods and improving our quality of life. Grape-seed proanthocyanidins may be good candidates for use as bioactive compounds against obesity, ageing, and related dysfunctions. Several studies have shown that GSPE has anti-inflammatory and anti-tumoral properties [29] and plays an important role in reducing hyperlipidemia [12,13]. Previous results from our group demonstrated that a dietary dose of 25 mg GSPE/kg of body weight was effective in improving obesity-associated intestinal damage and decreasing intestinal inflammation when administered as a corrective treatment in rats [30]. However, this dietary dose was not effective in decreasing body weight. In contrast, our group recently demonstrated that a dose of 500 mg/kg BW of GSPE is effective in

reducing food intake in both lean and obese young rats [31] as well as in aged, chow-fed rats [15,32] and, therefore, helps to reduce body weight.

Additionally, from a functional food perspective, the most appropriate way for a compound to be effective as an anti-obesity agent is to prevent and/or approach the problem at the initial stages. As stated before, we have previously demonstrated the effectiveness of these preventive treatments of GSPE in young rats, and, now, the same hypothesis might be applied for the elderly. As far as we know, this study has, for the first time, assessed the effects of GSPE on most metabolic risk factors associated with obesity in aged rats. Our results support the hypothesis that grape-seed proanthocyanidins are effective in preventing certain metabolic syndrome features induced by an obesogenic challenge during ageing.

The animals in this study received an unhealthy, highly palatable, energy-dense human cafeteria diet. We have shown that the obesogenic diet administered to aged rats induced obesity and disturbed glucidic and lipidic profiles. More interestingly, a dose of 500 mg/kg BW of GSPE can prevent certain metabolic disruptions in aged rats, as has previously been demonstrated in young rats [15,31,33].

In our experiment, GSPE-treated rats in both the CAF PRE and CAFMONTHLY groups showed a decrease in body weight gain compared to rats in the CAF group. These results were previously observed by our group in a 17-week-experiment with young, female rats subjected to the same feeding conditions, GSPE dose, and administration method [31].

Although the durations of the two experiments are not comparable, aged rats fed the cafeteria diet had a higher body weight gain in a shorter period than young rats fed the same diet, indicating that aged rats are more susceptible to obesity. Various animal studies have also demonstrated that ageing is associated with higher body weight gain and fat mass accumulation. In agreement with our results, 16-month-old male rats showed greater body weight and adiposity than their young counterparts both under a normal chow diet and a high-fat diet before and after surgery or dietary interventions [34]. Interestingly, our results show that simultaneous GSPE treatment has the same efficacy in reducing body weight regardless of age. On the other hand, when administered as a preventive treatment, GSPE was not as effective in aged rats

as it was in young rats. Despite the fact that GSPE- specific mechanisms of action to reduce adiposity and body weight gain have not been fully elucidated, out results in the present study suggest that effects of GSPE could be partially mediated by the decrease in food intake. Moreover, literature suggests that GSPE could induce white adipose tissue browning probably by acting as a scavenger receptor of free radicals, thus leading to a reduction of body weight [20].

GSPE is also widely reported to be a lipolytic agent [13,35,36]. For example, according to results obtained previously in young, female rats [31,37], in this work, GSPE reduced fat accumulation in the mesenteric adipose tissue of obese, aged rats when administered together with the cafeteria diet. However, no consensus could be established on the effect of GSPE on fat accumulation when administered preventively, which suggests that age may affect the long-term GSPE effect seen previously in young rats [31]. Dietary polyphenols, such as resveratrol and quercetin, are believed to promote longevity and life expectancy [38], while scientific evidence suggests they can also modulate epigenetic patterns [39,40]. Our group has also shown that the long-lasting effects of GSPE in young rats are mediated, at least partially, by epigenetic modifications in the intestine [41]. In the present study, the loss of long-lasting GSPE effects in aged animals may be due to the deep, epigenetic modifications that are linked to the ageing process [40]. Our results therefore suggest that aged animals are more prone to obesity and difficulty in losing weight. This may be due to age-related changes in body fat distribution and functional impairments [4,42] for which a short preventive GSPE treatment is insufficient to compensate for the homeostasis disruption associated with senescence.

In previous studies of young, female rats [37,43], all GSPE treatments ameliorated dyslipidaemia and the insulin-resistant state of obese rats. Other studies have also shown that GSPE protects against disorders in mice induced by a high-fat diet [44]. Contrary to our results, a dietary dose of GSPE (25 mg/kg BW) managed to reduce the plasma levels of triglycerides, glucose, and insulin in cafeteria-diet-fed male rats [45], while puerarin [46], resveratrol [47], and procyanidin B2 [17] ameliorated hyperglycaemia and hyperinsu- linemia caused by an obesogenic diet. However, none of the above studies applied the double challenge of ageing and obesity, two factors that contribute to the dyslipemic and insulin-resistant phenotype. Indeed, our results

may indicate that the cafeteria diet has a stronger effect on aged animals than on young ones, since aged animals have a marked increase in glucose and insulin levels in plasma probably due to the decline in adaptive homeostasis associated with age. The noticeable effect of the cafeteria diet on aged animals may therefore counteract the positive effects of GSPE on re-establishing homeostasis.

Together with inflammation and oxidative stress, hepatic steatosis is the basis for the pathophysiology of NAFLD Our results indicate that GSPE is effective in protecting against ectopic fat accumulation in the liver when administered simultaneously with the cafeteria diet. According to the literature, other bioactive compounds, such as procyanidin B2 [17,48], polyphenol-rich extract from cranberries [49], and myricetin [50], are good candidates for reducing fat accumulation in the liver. Specifically, resveratrol is one of the most promising polyphenols for improving lipid droplet accumulation in the liver in both young and aged male mice [51]. Several mechanisms, such as binding to bile acids [49,52], regulating the lysosomal pathway and redox state [53], activating free fatty acid β-oxidation, and modulating factors involved in lipid metabolism [54], are reported to be responsible for improving hepatic steatosis by dietary polyphenols. Some other authors pointed out that GSPE could influence expression of genes involved in different signaling pathways, such as glycolysis, insulin, or inflammatory pathways, via modulating microRNA expression in vitro [55]. Moreover, hepatocyte senescence increases with age, thus contributing to hepatic fat accumulation and steatosis [56]. Although the mechanisms behind this effect have not been explored in this article, modulation of the lipid metabolism and/or cellular senescence in the liver cannot be ruled out.

Contrary to previous results from studies that compared obese, young rats with lean rats [31,37], the cafeteria diet had no effect on increasing inflammatory response through an increase in the production of pro-inflammatory cytokines, especially TNF-α and IL- 6. Interestingly, ageing had no effect on the serum levels of TNF-α or IL-6 in obese rats. These cytokines are usually considered biomarkers of ageing in humans [57], especially IL-6, IL-8, IL-1β, and TNF-α, since they are strong predictors of age-related morbidity and mortality [58,59]. Although relatively little is known about the identification of inflammatory biomarkers in rodent models of ageing, in agreement with our results, Gordon et al. [60] found that only 5 out of 58 pro-inflammatory-

related molecules were modified in 24-month- old rats compared to 4-month-old rats. IL-6 and TNF-αwere unchanged, and only C-reactive protein was increased.

Although most flavonoids and other bioactive compounds are reported to have an anti-inflammatory effect, in our model, we saw no such effect with GSPE. These results might be related with the decline in immunological response associated with immunosenescence during ageing [61]. Immunosenescence is defined as a progressive and overall diminution of immune functions that affect all cells and organs, where defects in the processing and presentation of antigens by the cells of the innate immune system contribute to diminished activation and stimulation of cells in the adaptive immune system [57]. Furthermore, the progressive involution of the thymus leads to a disturbed balance and function of naïve, memory, and effector T cells, thus promoting a latent pro-inflammatory status in the elderly, called inflammageing [58]. This progressive proinflammatory state in the elderly has been described as elevated plasma concentrations of IL-6, IL-1β, and TNF-α, among other cytokines [62]. The inflammageing process seems to call in question the functional defects observed in innate immune cells. However, it is believed that chronic, subclinical inflammation is caused by the chronic antigenic stress that impinges throughout life upon innate immunity and/or by the partial inability of the aged immune system to eliminate certain pathogens. This could lead to chronic, yet inefficient, innate immune responses and has potential implications for the onset of inflammatory diseases [63,64].

However, as previously stated, we have not found this low-grade, pro-inflammatory state in our model of obese/aged rats nor as an effect of GSPE treatment. A hypothesis to explain these results might be the fact that the rats used in our experiment were not under any substantial chronic stimulation of the innate immune system, as they were grown in the animal house during their whole life. Without this chronic stimulus, it is possible that the pro-inflammatory state in the elderly was not developed. From this perspective, our model is not ideal to study immunosenescence. In any case, better understanding of age-associated changes in the immune system should enable the development of more effective strategies to promote a healthy ageing.

In summary, GSPE treatment is not as effective in old age as it is in youth. This is probably due to the great metabolic disruption associated with the ageing process and the inability to respond quickly to homeostatic fluctuations. With this in mind, GSPE should be administered before the normal functioning of the organism declines over time.

5. Conclusions

In conclusion, administering GSPE at a dose of 500 mg/kg BW prevents the development of certain unhealthy states related to obesity and ageing. Simultaneous treatment with GSPE is effective in reducing body weight gain, adiposity, and liver steatosis caused by cafeteria-diet consumption.

Further studies are needed to determine the potential memory effect of GSPE during ageing and under an obesogenic challenge. The final goal is to translate these approaches to human nutrition by developing new strategies based on bioactive agents to improve the quality of life of elderly people. However, the time of administration and optimal dose must be ascertained.

Author Contributions: M.S.-C.: writing—original draft, data curation, methodology, software. A.M.- G.: methodology. C.G.-B.: methodology. E.R.-G.: investigation, conceptualization. M.B.: investigation, conceptualization, resources. M.P.: conceptualization, funding acquisition, project administration. A.A.: conceptualization, funding acquisition, project administration. X.T.: writing—review & editing, formal analysis, supervision. R.B.-D.: writing—review & editing, formal analysis, supervision. All authors have read and agreed to the published version of the manuscript.

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UNIVERSITAT ROVIRA I VIRGILI
ROLE OF FLAVONOIDS IN THE MODULATION OF INTESTINAL ALTERATIONS ASSOCIATED WITH METABOLIC CHALLENGES: OBESITY AND AGING
Marta Sierra Cruz

MANUSCRIPT 3

Effects of grape seed proanthocyanidin extract on lipopolysaccharide translocation and trafficking from the gut to tissues

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Abstract: Diet-associated alterations of the intestinal barrier and gut microbiota promote intestinal LPS translocation from the lumen to the lamina propria through different pathways, leading to an increase in LPS levels in the plasma known as metabolic endotoxemia.

Previous studies have demonstrated that a pharmacological dose of grape seed proanthocyanidin extract (GSPE) can reduce metabolic endotoxemia of obese rats. In the current study, we aimed to evaluate GSPE modulation of LPS translocation and the underlying mechanisms. To do this, we performed both an *in vitro* experiment with Caco-2 cells and an *in vivo* experiment with Wistar female rats fed a cafeteria diet. GSPE was effective in regulating intestinal permeability through the modulation of CD36 and the SRB1-mediated endocytosis pathway, as well as the gut microbiota interaction with the endocannabinoid system through epigenetic mechanisms. Our results confirm that GSPE is able to ameliorate intestinal dysfunction and metabolic endotoxemia caused by an excess of dietary lipids by modulating the endotoxin-translocation pathways.

Keywords: intestinal permeability, metabolic endotoxemia, proanthocyanidins, gut microbiota, systemic inflammation

1. Introduction

The gastrointestinal tract (GI) is a high-surface organ of the body. Its primary function is nutrient absorption but it is also a selective barrier and is involved in the immune defence, metabolism and endocrine functions [1].

Dietary pattern is a relevant factor affecting gut homeostasis. Under healthy dietary conditions, the intestinal barrier prevents the passage of undesirable compounds such as endotoxins from the lumen to the lamina propria [2]. In contrast, consumption of high-fat/high-sugar diets affects the integrity of the intestinal barrier as well as the microbiota composition and functionality (dysbiosis). Lipopolysaccharide (LPS; a type of endotoxin) is a major component of the outer membrane from both commensal and pathogenic gram-negative bacteria, and it plays a key role in host-pathogen interactions with the immune system. Low concentrations of LPS in the blood are associated with good maintenance of the immune system [3]; however, dietary patterns linked to dysbiosis translate into a greater passage of LPS from the intestinal lumen towards the *lamina propria*, and consequently, into the blood circulation. This phenomenon is known as metabolic endotoxemia. This condition is characterized by the activation of the immune system, thus inducing systemic inflammation by increasing pro-inflammatory secretion of TNF-α and IL-6, especially in tissues involved in the metabolism of sugars and lipids, such as the liver and adipose tissue [2]. This situation contributes to the development of metabolic diseases, including type 2 diabetes, atherosclerosis and cardiovascular disease [3,4].

The precise pathway by which LPS is transported through the gut barrier remains controversial. However, currently, different mechanisms for LPS transport across the intestinal barrier have been proposed: (1) paracellular transport by passive diffusion associated with an increase in gut permeability due to tight junction alterations [1]; (2) a transcellular pathway through cell-associated antigen passage as intestinal-epithelial microfold cells or goblet cells that are implicated in transporting antigens from the intestinal lumen to immune cells [5]; (3) receptor mediated endocytosis through clathrin- or caveolin-dependent transcytosis in enterocytes and colonocytes [6]; or (4) the chylomicron-associated pathway in which LPS is transported in newly released chylomicrons (CMs) during the post-prandial period [4,5]. Indeed, intestinal

epithelium cells of the proximal intestine express the cluster of differentiation 36 (CD36) and scavenger receptor class B type I (SRB1). These are both involved in endocytosis and CM-associated pathways by uptaking fatty acids, triglycerides and other dietary lipids [4]. They act as sensors of dietary lipids, thus leading to an optimization of the size of CMs produced during the postprandial stage [5].

Traditional approaches to deal with obesity and its related metabolic endotoxemia were based on weight loss through eating a healthy diet and practising sport. In recent years, new anti-obesity treatments based on nutrition supplementation have been studied. Bioactive compounds, particularly flavonoids, are chemical components found in small quantities in food and have demonstrated beneficial effects on inflammatory pathways, barrier integrity and microbiota composition [7]. The most abundant flavonoids in the Western diet are proanthocyanindins (PACs), also known as condensed tannins [8]. PACs are oligomers and polymers of monomeric flavan-3-ols mainly found in foods and beverages of vegetal origin that are ingested daily, such as grapes, cocoa, chocolate, red wine and green tea [8]. PACs have been attributed to a wide range of biological activities as they modulate pathways involved in chronic inflammation, lipid homeostasis, energy metabolism, apoptosis and cell cycle arrest [8]. In relation to their function in lipid metabolism, PACs repress lipoprotein secretion, inhibit the absorption of dietary lipids and reduce CM secretion by enterocytes [9,10]. Therefore, PACs have been attributed hypotrigliceridemic and hypolimidemic effects [9,10].

In previous studies, we demonstrated that PACs are involved in maintaining the intestinal barrier [11] and reducing metabolic endotoxemia acting on the paracellular pathway in CAF-fed rats [12]. Now, with the aim of obtaining a more holistic view of the effects and mechanisms of GSPE for modulating LPS transport, we have studied the receptor-mediated endocytosis and CM-associated pathways. In this study, PACs are evaluated as possible modulators of LPS translocation through the intestinal barrier using both *in vitro* and *in vivo* models of intestinal dysfunction.

2. Materials and methods

2.1. Proanthocyanidin Extract

The grape seed extract enriched in proanthocyanidins (GSPE) was provided by *Les Dérivés Résiniques et Terpéniques* (Dax, France). According to the manufacturer, the GSPE composition used in this study contains monomers (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5-13 units; 31.7%) of flavan-3-ols.

2.2. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/ml glucose, glutamine, 0.5 g/L trypsin-versene mixture solution and penicillin-streptomycin were purchased from Lonza Bioscience (Switzerland). HEPES buffer, Fetal Bovine Serum (FBS), fungizone, lipopolyssacharide (LPS) from *Escherichia coli O111:B*, oleic acid, sodium taurocholate, egg lecithin and phosphate buffer saline (PBS) were all purchased from Sigma Aldrich (Madrid, Spain). Sodic pentobarbital was purchased from Fagron Iberica (Barcelona, Spain). Heparin was provided by Deltalab (Barcelona, Spain). Total RNA was isolated from frozen intestinal segments using Trizol reagent (Ambion, MA, USA) according to the manufacturer's protocol.

2.3. Lipid mixture and GSPE solution preparation

A 10X lipid mixture (LM) was prepared by mixing oleic acid, sodium taurocholate and egg lecithin (OA:NaTC:LC = 20mM:10 mM:13.6mM) in PBS 1X to prepare fatty acid micelles. To ensure complete homogenization, the solution was maintained in agitation overnight at room temperature and then sterilized by filtration with a syringe coupled to a 22 µm pore-size filter. 10X LM stock solution was stored at -20ºC prior to use.

GSPE was frozen upon receipt at -80ºC until the treatment day and kept in darkness. On the day of the assay, the GSPE solution was prepared freshly in 10% ethanol in pyrogen-free water.

2.4. Cell culture

Human epithelial colorectal adenocarcinoma Caco-2 cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC). Cells were cultured and maintained in an incubator at 37ºC and 5% CO_2 in DMEM 4.5 g/mL glucose supplemented, 0.02 mM glutamine,1 U/mL-1 µg/mL penicillin/streptomycin solution, 0.1 mM HEPES, 0.1% FBS and 0.01% fungizone. Cells were split at a 1:4 ratio when they reached 70-80% confluence using 0.5 g/L trypsin-versene mixture solution. The medium was changed every two-three days.

Cells were differentiated for 21 days using transwell inserts (MilliCell, Northeim, Germany) in 6-well and 12-well plates (Greiner Bio-one, Madrid, Spain) in order to imitate the thin epithelial-cell monolayer. In 6-well plates, 200,000 cells/2 ml were seeded at the upper part of the insert of each well and, 65,000 cells/0.4 ml were seeded in the 12-well plates. Basolateral medium contained 1 ml and 2.5 ml of supplemented DMEM for 12-well and 6-well plates, respectively.

2.5. In vitro chylomicron production

Caco-2 cells were treated with lipid mixture to induce *in vitro* chylomicron production. Colourless medium was used for colorimetric determinations. The experimental medium was the same cell culture medium described in 2.4; however, it was not supplemented with FBS in order to avoid possible interferences of TAG contained in it.

Firstly, 10X LM stock solution prepared previously was thawed and diluted with experimental medium. After the cells were washed with PBS 1X, the different study treatments were added: (1) Control containing experimental medium; (2) LPS (1 µg/ml); (3) LM; (4) LM + LPS; (5) LPS + GSPE (250 µg/ml); (6) LM + GSPE; and (7) LM + LPS + GSPE. After four hours of incubation, apical and basolateral media were collected and stored at -80ºC until analysis.

2.6. Lipoprotein fractionation

NaCl density gradient ultracentrifugation was used to isolate the CM-rich fraction from other types of lipoproteins. Basolateral media from 6-well plates were mixed with the corresponding amount of NaCl to obtain a solution with a density of 1.006

g/mL. This density mixture was then carefully overlaid with 500 µl of pyrogen-free water and subjected to ultracentrifugation (16,000 rpm, 30 minutes, 4ºC; Beckman Coulter, JA-25.15 rotor). The top 500 µl was carefully isolated to obtain a CM enriched fraction. Samples were stored in pyrogen-free tubes at -80ºC prior to determinations.

2.7. Animal models

Female Wistar rats (240-270g) were purchased from Charles River Laboratories (Barcelona, Spain). After one week of adaptation, the rats were individually caged in animal quarters at 22ºC with a 12h light/12h dark cycle and were fed *ad libitum* with a standard chow diet (Panlab 04, Barcelona, Spain) and tap water. After the acclimation period, rats were randomly distributed into four experimental groups (n=7-10/group). The control group (STD) was fed only with a standard chow diet. In addition to the chow diet, the other three groups received a cafeteria diet as a model of a high-fat/high-sucrose diet (CAF groups). Two CAF groups were also supplemented with an oral dose of 500 mg GSPE/kg b.w. at different times. The preventive treatment group (PRE) was administered GSPE during 10 days before the cafeteria diet challenge, whereas the simultaneous intermittent treatment-CAF (SIT) group received GSPE together with the cafeteria diet every other week (Supplementary material, Figure 1).

The cafeteria diet consisted of bacon, sausages, biscuits with pâté, carrots, muffins and sugared milk to induce voluntary hyperphagia. This diet was offered freshly every day to the animals for 17 weeks, which was the entire duration of the study. The energy content of each diet has been described previously by *Ginés et al*. [13].

GSPE was dissolved in tap water and was administered orally by gavage to the rats at 18:00h for each treatment in a volume of 500 µL, one hour after removing all available food. Non-supplemented animals received tap water as a vehicle.

All procedures involving the care and use of animals in this work were reviewed and approved by The Animal Ethics Committee of the Universitat Rovira i Virgili (code: 0152S/4655/2015).

2.8. Blood and tissue collection

At the end of the study, animals were fasted for 4 h, anesthetized with sodic pentobarbital (70 mg/kg body weight) and exsanguinated from the abdominal aorta. The blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Plasma was obtained by centrifugation (1500 xg, 15 min, 4ºC) and stored at -80ºC until analysis. Mesenteric adipose tissue (mWAT), liver, and gut sections were rapidly removed, weighed and frozen in liquid nitrogen before storage at -80ºC until analysis.

2.9. Morphometric and biochemical parameters

LPS, triacylglycerides (TAG) and insulin concentration in plasma, and the HOMA-IR index were determined as previously described [12,13]. LPS levels in cell culture media, liver and mesenteric adipose tissue homogenates were measured using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Spain). A colorimetric enzyme commercial kit was used to measure the TAG concentration (QCA, Amposta, Spain). The apolipoprotein B48 concentration in basolateral media was quantified using a commercial ELISA kit (MyBiosource, Brussels, Belgium).

2.10. Quantitative real-time PCR

Total mRNA was isolated from 50 mg of duodenum, ileum and colon using Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. RNA from Caco-2 cells was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was obtained by reverse transcription of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Madrid, Spain) following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qPCR) was carried out in the Bio-Rad CFX96 Real-time PCR Systems (Bio-Rad Laboratories, Barcelona, Spain). All samples were run in duplicate in 96-well reaction plates.

The gene expression of Scavenger Receptor class B type 1 (SRB1 or *Srb1* for human and rat, respectively) and Cluster of Differentiation 36 (CD36 or *Cd36* for human and rat, respectively) was measured with both *in vitro* and *in vivo* experiments. Cannabinoid receptor 1 gene expression (*Cnr1*) was also determined in rat colon

samples. For rat samples, the iTaq Universal SYBR® Green Supermix (Bio-Rad) was used, together with the respective forward and reverse primers (Biomers, Germany) for the targeted rat genes (Supplementary material, Table 1). The results were normalized with respect to the mRNA levels of Hypoxanthine-guanine Phosphoribosyl Transferase (*HPRT or Hprt* for human and rat, respectively) and cyclophilin-E (*Ppia*) genes, used as housekeeping controls. The relative mRNA expression levels were calculated following the $2^{-\Delta\Delta Ct}$ method. The identity and purity of the amplified products were assessed by melting curve analysis.

For cell culture samples, we used the TaqMan Universal PCR Master Mix (Applied Biosystems), together with the respective specific TaqMan probes (Applied Biosystems): Hs00354519_m1 for *CD36*, Hs00969821_m1 for *SRB1* and Hs02800695_m1 for *HPRT*. The relative amount of mRNA was normalized to the *HPRT* as the endogenous control gene.

Reactions with the iTaq Universal SYBR® Green Supermix were performed using the following thermal profile: 30 seconds at 95ºC, 40 cycles of 5 seconds at 95ºC and 30 seconds at 60ºC. Reactions with the TaqMan Universal PCR Master Mix were performed as previously described by *Gil-Cardoso et al*. [12]. The relative mRNA expression levels were calculated following the $2^{-\Delta\Delta Ct}$ method. Zoonulin-1 (*Zo-1*), Occludin-1 (*Ocln*), Claudin-1 and Jam-A gene expression were previously determined by *Gil-Cardoso et al.* [12].

2.11. DNA methylation analysis

Genomic DNA was extracted from colon samples using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). DNA was subjected to bisulfite modifications using a commercially available kit (Zymo Research, Irvine, CA, USA). After that, DNA methylation analysis was carried out by pyrosequencing. Primers for rat *Cnr1*, the gene coding for the cannabinoid receptor type 1 (CNR1), targeting eight CpG sites, were generated according to Pyro Mark Assay design Assay Software version 2.0 (Qiagen). Bisulfite-treated DNA was amplified using a PyroMark PCR kit (Qiagen) according to the manufacture's protocol. The PCR protocol was as follows: 95ºC for 15 min, followed by 45 cycles of 94ºC for 30s, 56ºC for 30s, 72ºC for 30s, and finally,

72ºC for 10 min. PCR products were checked in an agarose electrophoresis. PyroMark Q24 (Qiagen, Hilden, Germany) was used to perform pyrosequencing methylation analysis and the PyroMark Q24 ID version 1.0.9 software (Qiagen) was used to calculate the methylation level. This software calculates the methylation percentage mC/(mC + C) (where mC is methylated cytosine and C is unmethylated cytosine) for each CpG site and makes quantitative comparisons possible. Quantitative methylation results were expressed both as a percentage of individual CpG sites and as an average of the methylation percentage of all the investigated CpG sites. Primer sets for the *Cnr1* pyrosequencing analysis are shown in Supplementary material (Table 2).

2.12. Short chain fatty acid quantification

Short chain fatty acids (SCFA) (propionic, isobutyric, butyric, isovaleric, and valeric) concentrations were determined in cecal content thawed at 4ºC.

2.13. Statistical analysis

The results are expressed as the mean value ± the standard error of the mean (SEM). For *in vitro* experiments, statistical comparisons between groups were assessed by a two-sided Student's t-test. For *in vivo* experiments, non-parametric Kruskal-Wallis and Mann-Whitney tests were used. Pearson's correlation coefficient was evaluated to assess relationships between different parameters. We performed a multiple linear regression analysis with backward variable selection was carried out to identify independent predictors of LPS circulating levels. Variables included in the model were OVA and TAG plasma levels, butyric acid in cecal content, and *Cldn-1*, *Cd36* and *Cnr1* gene expression in different gut sections. These variables presented strong correlation coefficients with LPS levels. We considered p-values < 0.05 as statistically significant. Analyses were performed with XLStat 2021.03.1 (Addinsoft, Barcelona, Spain).

3. Results

GSPE as a modulator of LPS transport across the intestinal barrier

Our group obtained previous results that demonstrated that consuming a CAF diet significantly induced an increase in LPS circulating levels in rats. We also found that a pharmacological dose of GSPE (500 mg/kg b.w.) was able to reduce metabolic

endotoxemia in both PRE and SIT groups [12]. The details of the experimental groups are shown in Supplementary material, Figure 1.

In order to study how GSPE modulates LPS transport, we first explored the associations between metabolic endotoxemia and variables related to barrier integrity and intestinal permeability. We found that the LPS concentration was negatively associated with TEER values in duodenum and with claudin-1 gene expression in ileum (Figure 1A and F, respectively) showing a relationship between metabolic endotoxemia and the paracellular pathway. Furthermore, the LPS concentration was strongly positively associated with the TAG concentration in plasma (Figure 1B), suggesting a lipoprotein-associated LPS transport. Finally, the fact that LPS and OVA levels in plasma were positively associated (Figure 1D), led us to investigate receptor mediated endocytosis because this is one of the mechanisms of OVA transport across the intestinal barrier.

GSPE modulation of CM-associated LPS transport

To study the potential modulation of GSPE on CM-associated LPS transport, Caco-2 cell CM-production was induced using lipids. We first quantified TAG levels in the CM-rich fraction. As observed in Figure 2A, we were able to induce *in vitro* chylomicron production up to 3-fold with respect to the control cells, although the differences were not statistically significant. GSPE supplementation seemed to reduce TAG levels ($p>0.05$).

Moreover, the LPS levels were also quantified in CM-rich fraction media to assess the amount of LPS that crosses the cell monolayer associated with CMs and the potential effect of GSPE-supplementation. The LPS levels were significantly higher in LPS-treated cells compared with the control (Figure 2B). Interestingly, when administered together with lipids, the LPS levels were slightly higher than LPS administration alone. This suggests an increase in LPS transport associated with CMs. GSPE decreased CM-associated LPS levels in the co-culture of LM+LPS but not when cells were cultured with LPS alone (Figure 2B).

Considering these results, we wondered if GSPE was modulating LPS transport by reducing CM production. For this reason, we measured the APOB-48 levels in the cell culture media. The APOB-48 levels were increased up to 4-fold in all treatments

containing LM in comparison with the control and non-stimulated cells, thus confirming CM secretion in our *in vitro* model. However, GSPE did not modify the APOB-48 levels (Figure 2C), which indicates that GSPE was not reducing LPS transport by decreasing CM synthesis. To assess whether GSPE induces a reduction in CM size, we calculated the TAG/APOB-48 ratio. As shown in Figure 2D, although not significant, GSPE limited CM size when treated with LM.

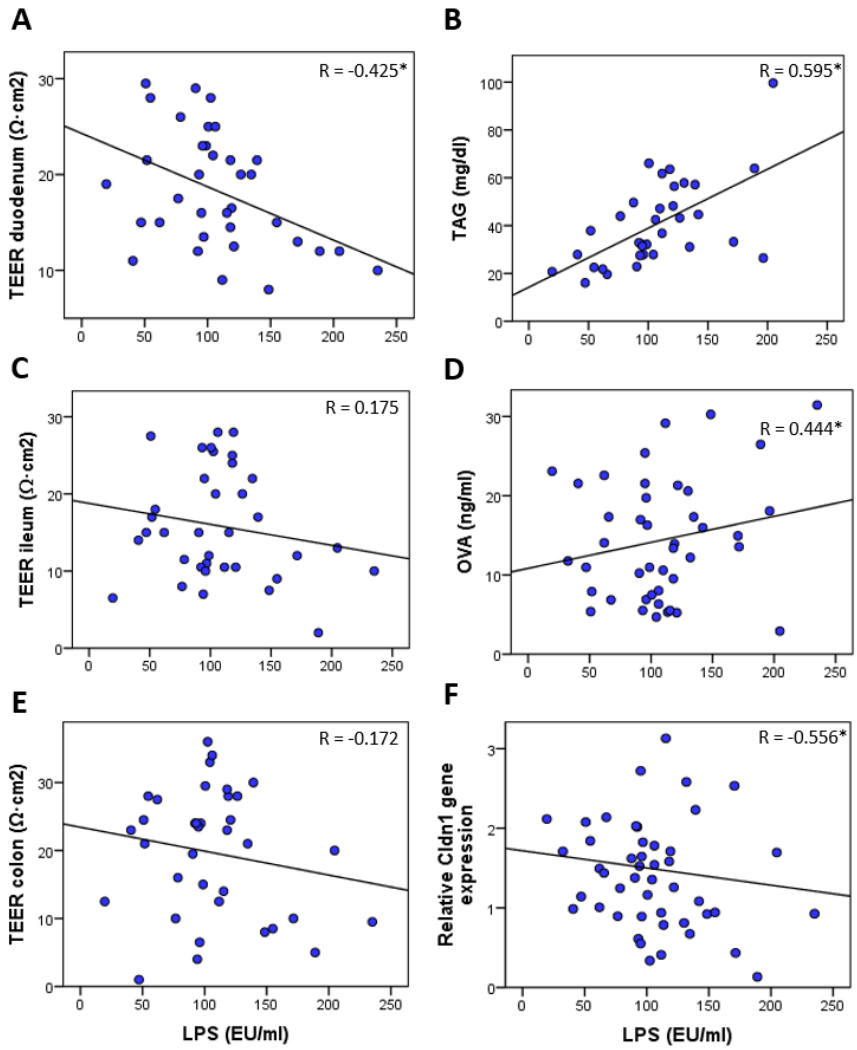

Figure 1. Pearson's correlations between LPS levels in plasma and different parameters: (A, C, E) Transepithelial Electrical Resistance (TEER); **(B)** Plasma triglycerides (TAG); **(D)** Ovalbumin (OVA); **(F)** Claudin-1 (*Cldn-1*) relative gene expression in the ileum; n = 35-54; *p < 0.05.

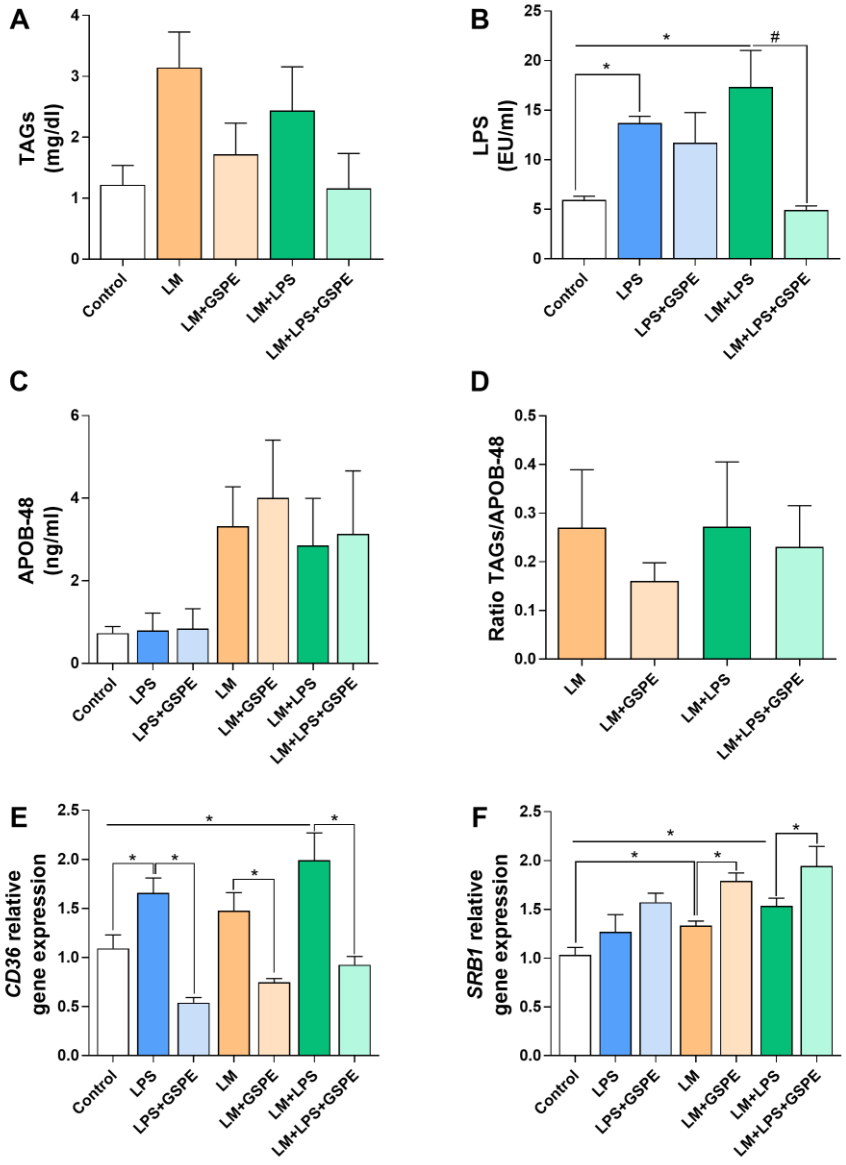

Figure 2. Caco-2 cells as an in vitro model for LPS intestinal translocation associated with CMs. Cells were stimulated with either LPS (1µg/ml), LM, or LM+LPS and also with or without GSPE (250 µg/ml). **(A-C)** TAGs, LPS and APOB-48 levels in the CM-rich fraction, respectively; **(D)** TAGs/APOB48 ratio; **(E-F)** *CD36* and *SRB1* gene expression levels, respectively. *Abbreviations: APOB-48, Apolipoprotein B48; CD36, Cluster of Differentiation 36; GSPE, Grape Seed Proanthocyanidin Extract; LM, lipid mixture; LPS, lipopolysaccharide; SRB1, Scavenger Receptor Class B member 1; TAGs, triglycerides.* Values are means ± SEM (n=2-9). *p < 0.05. #Trends: 0.05 < p-value < 0.1.

GSPE modulation of LPS transport through receptor-mediated endocytosis

We studied the receptor-mediated endocytosis pathway by evaluating *CD36* and *SRB1* gene expression in Caco-2 cells. LPS treatment induced a significant increase in *CD36* expression. Moreover, when cells were treated with a mixture of LM+LPS, the mRNA levels were even higher compared to individual treatments. GSPE induced a significant decrease in *CD36* expression compared to the respective controls (Figure 2E). With respect to *SRB1*, LM treatment increased its expression levels compared to the control. Similar to the results observed for *CD36*, the combination of LM+ LPS induced a higher increase in *SRB1* expression. Contrary to *CD36*, GSPE increased *SRB1* mRNA levels when administered together with LM and with the co-culture with LM+LPS (Figure 2F).

To validate these *in vitro* results, we measured *Cd36* and *Srb1* gene expression in the duodenum, ileum and colon of obese rats. In duodenum, although changes were not significant, *Cd36* expression was higher in the PRE group compared to the CAF group (Figure 3A) and *Srb1* did not show any difference between treatments (Figure 3B). In the ileum, CAF-fed rats showed an increase in *Cd36* mRNA levels with respect to the control, and the simultaneous treatment with GSPE tended to reduce mRNA levels (Figure 3C). In contrast, the CAF diet seemed to induce a reduction in *Srb1* mRNA levels compared with the control, and GSPE had no effect on gene expression (Figure 3D). No changes in gene expression were observed in the colon (Figure 3E-F).

GSPE modulates LPS accumulation in the liver

In previous animal studies, we showed that the CAF diet provoked metabolic endotoxemia [12]. Moreover, it has been described that metabolic endotoxemia is directly associated with insulin resistance due to LPS tissue accumulation, especially in liver and white adipose tissue [14]. In order to assess LPS accumulation, we measured LPS levels in liver and MWAT homogenates. In the liver, the CAF diet induced a higher accumulation of LPS compared to the control animals. GSPE significantly decreases LPS accumulation in the liver when administered both preventively and simultaneously with the CAF diet, and was statistically different in the SIT group (Supplementary material, Figure 2A). No changes were observed in the LPS levels in adipose tissue (Supplementary material, Figure 2B).

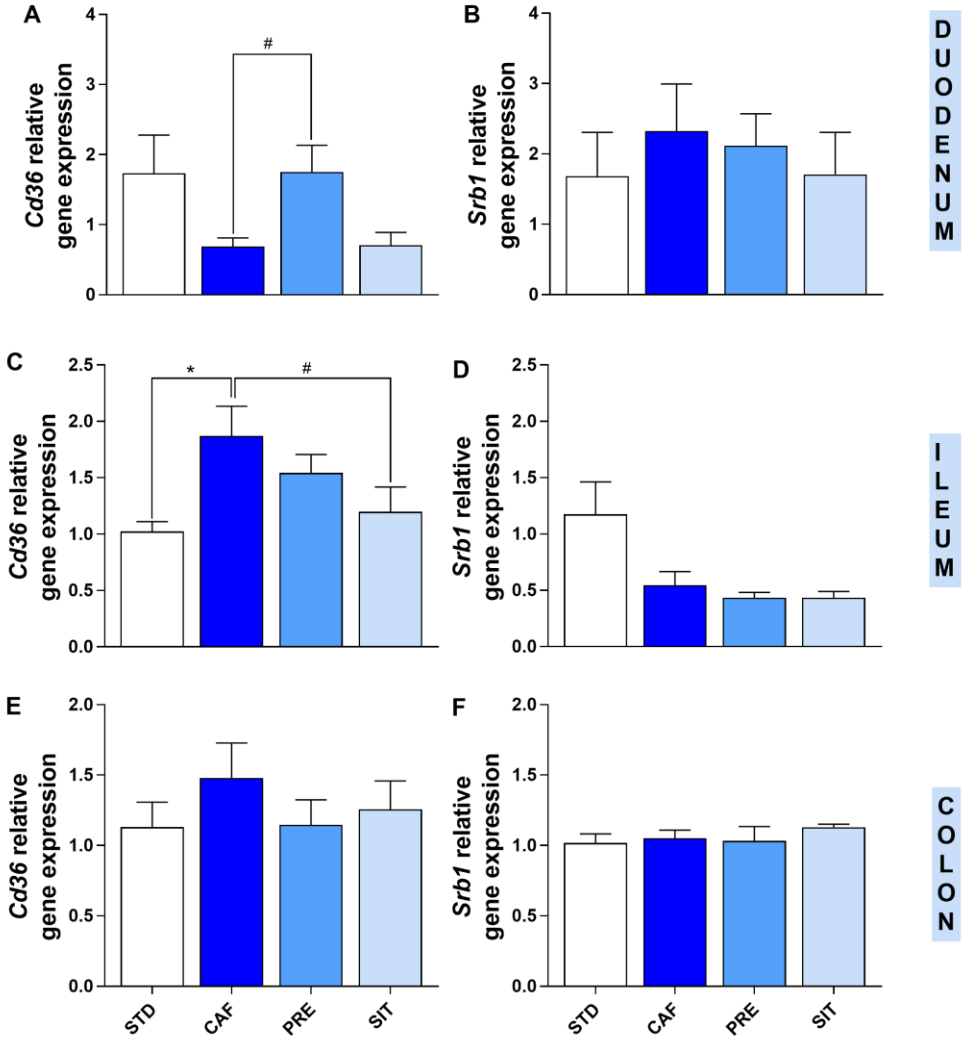

Figure 3. The effect of GSPE treatment on *Cd36* and *Srb1* gene expression in the intestine of diet-induced obese rats. (A-B) Gene expression in the duodenum; **(C-D)** Gene expression in the ileum; **(E-F)** Gene expression in the colon. The expression of target genes was normalized to *Hprt*. *Abbreviations: Cd36, Cluster of Differentiation 36; Srb1, Scavenger Receptor Class B member 1.* Values are means ± SEM (n=4-10). *p < 0.05. #Trends: 0.05 < p-value < 0.1.

GSPE epigenetic modulation of the endocannabinoid system

Given our results, in which intestinal permeability is affected by the CAF diet, and GSPE supplementation seems to have a modulatory effect, we aimed to determine its mechanism of action. One potential mechanism is the modulation of the endocannabinoid system. *Cnr1* activation regulates intestinal permeability and inflammation [15], especially in a context of obesity [16]. For this reason, we studied whether GSPE could be modulating intestinal permeability and LPS translocation at this level in our diet-induced obesity animal model.

We evaluated *Cnr1* gene expression in the colon because it is the most abundant LPS-containing intestinal segment due to the presence of microbiota. The CAF diet induced an increase in *Cnr1* mRNA levels compared with the STD group. Moreover, GSPE simultaneous supplementation reduced *Cnr1* expression (Figure 4A).

To further study the possible mechanism of GSPE modulation on *Cnr1* expression in the colon, we performed an epigenetic analysis of the *Cnr1* promoter's CpG site methylation (Figure 4B). Although no changes in the average methylation were observed (Figure 4C), the methylation of the promoter in position 7 was higher in the SIT group than in the CAF group (Figure 4D). This result could partially explain the decrease in *Cnr1* gene expression (Figure 4A). Furthermore, preventive supplementation with GSPE also reduced the DNA methylation pattern of the *Cnr1* promoter with respect to the CAF rats in positions 4 and 6 (Figure 4D).

Gut microbiota as a modulator of the endocannabinoid system

Gut microbiota and derived short-chain fatty acids (SCFAs) can be altered by the consumption of sugars and saturated fats [2]. Moreover, gut microbiota can modulate intestinal permeability and LPS translocation through the interaction with ECS [15,17]. Therefore, we wanted to evaluate the possible correlations between some cecal SCFAs and the expression of *Cnr1* and the methylation pattern of its promoter (Table 1). The expression of *Cnr1* was negatively correlated with methylation levels in positions 2 and 5. We also found that positions 2 and 5 were positively correlated with the isobutyric acid levels, and position 8 with isovaleric acid. Moreover, we also found some negative correlations between positions 2, 3, 4, 6, and 7 with valeric acid in the cecal content (Table 1). In addition, the methylation of positions 2, 3, 4 and 5

were negatively associated with insulin resistance indexes such as HOMA-IR and insulin levels (Table 1).

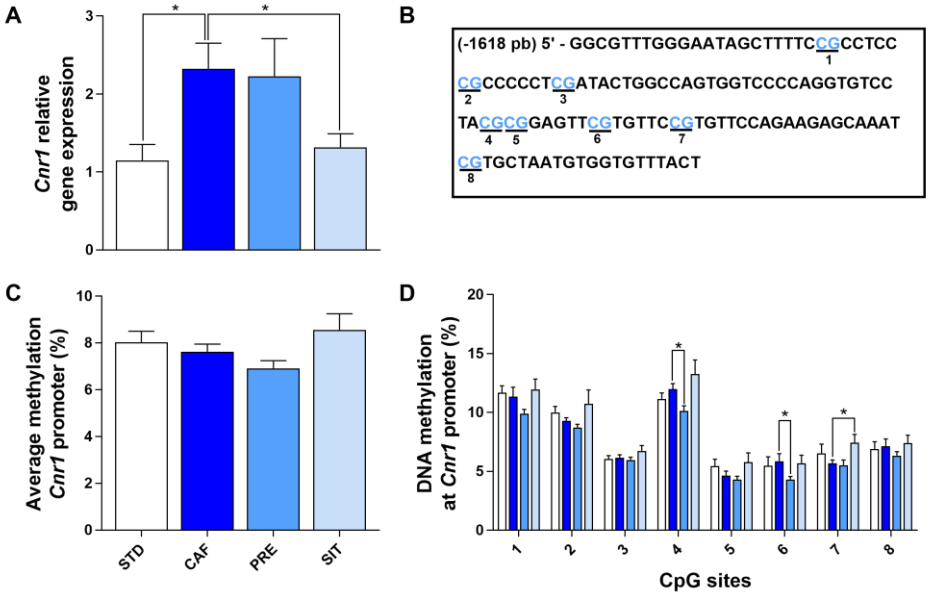

Figure 4. Simultaneous treatment with GSPE modulates *Cnr1* gene expression, possibly through epigenetic mechanisms. (A) *Cnr1* gene expression; **(B)** Localization of the CpG sites (numbered from 1 to 8) of the *Cnr1* promoter; **(C)** Average DNA methylation from the CpG sites of a region of *Cnr1* promoter; **(D)** DNA methylation on 8 CpG sites of a region of the *Cnr1* promoter. The expression of target genes was normalized *Ppia*. *Abbreviations: Cnr1, Cannabinoid receptor type 1.* Values are means ± SEM (n=3-9). *p < 0.05

Table 1. Correlation coefficients of cannabinoid receptor 1 gene expression and methylation values compared with cecal microbiota derived SCFAs and markers of intestinal permeability and insulin resistance.

Variables	***Isobutyric***	***Butyric***	***Isovaleric***	***Valeric***	***Insulin***	***HOMA-IR***	***OVA***	***LPS***	***Colon Cnr1 gene expression***
Colon Cnr1 gene expression	0.263	-0.225	0.198	0.283	0.266	**0.428***	**0.496***	0.132	
Cnr1 promoter methylation									
Position 1	0.088	0.101	-0.278	-0.095	-0.218	-0.306	-0.061	0.224	-0.382
Position 2	-0.009	-0.041	**0.524***	**-0.427***	**-0.623***	**-0.734***	-0.078	0.168	**-0.403***
Position 3	0.344	-0.249	0.295	**-0.481***	**-0.469***	**-0.542***	-0.177	0.100	-0.240
Position 4	0.260	-0.234	0.137	**-0.505***	**-0.465***	**-0.517***	0.070	0.215	-0.261
Position 5	-0.023	0.110	0.039	-0.284	**-0.424***	-0.378	-0.281	0.025	**-0.481***
Position 6	**0.573***	-0.207	-0.092	**-0.435***	-0.306	-0.333	-0.059	0.214	-0.047
Position 7	0.035	-0.117	0.137	**-0.569***	-0.428	-0.426	-0.312	-0.022	-0.410
Position 8	**0.439***	-0.202	0.042	-0.412	-0.403	-0.363	0.031	0.058	-0.142

Abbreviations: Cnr1: Cannabinoid receptor type 1; LPS: lipopolysaccharide; OVA: ovalbumin. n = 31-54; *p-values < 0.05

Gut microbiota SCFAs are associated with intestinal permeability

With this established correlation between the *Cnr1* methylation pattern and SCFAs, we aimed to evaluate the possible associations between SCFAs and intestinal permeability, LPS transport and tissue accumulation (Table 2). *Srb1* expression levels were negatively correlated with propionic acid and positively with butyric acid. Interestingly, accumulation of TAGs in the liver, plasma LPS and *Cd36* gene expression in the colon were positively associated with isobutyric acid levels. Moreover, butyric acid was negatively correlated with plasma OVA levels and positively with *Srb1* gene expression in the colon. Valeric acid was positively associated with the accumulation of LPS in the mesenteric adipose tissue (Table 2).

Paracellular pathway, receptor-mediated endocytosis and gut microbiota as major contributing factors of increased metabolic endotoxemia

With this scenario, we decided to generate a multiple regression model to explain the major factors studied that contribute to intestinal LPS translocation and subsequent metabolic endotoxemia. The independent variables were OVA and TAG levels in plasma, *Cldn-1*, *Cd36* and *Cnr1* intestinal gene expression, and butyric acid cecal concentration. Applying the backward exclusion method, we obtained a reduced model to explain LPS accumulation in plasma through OVA levels, *Cd36* gene expression in the ileum and butyric acid concentration. Hence, the most important factors determining LPS translocation are related to paracellular, receptor-mediated endocytosis and CM-associated pathways, as well as gut microbiota, with a corrected R^2 of 0.767 (Supplementary material, Table 3).

Table 2. Correlation coefficients of short chain fatty acids with intestinal permeability and systemic inflammation markers

Variables	***Propionic***	***Isobutyric***	***Butyric***	***Isovaleric***	***Valeric***
Plasma triglycerides (mg/dl)	-0.318	-0.224	0.032	-0.177	-0.276
Liver triglycerides (mmol/g tissue)	0.140	**0.485***	-0.144	-0.300	0.341
Plasma Ovalbumin (ng/ml)	0.279	0.354	**-0.459***	-0.033	0.068
Plasma LPS (EU/ml)	0.106	**0.445***	-0.169	0.104	-0.077
LPS in MWAT (pg/g tissue)	-0.156	-0.015	0.233	-0.184	**0.562***
Ileal gene expression					
Scavenger receptors					
Cd36	-0.099	**0.582***	-0.335	0.043	-0.245
Srb1	**-0.501***	-0.104	**0.608***	-0.050	0.050

Abbreviations: Cd36: Cluster of Differentiation 36; LPS: lipopolysaccharide; MWAT: mesenteric adipose tissue; Srb1: Scavenger Receptor class B type 1. n = 35-54; *p-values < 0.05

4. Discussion

The excessive consumption of dietary sugars and fat causes intestinal barrier dysfunction, provoking metabolic endotoxemia and systemic inflammation. New therapeutic approaches are needed to treat metabolic endotoxemia, and numerous studies have found that proanthocyanidins play an important role in protecting the intestinal barrier, especially through their anti-inflammatory activity. This study aims to use both *in vitro* and *in vivo* experimental approaches to elucidate how GSPE could modulate intestinal LPS translocation not only through the paracellular pathway but also by receptor-mediated endocytosis and chylomicron-associated transport in a context of obesity.

Our group obtained previous results that demonstrated that a dose of 500 mg/kg of GSPE was capable of reducing the plasma levels of both LPS and OVA, as well as ameliorating intestinal damage in the duodenum and ileum caused by consuming a CAF diet [12]. Now, we have found that LPS levels are associated with TAGs in plasma in our *in vivo* model for diet-induced obesity, in agreement with results obtained in obese humans and HFD-fed mice [18,19]. This association reinforced the idea that LPS is being transported associated with CMs. Moreover, we also found that LPS and OVA circulating levels were positively correlated, suggesting that they might be transported by similar pathways across the intestinal barrier, such as receptor-mediated endocytosis and paracellular pathways [20]. We found that ileal claudin-1 gene expression and TEER values in the duodenum were negatively correlated with LPS plasma levels. Regardless of the mechanism responsible for LPS translocation, any dietary intervention able to modulate the LPS transport across the intestinal barrier, and thus reduce the metabolic endotoxemia, would be of great interest.

Postprandial lipemia dysregulation is an important factor for cardiovascular diseases. Regarding LPS transport associated with CMs, we observed that GSPE seems to reduce LPS passage *in vitro*. Several studies have demonstrated the protective effect of GSPE as it reduces hyperlipidemia [13] and metabolic endotoxemia in cafeteria-fed animal models [12,21]. Our results also support the findings of *Quesada et al.*, who reported that a dose of 250 mg GSPE/kg b.w. reduced TAG levels associated with CM in male rats fed with a chow diet after lard oil administration [9]. The GSPE hypotriacylglycerolaemic effect has been previously demonstrated [22]; however,

whether the lipoprotein amount or size was modified, has not yet been elucidated. Although our results are not conclusive, they suggest that GSPE reduces TAG transport by reducing CM size, as APOB-48 levels were not affected by GSPE treatment. Taking all these results into account, we can hypothesize that GSPE might be modulating LPS transport associated with CMs in an indirect way by reducing CM size. *Hayashi et al.* demonstrated that consuming a high fat diet had no effect on increasing CM production but it did affect the size of rats [23]. *Yaman et al.* recently found that humans with a high postprandial TAG response showed higher levels of TAG, APOB-48 and a bigger CM size [24]. Therefore, although our results need to be confirmed in future studies, this potential effect of GSPE might have an impact on both hypertriglyceridemia and metabolic endotoxemia management.

With respect to receptor-mediated endocytosis, the gene expression results were inconsistent between the *in vitro* and *in vivo* experiments. In the *in vitro experiment*, GSPE was able to reduce *CD36* gene expression. In animal models, the CAF diet increased *Cd36* expression in the ileum, and synchronic treatment with GSPE reduced it, as seen in the *in vitro* model. These results support that Caco-2 cells spontaneously change their morphological and functional features during the differentiation process, exhibiting a phenotype closer to small intestine enterocytes than colonocytes, as previously reported [25]. Moreover, as it has been described in the literature, *Cd36* is mainly expressed in the proximal small intestine (duodenum and jejunum), where lipid absorption and CM production take place [26,27]. Our results probably suggest that the non-absorption of fatty acids in the duodenum is being compensated in the ileum. Interestingly, the *SRB1* expression profile was the opposite to the *CD36 in vitro*. SRB1 is described as a multifactorial receptor, displaying positive and negative effects depending on the context. For example, upregulation of SRB1 expression constitutes a risk factor for obesity, whereas it plays a protective role in diabetes [28]. In this study, GSPE increased *SRB1* gene expression *in vitro,* supporting previous results from experiments with obese rats treated with other antioxidants [29]. We could also hypothesize that the increase in *SRB1* expression is a possible compensatory mechanism due to the reduction in *CD36* expression, and not related to the GSPE treatment.

Previous reports have shown that ECS controls the gut barrier function through a CNR1-dependent mechanism and obesity is characterized by ECS dysregulation [30], leading to a higher intestinal permeability and metabolic endotoxemia [15,17]. In our study, we found that the CAF diet increased *Cnr1* expression in the colon. Moreover, we demonstrated that GSPE modulates DNA methylation of the *Cnr1* promoter in the colon of CAF-fed rats. *D'Addario et al.* previously demonstrated *Cnr1* regulation by promoter methylation in animal models of anorexia nervosa [31] and obesity [30]. Moreover, the epigenetic regulator resveratrol modulates gut microbiota via interaction with ECS in a HFD-induced obesity model [15]. Taken together, our results suggest that GSPE might modulate intestinal permeability through epigenetic mechanisms at the endocannabinoid system (ECS) level. Moreover, the methylation profile of the *Cnr1* promoter was negatively associated with insulin and the HOMA-IR values. This supports previous results that positively correlated gut permeability with insulin resistance [32,33].

We found interesting associations between SCFAs with intestinal permeability and metabolic endotoxemia-related parameters. SCFAs are products derived from bacterial fermentation of undigested carbohydrates, which play an important role in human health and metabolism. Butyric acid was negatively correlated with OVA plasma levels and positively with *Srb1* expression in the ileum. Our results support the idea that butyric acid is crucial in maintaining gut barrier integrity, especially avoiding LPS translocation through the paracellular pathway [34]. In contrast to the literature, we found that butyric acid did not have a protective effect against LPS-translocation via SRB1 [35,36]. Moreover, in contrast to butyric acid, the isobutyric levels were positively correlated with the TAG and LPS levels in plasma, as well as with the *Cd36* gene expression in the ileum. Then, increased isobutyric levels might be harmful for intestinal barrier integrity, promoting LPS translocation through both paracellular and receptor-mediated endocytosis pathways. In fact, high levels of isobutyric acid have been associated with negative effects in patients with cancer and metabolic syndrome [37]. Valeric acid was positively correlated with LPS levels in adipose tissue and negatively correlated with the *Cnr1* methylation pattern. In contrast to butyric acid, propionic acid was negatively associated with *Srb1* expression in the ileum, so that it exhibited a beneficial anti-inflammatory effect, as previously described [35,36]. Moreover, since dietary flavonoids are degraded by

intestinal microbiota in the colon, the microbiota-derived metabolite profile depends on the flavonoids ingested. In accordance with our results, it has been previously demonstrated that GSPE decrease butyric acid levels in cecal content CAF-diet fed animals [38]. All things considered, since the microbiota is linked to intestinal homeostasis, we propose GSPE as an interesting and promising candidate for modulating dysbiosis and intestinal permeability.

In previously published research by our group, we found that our *in vivo* model for obesity showed a slight insulin resistance [13]. It has been described that gut permeability is directly associated with LPS tissue accumulation and insulin resistance, especially in liver and adipose tissue, where LPS exerts its cytotoxic activity [14]. We found that LPS levels were five times higher in adipose tissue than in the liver. It has been described that the liver has mechanisms to detoxify LPS that are not present in adipose tissue [39,40], such as the disposal of LPS through bile secretion, or enzymes in liver Kupffer cells that detoxify LPS [6]. In our experimental animals, the CAF diet impaired these mechanisms, so that LPS levels increased in the liver; however, GSPE was able to restore this function. Interestingly, this lower LPS accumulation in the liver could be a sign of good hepatic functioning. In fact, in accordance with these results, we reported in previous studies that the SIT group had a reduced TAG accumulation in the liver compared to the CAF group. This supports that GSPE improves the hepatic function and reduces the risk of a low grade of inflammation and hepatic steatosis [13].

The proposed GSPE protective mechanisms against metabolic endotoxemia are shown in Supplementary material, Figure 3. In conclusion, this is the first study that evaluates how GSPE can modulate metabolic endotoxemia considering several pathways, and which studies potential action mechanisms such as the modulation of gut microbiota and ECS. We suggest that *Cnr1* could be a major therapeutic target for treatment of metabolic endotoxemia, and GSPE could be used as a prebiotic to regulate health and metabolic changes via SCFA production. Further studies are needed to explore new potential targets of GSPE in the modulation of metabolic endotoxemia and to translate this to human nutrition.

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Author contributions: Marta Sierra-Cruz: Writing - original draft, Data curation, Methodology, Software. **Alba Miguéns-Gómez:** Methodology, Software. **Esther Rodríguez-Gallego:** Research, Conceptualization**. Claudio D'Addario**: Review and editing. **Martina Di Bartolomeo**: epigenetic methodology. **Mayte Blay:** Research, Conceptualization, Resources. **Montserrat Pinent**: Conceptualization, Funding acquisition, Project administration. **Raúl Beltrán-Debón**: Writing - review & editing, Formal analysis, Supervision. **Ximena Terra**: Writing - review & editing, Formal analysis, Supervision.

Supplementary material: figures

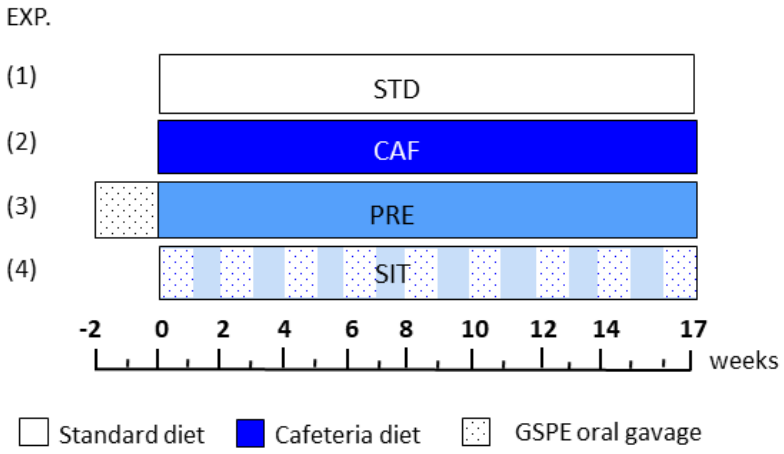

Figure 1. Schematic diagram of the experimental design. All animals were subjected to adaptation to the environment and to oral gavage during one week before the onset of the experiment. **(1) STD:** rats receiving the standard diet during the whole experiment; **(2) CAF:** rats receiving the standard diet before the cafeteria diet intervention; **(3) PRE:** rats receiving a preventive treatment with GSPE for 10 days before the cafeteria diet challenge; **(4) SIT:** rats receiving GSPE treatment simultaneously and intermittently with the cafeteria diet every other week. *Abbreviations: CAF, cafeteria diet; GSPE, Grape Seed Proanthocyanidin Extract; STD, Standard diet.*

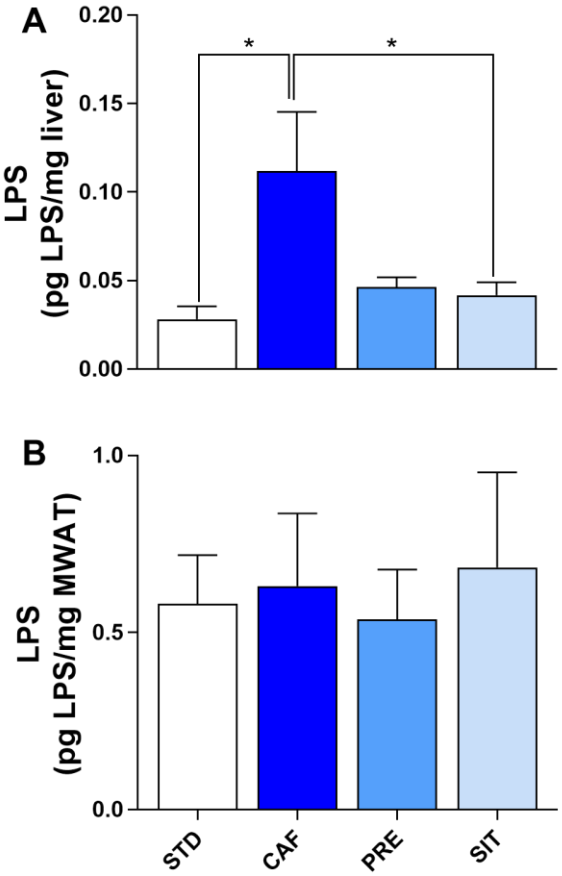

Figure 2. GSPE decreases LPS accumulation in the liver. LPS quantification in **(A)** the liver and **(B)** MWAT homogenates. *Abbreviations: LPS, lipopolysaccharide; MWAT, mesenteric adipose tissue.* Values are means ± SEM (n=3-9). *p < 0.05

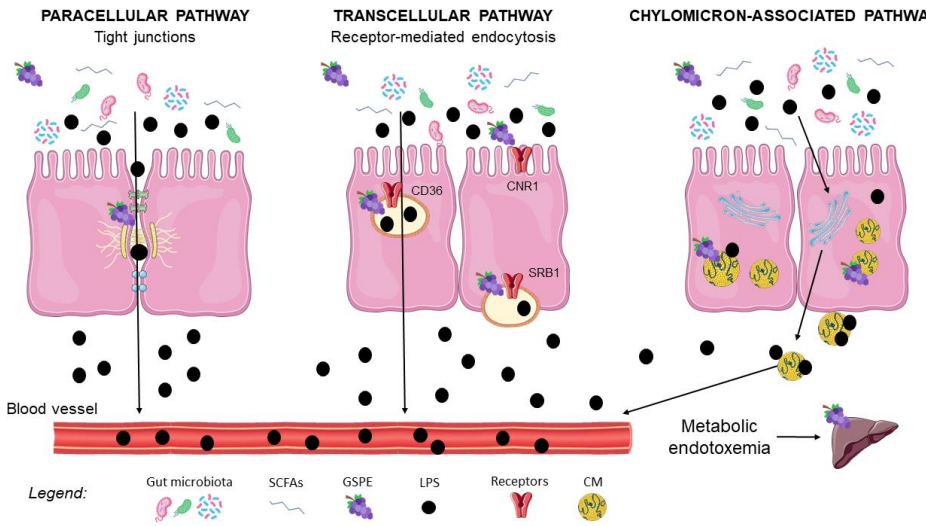

Figure 3. LPS transport pathways across intestinal epithelial cells and proposed GSPE protective mechanisms. Consuming high-fat/high-sucrose diets provokes large changes in microbiota composition and function (dysbiosis), leading to the release and translocation of LPS through the intestinal barrier. The paracellular pathway permits LPS translocation by passive diffusion due to tight junction alterations; the transcellular pathway is mediated by specific scavenger receptors for dietary fatty acids such as CD36 and SRB1; and the lipoprotein-associated pathway permits LPS translocation through newly released chylomicrons during post-prandial period. LPS accumulation in blood circulation leads to metabolic endotoxemia and consequent inflammation of peripheral tissues, especially the liver. GSPE modulates metabolic endotoxemia via CNR1 by changing microbiota and SCFA profiles, *Cd36* and *Srb1* gene expression, affecting the metabolism of chylomicrons as well as reducing hepatic LPS accumulation. *Abbreviations: CD36, Cluster of Differentiation 36; CM, chylomicron; CNR1, Cannabinoid Receptor Type 1; GSPE, Grape seed proanthocyanidin extract; LPS, lipopolysaccharide; SCFAs, short chain fatty acids; SRB1, Scavenger Receptor Class B Type 1.*

Supplementary material: tables

Table 1. Primer sequences used for Quantitative real-time PCR of rat tissues

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
Cd36	GTCCTGGCTGTGTTTGGA	GCTCAAAGATGGCTCCATTG
Srb1	AGCCCCACTTCTACAATGCT	TGGCTCGATCTTCCCTGTTT
Cnr1	TCGACAGGTACATATCCATTCACA	GAGAGGCAACACAGCGATTACTACT
Hprt	TCCCAGCGTCGTGATTAGTGA	CCTTCATGACATCTCGAGCAAG
Ppia	CCAAACACAAATGGTTCCCAGT	ATTCCTGGACCCSSSSCGCT

Abbreviations: Cd36, Cluster of Differentiation 36; Cnr1, Cannabinoid receptor type 1; Hprt, Hypoxanthine-guanine phosphoribosyl transferase; Ppia, cyclophilin-E.; Sr-b1, Scavenger receptor class B type 1.

Table 2. Primer sets used for pyrosequencing

Rat	*Cnr1*	Forward	5' – AGAAGGGTAAGATTTGGTATAGTG – 3'
		Biot-Reverse	5' – AACTATACAACTAAATAAACACCACATTA – 3'
		Sequencing	5' – GTGGAGTTTGGGAATAGTTT – 3'

Table 3. Multiple regression mathematical model to explain and predict metabolic endotoxemia and the relevance of intestinal permeability-related variables.

	Unstandardized coefficient	Typified coefficient	p-value
Model	**B (Confidence interval of 95%)**	**Beta**	
1			
OVA	10.018 (-38.494 – 58.530)	1.489	0.468
TAGs	0.178 (-6.030 – 6.386)	0.074	0.913
Cldn-1 gene expression (ileum)	25.253 (-438.310 – 488.815)	0.273	0.836
Cd36 gene expression (ileum)	95.312 (-263.955 – 454.580)	1.171	0.372
Cnr1 gene expression (colon)	-12.718 (-160.805 – 135.368)	-0.280	0.747
Butyric	2.880 (-10.645 – 16.404)	1.205	0.456
2			
OVA	10.989 (-10.027 – 32.004)	1.634	0.195
Cldn-1 gene expression (ileum)	30.580 (-226.776 – 287.936)	0.331	0.730
Cd36 gene expression (ileum)	100.539 (-87.000 – 288.077)	1.236	0.187
Cnr1 gene expression (colon)	-14.983 (-90.854 – 60.887)	-0.330	0.574
Butyric	3.172 (-2.203 – 8.546)	1.328	0.157
3			
OVA	8.639 (3.142 – 14.136)	1.284	0.012*
Cd36 gene expression (ileum)	80.514 (16.887 – 144.141)	0.990	0.025*
Cnr1 gene expression (colon)	-7.085 (-35.373 – 21.203)	-0.156	0.525
Butyric	2.673 (0.080 – 5.265)	1.119	0.046*
4			
OVA	8.953 (4.261 – 13.645)	1.331	0.040*
Cd36 gene expression (ileum)	87.653 (37.781 – 137.526)	1.077	0.006*
Butyric	3.016 (1.086 – 4.945)	1.262	0.010*

Abbreviations: Cd36: Cluster of Differentiation 36; Cnr1, Cannabinoid receptor class B type 1; OVA, ovalbumin; TAGs, triglycerides. *p-value < 0.05

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MANUSCRIPT 4

Maintenance of intestinal barrier function in aged rats fed with a grape seed proanthocyanidin extract supplemented cafeteria diet

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In preparation

UNIVERSITAT ROVIRA I VIRGILI
ROLE OF FLAVONOIDS IN THE MODULATION OF INTESTINAL ALTERATIONS ASSOCIATED WITH METABOLIC CHALLENGES: OBESITY AND AGING
Marta Sierra Cruz

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Abstract: Cafeteria diet (CAF diet) was proved to induce intestinal alterations and metabolic endotoxemia in young obese rats. Here, we explore the intestinal barrier function in aging in a context of obesity and evaluate the possible beneficial effects of a grape-seed proanthocyanidin extract (GSPE) in 21-month-old female Wistar rats. Animals were fed a 11-week obesogenic diet and treated with 500 mg/kg body weight for 10 days before CAF diet onset or synchronically to the obesogenic challenge. Plasma lipopolysaccharide (LPS), mRNA levels of gut integrity-related genes and an *ex vivo* gut barrier integrity assay of different intestinal sections were performed. This study provides evidence of the maintenance of intestinal barrier function in the elderly under both healthy and obesogenic conditions and that GSPE could be useful as a complement for healthy aging process

Keywords: intestinal permeability, tight-junctions, obesity, aging, mucus, proanthocyanidins.

1. Introduction

The intestine is the largest surface in the body [1]. The intestinal barrier must ensure the absorption of nutrients but, at the same time, avoid the translocation of macromolecules, including microbial components and pro-inflammatory agents by acting both as a physical and biochemical barrier [2,3].The first layer of defence is the mucus layer, composed by highly glycosylated proteins and secreted by goblet cells [4]. The mucus lubricates the progression of luminal content, and acts as a physical barrier to capture gut microbiota and derived harmful antigens [5].

Several factors such as diet, sedentary life style, drug abuse or aging affect intestinal homeostasis and can cause intestinal disruption [1,2,6]. Consequently, the microbiota-derived antigens, especially lipopolysaccharide (LPS), cross the intestinal barrier, reach the bloodstream and trigger a systemic inflammatory response known as endotoxemia [1,7]. Particularly, high-fat/high-sugar diets have been widely demonstrated to cause intestinal dysfunction. These alterations include an increase in intestinal permeability by affecting tight-junction (TJ) proteins [8], gut microbiota alterations and, finally, metabolic endotoxemia in animal models of diet-induced obesity [9–11]. However, literature regarding the role of aging in intestinal dysfunction remains controversial. Some authors demonstrated that the supplementation with different intestinal bacteria prevents from age-related decline of mucus thickness in colon in a murine genetic model for aging [12–14]. Others did not find any intestinal alteration during aging [15]. Together, intestinal dysfunction is directly associated with the onset and development of metabolic, intestinal and neurodegenerative disorders such as non-alcoholic fatty liver disease, Chron disease and Alzheimer's disease, respectively [16–19]. Thus, maintaining the intestinal barrier integrity remains essential to preserve intestinal health and avoid the appearance of severe associated diseases.

Numerous strategies have been developed to prevent, improve and/or delay the metabolic alterations derived from obesity and aging. Polyphenols constitute a very interesting field of study due to their previously demonstrated anti-oxidant, anti-inflammatory and anti-aging properties [20]. One of the most relevant groups of polyphenols are proanthocyanidins (PACs) from dietary fruits and vegetables. We have previously evaluated the effects of a pharmacological dose of GSPE on young rats

under an obesogenic challenge and found that GSPE reverted several features of the metabolic syndrome [21], intestinal permeability and metabolic endotoxemia [22–24]. Moreover, GSPE treatment in aged rats was able to reduce hepatic steatosis but was not as effective against metabolic alterations as in young counterparts [25].

Since the intestinal homeostasis remains essential for human health, to understand the mechanisms for maintaining intestinal integrity and functionality it is essential to avoid intestinal-associated pathologies and ameliorate lifespan. As far as we know, this is the first study to evaluate the effects of a proanthocyanidin extract against intestinal alterations in a double-hit animal model for obesity and aging.

2. Materials and methodology

2.1. Proanthocyanidin extract

The grape seed extract rich in proanthocyanidins (GSPE) was provided by *Les Dérivés Résiniques et Terpéniques* (Dax, France). According to the manufacturer, the GSPE used in this study (Batch number: 207100) contains a total procyanidin content of 76.9% which consist of a mixture of: monomers (23.1%), dimers (21.7%), trimers (21.6%), tetramers (22.2%) and pentamers (11.4%) of flavan-3-ols.

2.2. Animal model

In this study, a total of 80 female Wistar female rats were used, 70 were 21 months old (weighing 300-350 g) and 10 were two months old (weighing 210-220 g). Rats were acquired from Charles River Laboratories (Barcelona, Spain). After one week of adaptation, the rats were individually caged in the animal quarters at 22°C with a 12-hour light/12-hour dark cycle and were fed *ad libitum* with a standard chow diet (Teklad 2014 Envigo, Barcelona, Spain) and tap water. After a period of acclimation, the aged animals were randomly distributed into 5 experimental groups (n=14) and were fed a standard chow diet *ad libitum*. The different combinations of diets and GSPE treatments are shown in Figure 1. The control groups (YOUNG and AGED) only received standard chow diet during the whole duration of the experiment. The PRE group received standard chow diet and was supplemented with a GSPE preventive treatment the 10 days before the onset of the experiment. The three remaining groups, in addition to the standard chow diet, received a cafeteria diet (CAF diet), as

a model of a high fat/high sucrose diet. The CAF diet consisted of bacon, sausages, biscuits with paté, carrots, muffins, and sugared milk, which induces voluntary hyperphagia [26]. This diet was offered freshly *ad libitum* every day to the animals for 75 days. Energy contents of meals offered to the animals were described by *Sierra-Cruz et al.* [25].

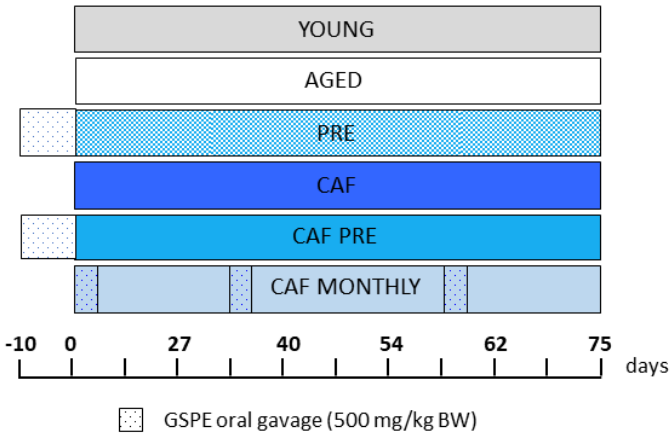

Figure 1. Schematic diagram of the experimental design. All groups were adapted to the environment and to oral gavage during one week before experiment started. YOUNG: two-month-old rats receiving standard chow diet; AGED: 21-month-old rats receiving standard chow diet; PRE: 21-month-old rats receiving standard chow diet and GSPE preventive treatment for 10 days; CAF: rats receiving a cafeteria diet; CAF PRE: rats receiving GSPE preventive treatment for 10 days before the cafeteria diet intervention started; CAF MONTHLY: rats receiving a 5-day GSPE treatment simultaneously with the cafeteria diet once per month. *Abbreviations: BW, body weight; CAF, cafeteria diet; GSPE, grape seed proanthocyanidin extract.*

The CAF group did not receive GSPE treatment. CAF PRE group received a preventive GSPE treatment during 10 days prior to CAF diet intervention, and CAF MONTHLY simultaneously with the CAF diet for 5 days once per month. GSPE was administered at a dose 500 mg/kg body weight. GSPE was dissolved in tap water and administered by oral gavage to the animals at 6 pm, three hours after removing all available food. The animals that were not supplemented with GSPE received water as a vehicle. Animals received fresh food one hour after GSPE administration.

2.3. Blood and tissue collection

At the end of the study, animals were fasted for 12 hours and euthanized by decapitation. The blood was collected using heparin (Deltalab, Barcelona, Spain) as anticoagulant. Plasma was obtained by centrifugation (1500g, 15 minutes, 4°C) and stored at −80°C until analysis. Both small and large intestine were dissected. Five-centimeter segments of the duodenum, ileum and colon were used for Ussing chamber assays. The rest of the tissue was rapidly removed, snap-frozen in liquid nitrogen, and stored at −80°C. All the procedures were approved by the Experimental Animal Ethics Committee of the Generalitat de Catalunya, Spain (Department of Territory and Sustainability, General Directorate for Environmental and Natural Policy, project authorization code: 10183).

2.4. Ussing chamber experiment

At the end of the experiment, rat intestine segments of duodenum, ileum and colon were immediately placed in cold oxigenated Krebs buffer (KRB), dissected to remove fat and muscular layers and placed in a 0.237 cm^2 aperture Ussing chambers (Dipl.-Ing. Mussler Scientific Instruments, Aechen, Germany). A total of 40 rats were used to do this experiment: YOUNG (n = 6), AGED (n = 6), PRE (n = 7), CAF (n = 7), CAF PRE (n = 7) and CAF MONTHLY (n = 7). First, apical and basolateral Ussing chamber compartments, representing intestinal lumen and blood circulation, respectively, were bathed with 2 ml of fresh KRB buffer containing 10 mM glucose [KRB-G] (Panreac, Barcelona, Spain) for stabilization during 15-20 minutes. Each gut segment was mounted by duplicate. Media from apical and basolateral sides were removed and replaced by KRB-G. In basolateral media, PBS containing FD4 (5.6 mg/ml) was also added. After 30 additional minutes, basolateral media was collected and stored at -80ºC until fluorescence analysis. Bathing solutions were at pH = 7.4 and continuously bubbled with a O_2/CO_2 (95%/5%) gas mixture and circulated in water-jacketed reservoirs kept at 37ºC.

2.5. Electrophysiological evaluation

Intestinal integrity and permeability were evaluated *ex vivo* by the measurement of transepithelial electrical resistance (TEER). To monitor the electrophysiological parameters in each Ussing chamber, a four-electrode system coupled to an external

six-channel voltage/current clamp electronic unit (Dipl.-Ing. Mussler Scientific Instruments, Aachen, Germany) was used. One pair of Ag/Cl electrodes were used for measuring the potential difference (PD) and another pair for the current passage. The spontaneous transepithelial PD was measured under open-circuit conditions after appropriate correction of fluid resistance. TEER (ohm·cm^2) was calculated every 30 min from the transepithelial PD and the short-circuit current in accordance with Ohm's law.

2.6. Paracellular transport of fluorescently labelled dextran

A 110 mg/ml stock solution of 4kDa-fluorescein isothiocyanate-dextran (FD4; TdB Consultancy AB, Uppsala, Sweden) was prepared in phosphate-buffered saline. FD4 was added apically to each Ussing chamber at a final concentration of 5.6 mg/ml and incubated for 30 minutes. The quantity of FD4 that crossed the basolateral compartment was measured by a PerkinElmer LS-30 fluorimeter ((Beaconsfield, UK) at λ_{ex} = 485 nm; λ_{em} = 540 nm) and compared with a FD4 standard curve.

2.7. Determination of plasma biochemical parameters

Plasma LPS levels were quantitatively measured using endotoxin detection system (Toxin Sensor™, Genscript, New Jersey, USA) based on a Limulus Amebocyte Lysate (LAL) colorimetric assay. Intestinal fatty acid-binding protein (iFABP) plasma levels were determined using commercial ELISA kit (KifeSpan Biosciences, Seattle, USA). The manufacturer's protocol was followed in all cases. Commercial ELISA kits were used to quantify plasma levels of intestinal fatty acid-binding protein (iFABP) (KifeSpan Biosciences, Seattle, USA) and interleukin-6 (IL-6) (Thermo Scientific, Spain).

2.8. Gene expression analysis

Total RNA was extracted from 50 mg of duodenum and both proximal and distal colon samples using Trizol (AMbion, USA) following the instructions as described previously by *Gil-Cardoso et al.* [27]. cDNA was obtained using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Madrid, Spain) following manufacturer's instructions. Quantitative real-time polymerase chain reaction (qPCR) amplification and detection was carried out in a qPCR system (CFX96 Touch-

Real Time PCR, Bio-Rad, Madrid, Spain). All samples were run in duplicate in 96-well reaction plates. Gene expression of claudin-2 (*Cldn2*), claudin-3 (*Cldn3*), tight junction protein zonula occludens-1 (*Tjp1*), and occludin-1 (*Ocel1*) were performed using TaqMan Universal PCR Master Mix and Taqman probes (Applied Biosystems, Madrid, Spain). The references of Taqman probes for rat are the following: Rn02063575_s1 for *Cldn2*, Rn00581751_s1 for *Cldn3*, Rn02116071_s1 for *Tjp1*, and Rn01420322_g1 for *Ocel1*. All the results were normalized respect to cyclophilin E (*Ppia*) (Rn00690933_m1). Gene expression of cluster of differentiation 36 (*Cd36*), scavenger receptor class B type 1 (*Srb1*), endocannabinoid receptor type 1 (*Cnr1*), endocannabinoid receptor type 2 (*Cnr2*), myosin light-chain kinase (*Mlck*), mucin-2 (*Muc2*) and cyclin-dependent kinase inhibitor p21 (*p21*) were carried out using Universal SYBR Green Supermix (Bio-Rad, Spain). mRNA expression levels were also normalized with respect to *Ppia* as endogenous control gene. Reactions with the iTaq Universal SYBR® Green Supermix were performed using the following thermal profile: 30 seconds at 95ºC, 40 cycles of 5 seconds at 95ºC and 30 seconds at 55-61ºC (depending on specific annealing temperatures), and 5 seconds at 65-95ºC with an increment of 0.5ºC. Reactions with the TaqMan Universal PCR Master Mix were performed as previously described by *Gil-Cardoso et al*. [23]. The relative mRNA expression levels were calculated following the $2^{-\Delta\Delta Ct}$ method. Primer sequences for the targeted SYBR rat genes are summarized in Table 1.

2.9. Statistical analysis

The data are represented as the mean ± standard error of the mean (SEM). Non-parametric statistical comparisons between groups were assessed by applying Kruskal-Wallis and Mann-Whitney tests. Analyses were performed with XLSTAT 2021.03.1 (Addinsoft, Barcelona, Spain). p-values < 0.05 were considered statistically significant.

Table 1. Primer sequences used for Quantitative real-time PCR of rat intestinal samples

Gene	Forward primer	Reverse primer
	Sequence (5'-3')	**Sequence (5'-3')**
Cd36	GTCCTGGCTGTGTTTGGA	GCTCAAAGATGGCTCCATTG
Srb1	AGCCCCACTTCTACAATGCT	TGGCTCGATCTTCCCTGTTT
Cnr1	TCGACAGGTACATATCCATTCACA	GAGAGGCAACACAGCGATTACTACT
Cnr2	GCCTGCAACTTCGTCATCTT	CCATGAGCGGTAGGTAGGAG
Mlck	CCCTTCCTTCTCTAGTGTTCTGA	AGCCTCACAGATGGATCGAG
Muc2	GATCCCGAAACCATGTCTGC	CCATTCACAACTGCCAGCTT
p21	AGAAGGGAACGGGTACACAG	ACCACCACATACCACACACA
Ppia	CCAAACACAAATGGTTCCCAGT	ATTCCTGGACCCSSSSCGCT

3. Results

3.1. The cafeteria diet affects differentially the integrity of intestinal sections

Sections of duodenum and colon were collected for *ex vivo* assessments. We evaluated intestinal integrity by measuring TEER values and FD4 concentration in basolateral compartment in an Ussing chamber equipment. FD4 concentration was significantly lower in CAF group compared to AGED in basolateral medium of duodenum section (Figure 2B). CAF diet significantly increases intestinal integrity in colon (Figure 2A). No changes were observed among groups for TEER in duodenum neither FD4 in colon (Figure 2). We did not observe an integrity loss associated with aging in this model, instead, we found reduced FD4 translocation associated with the CAF diet.

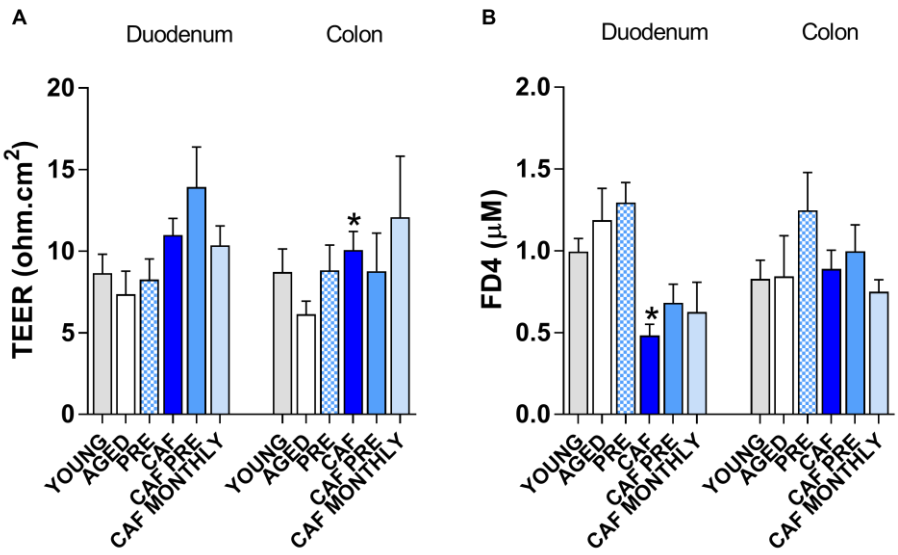

Figure 2. Intestinal permeability assessment in duodenum and colon. (**A**) Evaluation of TEER values and (**B**) quantification of FD4 transport across the intestinal barrier in duodenal and colonic mucosal preparations mounted in an Ussing chamber system. Values are represented as the means ± SEM. *p < 0.05 compared to AGED group. *Abbreviations: CAF, cafeteria diet; FD4, FITC-Dextran 4 kDa; GSPE, grape seed proanthocyanidin extract; TEER, transepithelial electrical resistance.*

3.2. Age and diet do not affect endotoxemia

The levels of circulating LPS were measured to assess endotoxin intestinal translocation (Figure 3). Age or diet did not affect endotoxemia. We did not observe any significant change among groups but LPS levels were generally lower in aged rats (Figure 3). CAF diet did not affect LPS levels, in line with the results regarding permeability. Interestingly, GSPE treatments seemed to reduce LPS concentration in plasma. In addition, IL-6 concentration in plasma was determined as a biomarker of systemic inflammation. Age significantly induced an increase in IL-6 levels compared with young animals (16.1 vs 35.2 pg/ml of IL-6, $p < 0.05$). However, systemic inflammation was not increased by CAF diet in these rats [25].

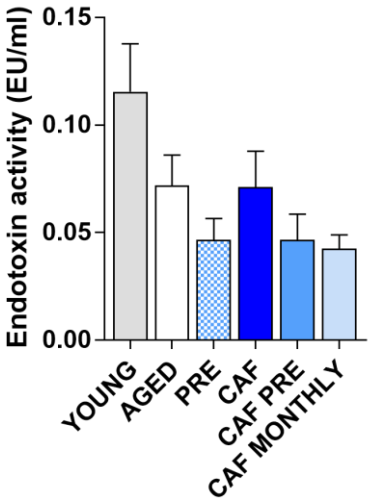

Figure 3. Plasma endotoxin levels. Values are represented as the means ± SEM.

3.3. The intestinal paracellular pathway: age, cafeteria diet and GSPE differential effects depending on the intestinal section

Tight-junctions, consisting on claudins, zoonulins, occludins and JAMs proteins, are related to the translocation of endotoxin through the intestinal paracellular pathway. Moreover, myosin light-chain kinase (MLCK) is a protein involved in controlling gut permeability by regulating TJ opening. In this study, we assessed the effects of aging, CAF diet and GSPE supplementation on TJ and *Mlck* gene expression in duodenum, proximal colon and distal colon. We did not observe any difference among treatments in the expression of TJ genes in duodenum (Figure 4B-E). However, both age and diet

induced a significant decrease in *Mlck* gene expression. GSPE, when administered preventively, was able to reverse this condition (Figure 4A). In proximal colon, GSPE significantly reduced *Cldn2* expression when administered previously to CAF diet intervention (Figure 5B). Moreover, *Cldn3* mRNA levels were significantly lower in YOUNG and CAF groups compared to AGED (Figure 5C). Indeed, GSPE preventive treatment also reduced *Cldn3* and *Ocel1* gene expression when administered to aged rats (Figure 5C). No changes in *Mlck* (Figure 5A) and *Tjp1* gene expression were observed (Figure 5E). GSPE synchronic supplementation to CAF diet reduced *Ocel1* gene expression compared to CAF group (Figure 5E). We did not observe any changes related to tight junctions in distal colon (Figure 6).

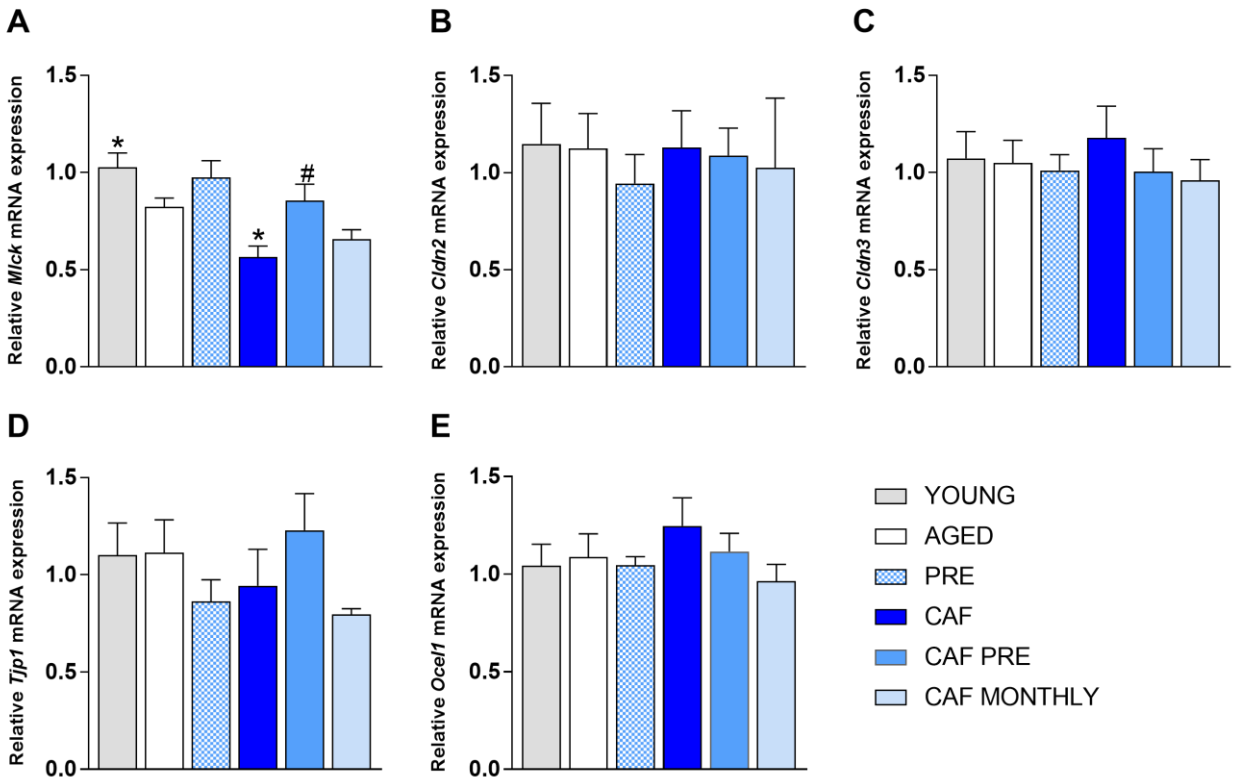

Figure 4. Expression of *Mlck* and TJ-related genes in the duodenum. The expression of target genes was normalized to cyclophilin A gene expression (*Ppia*). Values are represented as the means ± SEM. *p < 0.05 compared to AGED group. #p < 0.05 compared to CAF group. *Abbreviations: CAF, cafeteria diet; Cldn2, claudin-2; Cldn3, claudin-3; GSPE, grape seed proanthocyanidin extract; Mlck, myosin-like chain kinase; Ocel1, occludin-1; Tjp1, tight-junction protein 1.*

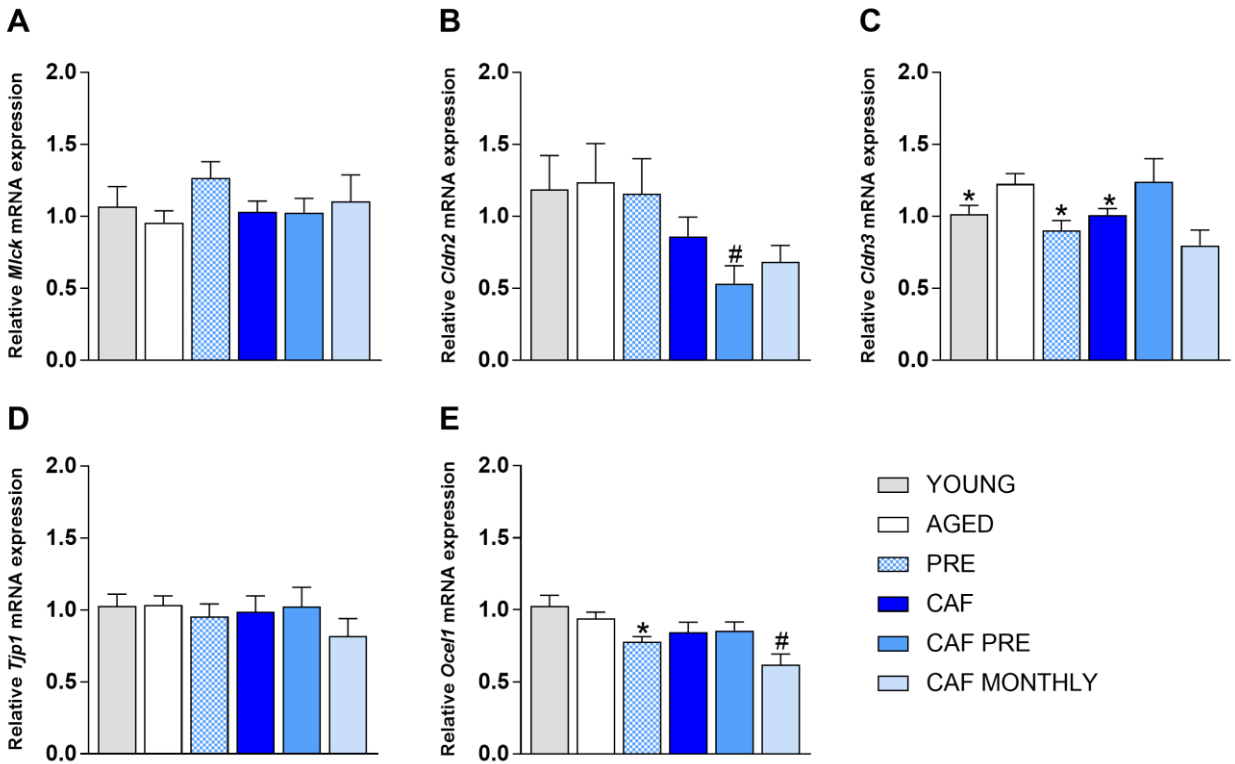

Figure 5. Expression of *Mlck* and TJ-related genes in the proximal colon. The expression of target genes was normalized to cyclophilin A gene expression (*Ppia*). Values are represented as the means ± SEM. *p < 0.05 compared to AGED group. #p < 0.05 compared to CAF group. *Abbreviations: CAF, cafeteria diet; Cldn2, claudin-2; Cldn3, claudin-3; GSPE, grape seed proanthocyanidin extract; Mlck, myosin-like chain kinase; Ocel1, occludin-1; Tjp1, tight-junction protein 1.*

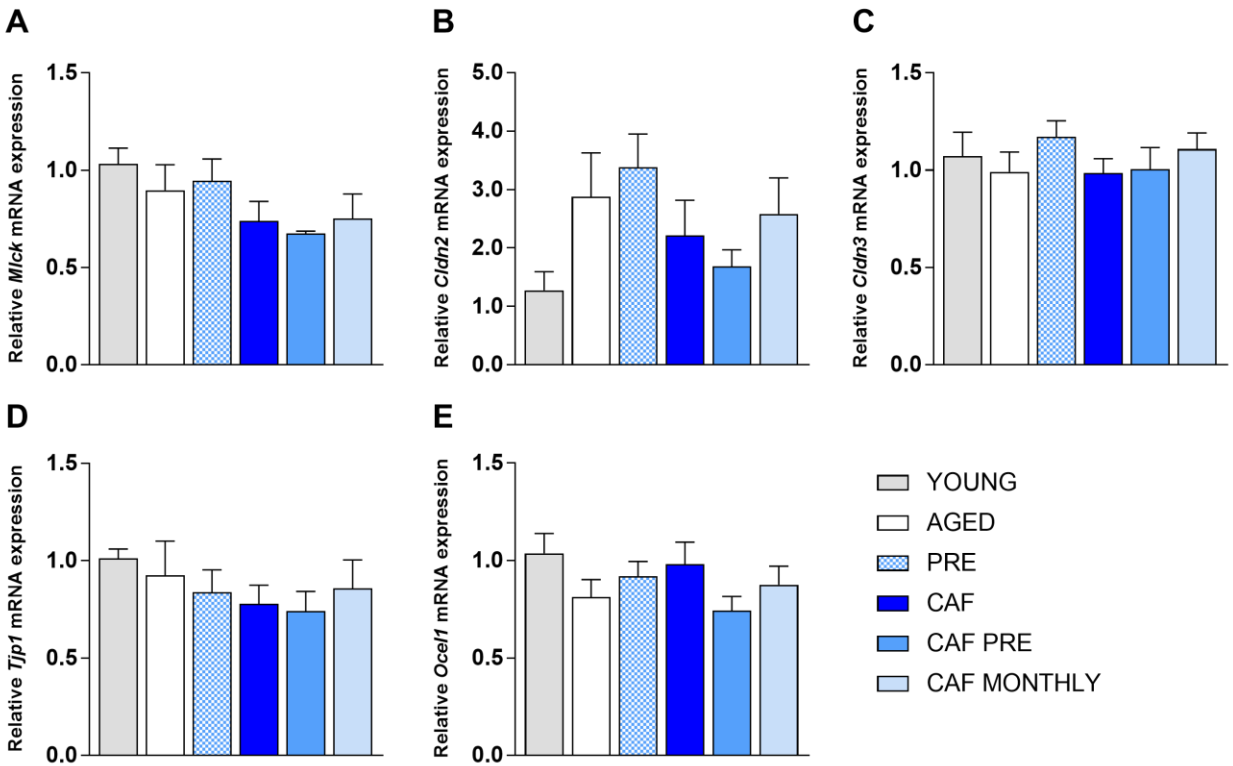

Figure 6. Expression of Mlck and TJ-related genes in the distal colon. The expression of target genes was normalized to cyclophilin A gene expression (*Ppia*). Values are represented as the means ± SEM. *Abbreviations: CAF, cafeteria diet; Cldn2, claudin-2; Cldn3, claudin-3; GSPE, grape seed proanthocyanidin extract; Mlck, myosin-like chain kinase; Ocel1, occludin-1; Tjp1, tight-junction protein 1.*

3.4. The intestinal transcellular pathway: gene expression of Cd36 in colon and Srb1 in duodenum are increased with age

Cd36 and *Srb1* are both receptors for dietary lipids and LPS. Both receptors are involved in the LPS intestinal transport through receptor-mediated endocytosis and chylomicron-associated pathways. In the present study, we measured *Cd36* and *Srb1* gene expression in small and large intestines. Although no changes in *Cd36* gene expression were observed in duodenum, *Cd36* mRNA levels were significantly increased with age in colon (Figure 7A). *Srb1* gene expression was increased in the duodenum of aged rats independently of the treatment. These differences were not significant (Figure 7B).

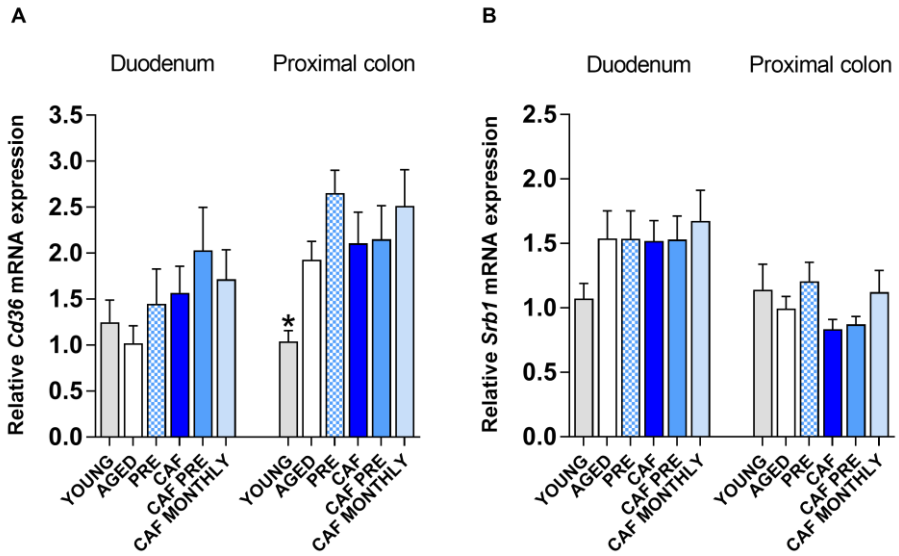

Figure 7. Gene expression of *Cd36* and *Srb1* in duodenum and proximal colon. The expression of target genes was normalized to cyclophilin A gene expression (*Ppia*). Values are represented as the means ± SEM. *p < 0.05 compared to AGED group. *Abbreviations: CAF, cafeteria diet; Cd36, cluster of differentiation 36; GSPE, grape seed proanthocyanidin extract; Srb1, scavenger receptor class B type 1.*

3.5. Endocannabinoid receptors are depleted with age in distal colon

Endocannabinoids modulate obesity-associated intestinal permeability and inflammation through CNR1 and CNR2. In this study, we aimed to evaluate ECS as a possible therapeutic target of GSPE in the modulation of the gut barrier function. We measured *Cnr1* and *Cnr2* gene expression in colon. mRNA levels in distal colon for both *Cnr1* (Figure 8A) and *Cnr2* (Figure 8B) genes were significantly reduced with age.

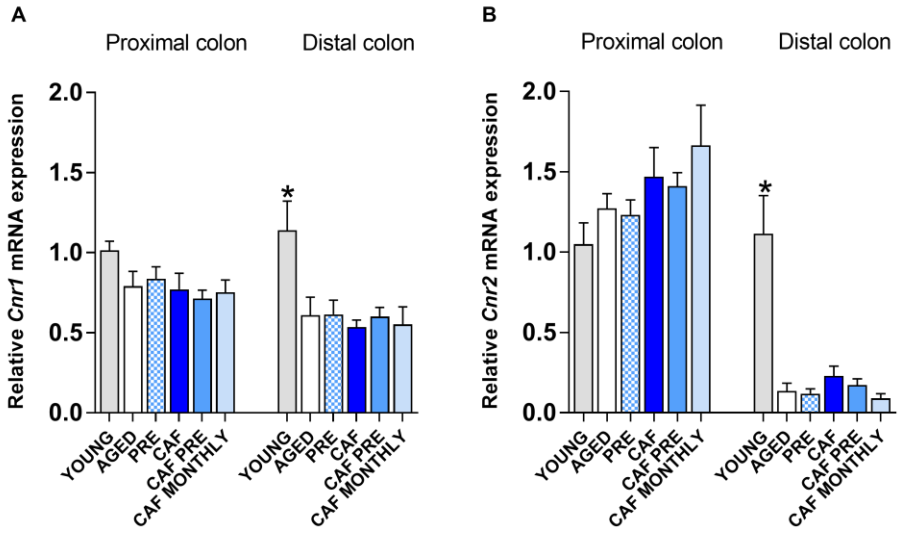

Figure 8. Expression of intestinal endocannabinoid system-related genes in proximal and distal colon. The expression of target genes was normalized to cyclophilin A gene expression (*Ppia*). Values are represented as the means ± SEM. *p < 0.05 compared to AGED group. *Abbreviations: CAF, cafeteria diet; Cnr1, cannabinoid receptor type 1; Cnr2, cannabinoid receptor type 2; GSPE, grape seed proanthocyanidin extract.*

3.6. The intestinal goblet cells: Muc2 gene expression in colon

Mucus layer is also crucial in maintaining intestinal barrier homeostasis and protecting from gut permeability. Goblet cells of intestinal mucosa secrete gel-forming proteins, mostly *Muc2*. *Muc2* was 3-fold more expressed in distal colon than in proximal colon (Figure 9). In distal colon, age significantly increased *Muc2* gene expression. Its expression was not affected by the diet nor the treatment with GSPE (Figure 9).

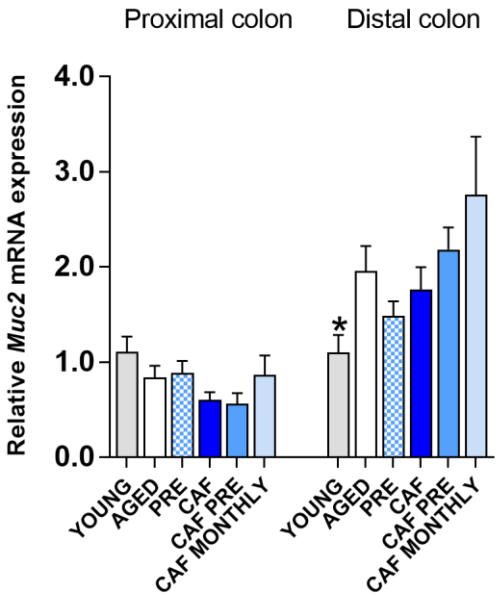

Figure 9. Gene expression of *Muc2* in proximal and distal colon. The expression of target genes was normalized to cyclophilin A gene expression (*Ppia*). Values are represented as the means ± SEM. *p < 0.05 compared to AGED group. *Abbreviations: CAF, cafeteria diet; GSPE, grape seed proanthocyanidin extract; Muc2, mucin-2.*

3.7. Age and diet do not induce intestinal cells senescence

The cyclin-dependent kinase inhibitor *p21* is a gene expressed in senescent cells causing growth arrest. We measured *p21* gene expression in duodenum, proximal colon and distal colon. No changes were observed in duodenum and proximal colon (Figure 10), however, GSPE synchronic treatment induced an increase in *p21* mRNA levels in distal colon compared to CAF group (Figure 10).

3.8. Cafeteria diet induces cellular damage in aged rats

Intestinal fatty acid-binding protein (iFABP) is a widely used biomarker for intestinal mucosal damage assessment. In this study, we evaluated iFABP concentration in plasma. Although the difference was not significant, iFABP concentration was clearly higher in CAF group compared with AGED group (Figure 11). Interestingly, GSPE supplementation prevented the intestinal damage induced by CAF diet. GSPE significantly reduced iFABP release into the bloodstream both when administered preventively and synchronically to CAF diet (Figure 11).

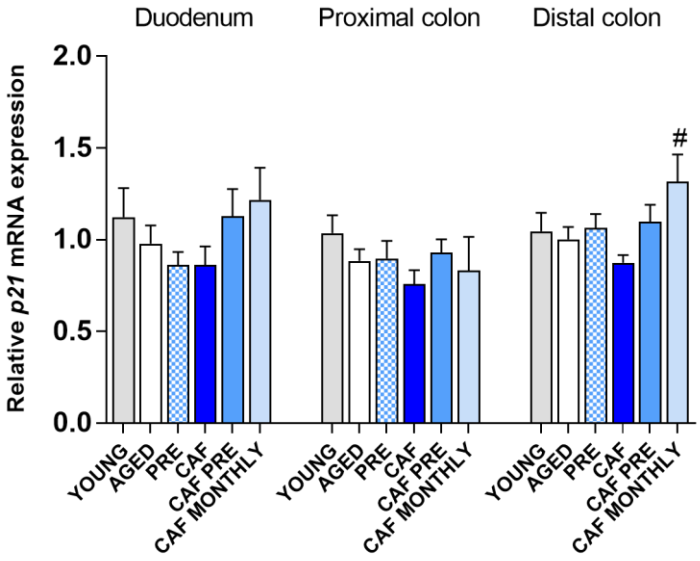

Figure 10. Gene expression of *p21* as a biomarker for intestinal cellular senescence in duodenum, proximal and distal colon. The expression of target genes was normalized to cyclophilin A gene expression (*Ppia*). Values are represented as the means ± SEM. #p < 0.05 compared to CAF group. *Abbreviations: CAF, cafeteria diet; GSPE, grape seed proanthocyanidin extract; p21, cyclin-dependent kinase inhibitor p21.*

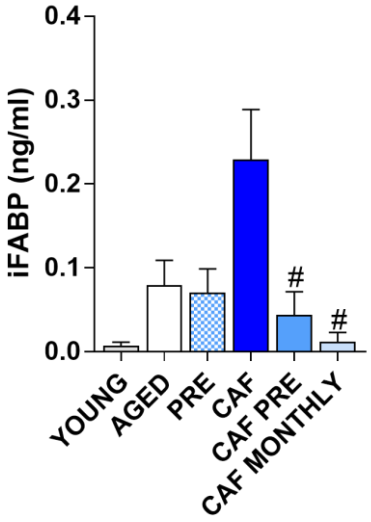

Figure 11. Plasma iFABP concentration. Values are represented as the means ± SEM. #p < 0.05 compared to CAF group. *Abbreviations: CAF, cafeteria diet; GSPE, grape seed proanthocyanidin extract; iFABP, intestinal fatty acid-binding protein.*

4. Discussion

Obesity and aging are accompanied by numerous metabolic and physiological alterations that predispose to disease. Among them, intestinal barrier function is one of the most affected. For that reason, many efforts have been directed in the recent decades to find new therapeutic approaches for the prevention and restoration of the intestinal barrier function. In this work, we wanted to characterize the function of the intestinal barrier in an animal model both of aging and obesity. We also aimed to study the beneficial role of GSPE against these challenges. In our animal models, both aging alone or combined with obesity, we show that intestinal barrier function is maintained. Contrary to previous results in young animals, CAF diet produced a decrease in barrier permeability in duodenum and colon from aged rats. Previous results from our group demonstrated that CAF diet induced a decrease in TEER values and an increase in FD4 gut permeation, leading to metabolic endotoxemia and intestinal inflammation in young obese rats [22–24]. Gut permeability is also increased in other animal models of diet-induced obesity [28] and human *ex vivo* models for DSS-induced colitis [29]. To date, little is known about the effect of aging in gut permeability and current literature is controversial. In accordance to our results, different studies have demonstrated that epithelial intestinal barrier is not impaired by aging itself in healthy humans [15,30,31]. In contrast, other studies have shown an age-associated decline in barrier function, having a higher gut permeability compared with young animals in experiments with non-human primates [14] and rodents [12,32].

Changes in TJ distribution are related with gut permeability [8,33]. Lower TJ expression leads to an increase in endotoxemia [23,34]. As mentioned before, MLCK is involved in the opening of TJs and its increased expression due to the diet is associated with increased TJ opening and, thus, increased permeability. Hence, TJ disruption and consequent endotoxemia have been pointed out as relevant factors in the development of metabolic diseases such as type 2 diabetes and NAFLD, even in healthy subjects [35]. In duodenum, CAF diet decreased intestinal permeability assessed by FD4 translocation. In accordance with that, *Mlck* gene expression was downregulated by both age and diet. In proximal colon, according to TEER values, *Cldn3* gene expression was upregulated by age and downregulated by diet. The

results regarding TJ expression in the literature are controversial Differently to our results, in 24-month-old male Wistar rats a decrease in TJP1 protein expression assessed by immunofluorescence in distal colon was found compared to 4-month-old counterparts [36]. In humans, no differences were found in TJP1 and OCEL1 expression between healthy elderly and young adults. However, in the same study, gut permeability was higher in aged patients of inflammatory bowel disease compared to young patients [15]. In a context of obesity, there is also conflicting data. Some studies showed that high-fat/high-sugar diets induced intestinal hyperpermeability by altering TJs and *Mlck* expression in rats [23,28] while some others demonstrated poor or none effects of diet in mice [9] and rats [22,24]. In light of our findings, the CAF diet cannot provoke severe intestinal damage in 24-month-old female Wistar rats and, what is more interesting, that exposition to the obesogenic challenge must be early in life to cause a measurable injury.

LPS is transported across the intestinal epithelium mainly by the paracellular pathway (due to TJ downregulation) and transcellular pathway through receptor-mediated endocytosis. Consequent endotoxemia has been widely associated to obesity and aging. Gut barrier dysfunction derived from CAF diet consumption facilitates LPS intestinal leakage, thus provoking metabolic endotoxemia and systemic inflammation [22,24,37,38]. In this study, endotoxin levels were not increased neither by age nor CAF diet. Contrary to this result, plasmatic levels of LPS and pro-inflammatory cytokines are known to be increased in the elderly, a phenomenon known as *inflammaging* [39]. In that sense, although we did not find an inflammatory response induced by CAF diet, we observed a low-grade inflammation associated to age attending to the higher IL-6 plasma concentration in aged rats compared with young counterparts.

Endocannabinoids are known to modulate gut permeability and inflammation [40] and have been involved in the development of neurodegenerative disorders [41]. Although CAF diet neither GSPE treatment did not have any effect in the expression of endocannabinoid receptors in our rats, age significantly reduced *Cnr1* and *Cnr2* gene expression in distal colon. Contrary to our results, CNR1 is upregulated in obese young human compared with aged subjects [42] as well as in experimental obese

animals [43]. In contrast with our results with GSPE, resveratrol improved gut barrier integrity and reduced inflammation through ECS by microbiota remodeling [44].

Additionally, mucus layer also changes with age and diet. In this study, we found that age induced an increase in *Muc2* gene expression in distal colon. Regarding the effect of aging on intestinal mucus, literature is also controversial. In elderly healthy people, mucus thickness seems to remain unchanged in duodenum [45], whereas is decreased in colon in a transgenic mice model of accelerated aging [13]. Moreover, previous results showed that *Muc2* gene expression was not different between young and old animals, however, mucin protein levels were higher in ileum of old mice [46]. Although we did not measure mucin production and goblet cells morphology in our rats, we can hypothesize *Muc2* could be regulated at post-transcriptional level.

Aging is also characterized by a progressive loss of intestinal homeostasis. In this study, aging did not affect iFABP levels in plasma, suggesting a good maintenance and renewal of enterocytes by intestinal epithelial stem cells. CAF diet seemed to increase intestinal damage due to the increase of iFABP in circulation and GSPE treatments improved this situation. Little is known about iFABP and metabolic-associated intestinal barrier alterations. *Lalande et al.* [47] showed that higher iFABP levels were associated to imbalanced glucose and lipid homeostasis in obese men with T2D. Several authors have demonstrated that iFABP is a good biomarker for severe intestinal alterations [48,49] but not for intestinal barrier dysfunction linked to obesity [50]. Surprisingly, iFABP results go in the opposite direction to the rest of the data in this study. Considering that these cafeteria diet-fed aged rats are insulin resistant, as previously demonstrated attending to glucose, insulin and HOMA-IR levels in plasma [25], we hypothesize that the increase in circulating iFABP in the CAF group is not due to intestinal damage but to a compensatory mechanism for lipid absorption in enterocytes. Hence, this opens up an interesting field of study around intestinal damage associated to metabolic disarrangements caused by the diet.

Polyphenols such as quercetin and curcumin have demonstrated to improve intestinal barrier function in *in vitro* models [51,52]. Moreover, contrary to our background in young obese rats, GSPE supplementation did not have any effect on reducing gut permeability, endotoxemia and intestinal damage associated to CAF diet

intervention in aged rats. Therefore, we could hypothesize that GSPE effectiveness strongly depends on the experimental model and the time of administration.

In summary, CAF diet is not as aggressive and GSPE treatment is not as effective in the elderly as in youth. We hypothesize that the good maintenance and welfare of animals, from birth to the onset of the study, have prevented the CAF diet from causing alterations in the intestinal barrier function at the same level as in young animals. If this is the case, GSPE treatments resulted unnecessary to correct this non-existent damage and should be administered before homeostatic fluctuations associated to age. Another hypothesis is that these aged rats present immunosenescence, and therefore are unable to respond to a second hit, in our case the obesogenic challenge, as happens with their vaccination response [53] or their deficient response to pathogen infections [54] mainly due to a failure in the adaptative immune response driven by both B and T cells. Longer treatments with the obesogenic challenge in these rats would be needed to evaluate their long-term response. In this scenario, GSPE potential protective effects might be deployed when administered during life to force a healthy aging process. Further research needs to be done in the field of proanthocyanidins as modulators of aging and obesity disarrangements, especially deeper in the mechanisms of action and as a complement for a healthy aging.

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Author contributions: Marta Sierra-Cruz: Writing - original draft, Data curation, Animal studies, sample processing, sample analysis. **Alba Miguéns-Gómez:** Animal studies, sample processing, sample analysis. **Esther Rodríguez-Gallego:** Research, Conceptualization**. Mayte Blay:** Research, Conceptualization, Resources. **Anna Ardévol:** Conceptualization, Funding acquisition, Project administration.

Montserrat Pinent: Conceptualization, Funding acquisition, Project administration. **Ximena Terra**: Writing - review & editing, Formal analysis, Supervision. **Raúl Beltrán-Debón**: Writing - review & editing, Formal analysis, Supervision.

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GENERAL DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
ROLE OF FLAVONOIDS IN THE MODULATION OF INTESTINAL ALTERATIONS ASSOCIATED WITH METABOLIC CHALLENGES: OBESITY AND AGING
Marta Sierra Cruz

GENERAL DISCUSSION

Obesity and aging are two of the most important factors in chronic metabolic diseases that have been increasing prevalence in population in the recent decades. In a simplified way, obesity is characterized by an imbalance between ingested calories and energy expenditure [1,2]. However, obesity is considered a multifactorial pathology where numerous factors such as diet, lifestyle, genetics, socioeconomic and cultural factors are involved. In addition, in Western societies, obesity is highly linked to the metabolic syndrome, a cluster of risk factors for disease such as T2D, NAFLD and cardiovascular diseases [3–5]. On the other hand, the population over 65 is growing very fast and the quality of life in Western countries is getting better, so life expectancy is increasing. Homeostasis disruption characterized by morphological, functional and biological decline during aging also increases the risk for disease, especially T2D and neurodegenerative disorders [6,7].

Consumption of diets rich in saturated fats and sugars constitutes a risk factor for the development of metabolic syndrome and associated diseases, as well as in the reduction of quality and life expectancy. In this sense, the consumption of more balanced diets such as the Mediterranean diet is a good alternative. In addition, it has been widely shown that caloric restriction is effective against obesity, since it reduces food intake and, with it, adiposity and body weight. Likewise, dietary restriction has been shown to be effective in murine animal models in increasing life span [8,9]. Moreover, Western diets consumption also promotes intestinal barrier and gut microbiota alterations and both obesity [10–12] and aging have been associated with disruptions in those factors [13,14]. Consequently, a loss in gut integrity provokes intestinal permeability and promote inflammation, increasing the risk for both intestinal and neurodegenerative disorders. Altogether, we are facing a double metabolic challenge that perhaps with the current therapeutic tools and approaches is not enough to reduce the prevalence of obesity as well as improve the quality of life in the elderly. In that sense, new strategies based on bioactive compounds are promising in targeting obesity and aging and have been gaining relevance in the last decades.

Bioactive compounds are components of some food, especially fruits and vegetables, represented in a small quantity [15]. PACs are one of the most relevant groups, which

widely demonstrated beneficial effects on human health acting as anti-inflammatory, anti-oxidant, anti-tumoral, anti-obesity, anti-aging and neuroprotective agents [16–21]. However, the possible protective properties of PACs against a double metabolic challenge as well as the underlying mechanisms in modulating intestinal permeability and metabolic endotoxemia have not been elucidated. Thus, the general objective of this thesis was to evaluate the effectiveness of GSPE and mechanisms by which PACs act in the long term on the immunoprotective function of intestinal tract by enhancing the metabolic homeostasis disruption caused by an obesogenic diet and aging.

The first objective was to characterize and evaluate the effect of a pharmacological dose of GSPE (500 mg/kg b.w.) on metabolic disarrangements associated to aging itself (**Manuscript 1**) and obesity in the elderly (**Manuscript 2**). We found that aged animals fed with standard chow diet showed greater body weight and adiposity compared to young animals. Similarly, CAF diet induced an increase in adiposity and body weight gain compared with lean aged rats. According to our results, 16-month-old male rats increased body weight and adiposity compared to young rats both receiving normal chow diet and a high-fat diet [22]. As described previously in other studies, a dose of 500 mg/kg b.w. of GSPE was effective on decreasing body weight by reducing food intake in both lean [23] and obese young rats [24]. According to these results, we showed that GSPE reduced adiposity and food intake when administered preventively for 10 days to chow-fed aged rats by exerting satiating properties. However, these GSPE effects were not translated into significant effects on body weight reduction, probably because animals have already reached adulthood. On one side, GSPE effects were maintained for several weeks after the end of administration in aged rats, thus exerting a caloric restriction mimetic effect [25]. In fact, different clinical studies have demonstrated that daily caloric restriction exerts long-term beneficial effects. An alternate-day fasting was able to avoid weight regain after 24 weeks after completing the intervention in adults with obesity [26]. Similarly, in a study with healthy overweight and obese subjects, body weight was maintained for one year after caloric restriction intervention [27]. Moreover, caloric restriction antioxidant effects were maintained for several days after the beginning of the diet in overweight and obese women [28]. On the other side, GSPE-preventive effect was not maintained in obese aged rats, suggesting that diet and age may affect synergistically the previous demonstrated long-term GSPE effect in young obese rats [24,29],

probably interfering with epigenetic mechanisms. In this sense, we previously demonstrated that GSPE exerts anti-obesity long-lasting effects through the epigenetic modulation of glucagon like peptide 1 receptor (GLP-1R) in the ileum [30]. In agreement with our results, GSPE itself [31] or synergistically with resveratrol [32] modulates DNA methylation and histone modifications in human breast and skin cancer cells, respectively.

Interestingly, GSPE was effective on reducing adiposity and body weight gain in aged rats when administered synchronically to CAF diet. Hence, we were able to reproduce previous results in a 17-week-CAF diet experiment with young female rats using same GSPE dose and administration method [24]. Aged obese rats increased body weight more quickly than young obese rats, thus suggesting a predisposition during aging to suffer from obesity and difficulty in losing weight. This may be explained by changes in fat distribution and functional alterations described in the elderly [33,34]. Moreover, GSPE ameliorated glucose metabolism by increasing HOMA-β index and maintaining a higher glucagon/insulin ratio when administered preventively to aged rats, according to previous studies in young rats [24,29,30]. These GSPE effects might be a mimetic effect of caloric restriction, according to previous studies with other polyphenols [35,36]. In contrast, GSPE was not able to improve insulin resistance by reducing glucose and insulin plasma levels, as well as increasing glucagon/insulin ratio when administered to obese aged rats. Contrary to these results, GSPE treatment improved dyslipidemia and insulin resistance induced by CAF diet consumption in young female rats [37,38]. In addition, GSPE synchronic treatment to CAF diet was effective in reducing ectopic fat accumulation in the liver. Other polyphenols such as resveratrol have been also effective in improving liver steatosis in both young and aged animals [39]. Moreover, resveratrol has been also demonstrated to reduce glycemia, HOMA index and hepatic lipid accumulation in a clinical trial of obese men [35]. In contrast to previous results, no signs of systemic inflammation were observed in aged rats due to the CAF diet. According to other authors [40], inflammatory markers like IL-6 and TNF-α were not modified in aged animals compared with young counterparts, probably due to senescence and immune system function decline in the elderly [41]. Due to the huge metabolic alterations and the inability to respond quickly to homeostatic fluctuations associated to aging, GSPE treatments were not sufficient to compensate homeostasis alterations associated with senescence and

obesity as it was in youth. In that way, we suggest administration of GSPE should be done before organism homeostasis decline.

Regarding this homeostasis disruption, one of the most affected organs is the gut. To date, there is sufficient scientific evidence that diet-induced obesity causes intestinal damage, causing a progressive loss of the barrier function and, with it, an increase in permeability. Consequently, products derived from the intestinal microbiota, especially LPS, cross the intestinal epithelium until they reach the lamina propria and the bloodstream, thus inducing the inflammatory process by interacting with immune system cells. Dietary flavonoids exert beneficial effects on inflammatory pathways, barrier integrity and microbiota composition [42]. Concretely, GSPE has been previously studied as a possible modulator of metabolic endotoxemia. Previous studies have demonstrated that GSPE reduces intestinal permeability by improving TJ-related gene expression and TEER values, as well as reducing OVA and LPS transport into the bloodstream via paracellular pathway caused by CAF diet consumption in young rats [37,38]. However, little is known about other LPS-associated mechanisms of transport: receptor-mediated endocytosis and chylomicron-associated pathways. Therefore, with the purpose of exploring how GSPE can modulate LPS transport across the intestinal barrier as well as derived metabolic endotoxemia in peripheral tissues, we designed a study using both *in vitro* and *in vivo* models for intestinal dysfunction **(Manuscript 3).**

We showed that CM-production is induced in Caco-2 cells monolayers when treated with lipids, reproducing dietary fat-effect during postprandial state, and that these CM were LPS loaded *in vitro*. This, together with the positive correlation between TAG and LPS levels in plasma in our *in vivo* model, suggested that endotoxin is being translocated associated to chylomicrons, in agreement with results from other authors [43]. Interestingly, GSPE reduced TAG and LPS translocation *in vitro*, confirming previous results obtained in both chow-fed [44] and CAF-fed rats [37,38]. Although we could not demonstrate the exact mechanism by which GSPE reduced LPS translocation via chylomicron-pathway, we suggest it could be by reducing chylomicron size, in agreement with the previous effect of proanthocyanidins against hyperlipidemia [45–47].

Regarding receptor-mediated endocytosis, we found controversial results between our two models of intestinal dysfunction. GSPE reduced *Cd36* gene expression in Caco-2 cells as well as in ileum of CAF-fed rats. Taking into account that Caco-2 cells spontaneously change from colonocyte to enterocyte typical phenotype [48–50] and that *Cd36* is mainly expressed in duodenum and jejunum, our findings could suggest that a compensatory lipid absorption process takes place in ileum. Interestingly, our results showed that CAF diet dysregulated endocannabinoid system in colon and GSPE was able to modulate it by epigenetic mechanisms. In fact, polyphenols are known to act as epigenetic modulators of different processes disrupted by obesity and aging such as food intake and satiety [30], autophagy [36], inflammation [51], tumour progression and cancer [52], among others. To further evaluate metabolic endotoxemia, we found that LPS accumulation was five times higher in adipose tissue than in the liver, with GSPE alleviating the effects of CAF diet. Hence, we suggest that the positive effect of GSPE on reducing LPS intestinal translocation is accompanied by a beneficial effect also in avoiding metabolic endotoxemia in liver. Previous results support that GSPE protects from metabolic endotoxemia in liver of high-fat diet-fed rats [47] and in adipose tissue of CAF diet-fed rats [53]. In the same way, other polyphenols, such as resveratrol, have demonstrated to reduce dyslipidemia, especially reducing triglycerides circulating levels as well as lipid accumulation in liver in diet-induced obese rodents [54–57].

All things considered, our findings suggest that GSPE might be modulating intestinal permeability and metabolic endotoxemia at several points: (1) reducing chylomicron size thanks to its effect on hyperlipidemia: (2) inducing epigenetic modifications at the endocannabinoid system level; (3) modulating receptor-mediated endocytosis pathway by affecting *Cd36* and *Srb1* gene expression; (4) acting as a prebiotic agent to modulate both gut microbiota and derived metabolites, especially SCFAs; and (5) maintaining hepatic function for correct mechanisms of endotoxin detoxification.

With this holistic view of the role of GSPE in modulating gut barrier function and metabolic endotoxemia in a context of obesity in youth, we wondered to characterize and explore if gut permeability is affected in the same way during aging. To do so, we performed a study with 21-month-old female Wistar rats fed with CAF diet **(Manuscript 4).**

To date, little is known about the effect of age in gut permeability. We found that the intestinal barrier function is maintained during aging. The current literature about gut barrier disruption and subsequent endotoxemia associated to age decline is controversial. In fact, some authors have found that the epithelial intestinal barrier is not impaired in healthy humans [58–60], in contrast with other findings in non-human primates [61] and rodents [14,62]. Contrary to previous results in young obese rats [37,38,63], CAF diet did not induce a loss in intestinal integrity attending to TEER and FD4 measurements in aged rats. In the same way, conflicting data is found regarding TJ expression changes due to age and diet. We found that both CAF diet and age decreased *Mlck* gene expression in duodenum. However, for *Cldn3* gene expression in proximal colon, diet and age exerted opposite effects. In this regard, no changes in TJP1 expression were found in healthy aged humans [60], in contrast to the results in 24-month-old male Wistar rats [64]. Similarly, in murine models of diet-induced obesity, some authors demonstrated an increase in the intestinal permeability by TJ alterations [37,65], while some others not [12,38,63].

Intestinal mucus composition and thickness have also been reported to change with age and diet. We found that *Muc2* gene expression was increased by age in distal colon. In the light of other findings, mucus production and features strongly depend on several factors such as the specific intestinal section, the genetics, the gut microbiota composition and function, as well as the model of study. In fact, controversial data is found between aged healthy people [66] and both natural [67] and transgenic [68] mice models for aging.

Differently to our previous results obtained in young obese rats [37,38], endotoxemia was not increased neither by age nor CAF diet consumption. Considering that endotoxin triggers both intestinal and systemic inflammation, the non-variation of plasma LPS concentration was in accordance with the non-observed changes in IL-6 and TNF-α circulating levels due to CAF diet **(Manuscript 3).** However, we observed higher IL-6 plasma levels in aged rats compared with young rats, thus indicating a low-grade inflammation associated to age which has been previously described in the literature as *inflammaging* [69,70].

Finally, effects of GSPE oral administration on modulating intestinal permeability and endotoxemia in aged rats, were not in the same line as those previously observed in

young rats [37,63,71]. Therefore, the good maintenance and welfare of animals during their whole life without receiving external factors disturbances could be a plausible explanation for these results. In this sense, we suggest that GSPE should be administered as a dietary supplement for a healthy aging as preventive, before the onset of the homeostatic fluctuations associated to age. Evidence derived from this thesis, together with the scarce information available so far on the effect of proanthocyanidins in a context of obesity and aging in animal models, opens an interesting field of research. Future pre-clinical and clinical studies are required to determine: (1) the precise pharmacological dose, (2) the timing of administration and (3) the specific mechanisms of action of GSPE. The final goal would be the GSPE administration as a dietary supplement for the treatment of metabolic and intestinal disorders, especially associated to obesity, aging and metabolic syndrome in Western societies.

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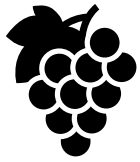


CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
ROLE OF FLAVONOIDS IN THE MODULATION OF INTESTINAL ALTERATIONS ASSOCIATED WITH METABOLIC CHALLENGES: OBESITY AND AGING
Marta Sierra Cruz

CONCLUSIONS

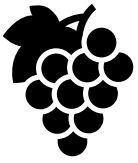
The main conclusions obtained from this doctoral thesis are:

1. **GSPE has preventive effects against age-associated metabolic changes. A preventive treatment with a pharmacological dose of GSPE (500 mg/kg b.w) is able to revert some aging-associated features.**
    - GSPE is effective in reducing food intake and body weight gain in the short-term.
    - Effects of GSPE are also observed 11 weeks after the treatment, limiting body weight gain and the increase in the insulinemia state.
    - Presence of tumours is reduced in the pre-treated rats with GSPE.

2. **GSPE has protective effects against obesity-associated metabolic changes in aged rats. A synchronic oral administration with a pharmacological dose of GSPE (500 mg/kg b.w.) alleviated some metabolic disruptions caused by an obesogenic diet in aged rats.**
    - GSPE prevents body weight increase mainly by reducing adiposity.
    - GSPE effectively reduces fat accumulation in liver.

3. **GSPE modulates intestinal permeability and metabolic endotoxemia caused by an excess of dietary lipids by:**
    - Modulating the intestinal endotoxin translocation through paracellular route, receptor-mediated endocytosis and chylomicron-associated pathways.
    - Epigenetically modifying the endocannabinoid system in colon.
    - Acting as a prebiotic, modulating intestinal microbiota and short-chain fatty acid production.
    - Avoiding LPS accumulation in liver, thus protecting hepatic function.

4. **Cafeteria diet affects differentially the gut barrier function in an age-dependent manner.**
    - Gut barrier function remains more invariable in aged rats fed with cafeteria diet than young counterparts.
    - The expression of genes modulating paracellular permeability are differently affected by age, cafeteria diet and GSPE depending on the intestinal section.
    - Age induces an increase in mucus production in distal colon.
    - Aged rats show signs of inflammaging but not low-grade inflammation associated to obesity.
    - Further research is needed to elucidate the therapeutical role of GSPE in aging.



ANNEX

UNIVERSITAT ROVIRA I VIRGILI
ROLE OF FLAVONOIDS IN THE MODULATION OF INTESTINAL ALTERATIONS ASSOCIATED WITH METABOLIC CHALLENGES: OBESITY AND AGING
Marta Sierra Cruz

ANNEX

LIST OF PUBLICATIONS

Sierra-Cruz M, Miguéns-Gómez A, Rodríguez-Gallego E, Blay M, Ardévol A, Pinent M, Terra X, Beltrán-Debón R. Maintenance of intestinal barrier function in aged rats fed with grape seed proanthocyanidin extract supplemented cafeteria diet. 2022, [manuscript in preparation]

Miguéns-Gómez A, **Sierra-Cruz M,** Rodríguez-Gallego E, Beltrán-Debón R, Blay M, Terra X, Pinent M, Ardévol A. Effect of an acute insect protein preload vs an almond preload on energy intake, subjective food consumption and intestinal health in healthy young adults. 2022, [manuscript submitted to Nutrients]

Miguéns-Gómez A, **Sierra-Cruz M,** Segú H, Beltrán-Debón R, Rodríguez-Gallego E, Terra X, Blay M, Pérez-Vendrel AM, Pinent M, Ardévol A. Administration of *Alphitobius diaperinus* or *Tenebrio molitor* before meals transiently increases food intake through enterohormone regulation in female rats. 2022**,** [manuscript submitted to Journal of Agricultural and Food Chemistry]

Sierra-Cruz M, Miguéns-Gómez A, Rodríguez-Gallego E, D'Addario C, Di Bartolomeo M, Blay M, Pinent M, Beltrán-Debón R, Terra X. Effects of grape seed proanthocyanidin extract on lipopolysaccharide translocation and trafficking from the gut to tissues. 2022, [manuscript submitted to Food Chemistry]

Sierra-Cruz M, Miguéns-Gómez A, Grau-Bové C, Rodríguez-Gallego E, Blay M, Pinent M, Ardévol A, Terra X, Beltrán-Debón R. Grape-Seed Proanthocyanidin Extract Reverts Obesity-Related Metabolic Derangements in Aged Female Rats. Nutrients. 2021 Jun 16;13(6):2059. doi: 10.3390/nu13062059. PMID: 34208508; PMCID: PMC8234113.

Grau-Bové C, **Sierra-Cruz M**, Miguéns-Gómez A, Rodríguez-Gallego E, Beltrán-Debón R, Blay M, Terra X, Pinent M, Ardévol A. A Ten-Day Grape Seed Procyanidin Treatment Prevents Certain Ageing Processes in Female Rats over the Long Term. Nutrients. 2020 Nov 27;12(12):3647. doi: 10.3390/nu12123647. PMID: 33260866; PMCID: PMC7759988.

Miguéns-Gómez A, Grau-Bové C, **Sierra-Cruz M**, Jorba-Martín R, Caro A, Rodríguez-Gallego E, Beltrán-Debón R, Blay MT, Terra X, Ardévol A, Pinent M. Gastrointestinally Digested Protein from the Insect *Alphitobius diaperinus* Stimulates a Different Intestinal Secretome than Beef or Almond, Producing a Differential Response in Food Intake in Rats. Nutrients. 2020 Aug 7;12(8):2366. doi: 10.3390/nu12082366. PMID: 32784756; PMCID: PMC7468914.

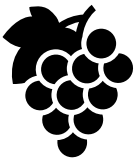
LIST OF POSTER COMMUNICATIONS

March 2022 – Participation in the I Nutraceuticals Conference: Bioactive compounds and nutraceuticals (Tarragona, Spain) – Poster presentation: Grape-seed proanthocyanidin extract reduces body weight and liver fat accumulation in aged female rats fed with cafeteria diet.

November 2021 – Participation in the I International Congress in Biomedical Sciences: Epidemics, endemics and persistent and emergent pandemics (Bucaramanga, Colombia) - Poster presentation: El extracto de uva rico en proantocianidinas reduce el peso corporal y la esteatosis hepática en ratas viejas alimentadas con dieta obesogénica.

June 2021 – Participation in the 4th European Summer School in Nutrigenomics (University of Camerino, Italy) – Poster presentation: Grape-seed proanthocyanidin extract reduces body weight gain and liver fat accumulation in aged female rats fed with cafeteria diet.

November 2020 – Participation in the Scientific program VII Spanish Nutrition Society Young Researcher's Meeting – Poster presentation: Role of GSPE in the modulation of metabolic changes associated to obesity and aging.



ACKNOWLEDGMENTS

UNIVERSITAT ROVIRA I VIRGILI
ROLE OF FLAVONOIDS IN THE MODULATION OF INTESTINAL ALTERATIONS ASSOCIATED WITH METABOLIC CHALLENGES: OBESITY AND AGING
Marta Sierra Cruz

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Obesity and aging have become issues of global concern due to their increasing prevalence in the population in recent decades. On one side, obesity is associated with a loss of intestinal barrier function as well as changes in the composition and functionality of the microbiota (dysbiosis). Together, this causes a greater transport of endotoxins into the bloodstream and, with it, a systemic inflammatory response that is directly related to the development of metabolic syndrome. Proanthocyanidins are bioactive compounds of natural origin present in foods of plant origin that have a protective effect against intestinal alterations associated with obesity induced by the consumption of high-fat/high-sugar diets. Specifically, proanthocyanidins reduce the translocation of endotoxins and act as an anti-inflammatory agent at the systemic level in young animal models. On the other side, it has been hypothesized that aging might derive in similar effects on the intestinal barrier functionality, but little is known about proanthocyanidin therapeutic effect during aging.

In that sense, the main objective of this thesis is to evaluate the role of proanthocyanidins in the modulation of metabolic and intestinal disruptions associated with obesity and aging. We found that a pharmacological dose of a grape seed proanthocyanidin extract (GSPE) can reduce body weight gain by reducing food intake when administered preventively to aged rats fed a standard diet. This dose of GSPE also reduces body weight gain by acting on food intake, as well as fat accumulation in the liver when administered synchronically to aged rats receiving cafeteria diet. Furthermore, this thesis aims to identify the mechanisms by which GSPE modulates metabolic endotoxemia using a dual *in vitro/in vivo* model of dietary lipids-induced intestinal dysfunction. We found that GSPE reduces intestinal permeability by modulating the translocation of endotoxins through different routes as well as the intestinal microbiota.

In conclusion, the administration of GSPE exerts protective effects against metabolic changes associated with age and obesity. Doses and administration time in humans must be determined in future clinical studies.