BIOACTIVITY OF FLAVANOLS ON THE MUCOSA OF THE INTESTINAL WALL: ENTEROENDOCRINE EFFECTS FOR PREVENTING DIET-INDUCED OBESITY AND ASSOCIATED PATHOLOGIES

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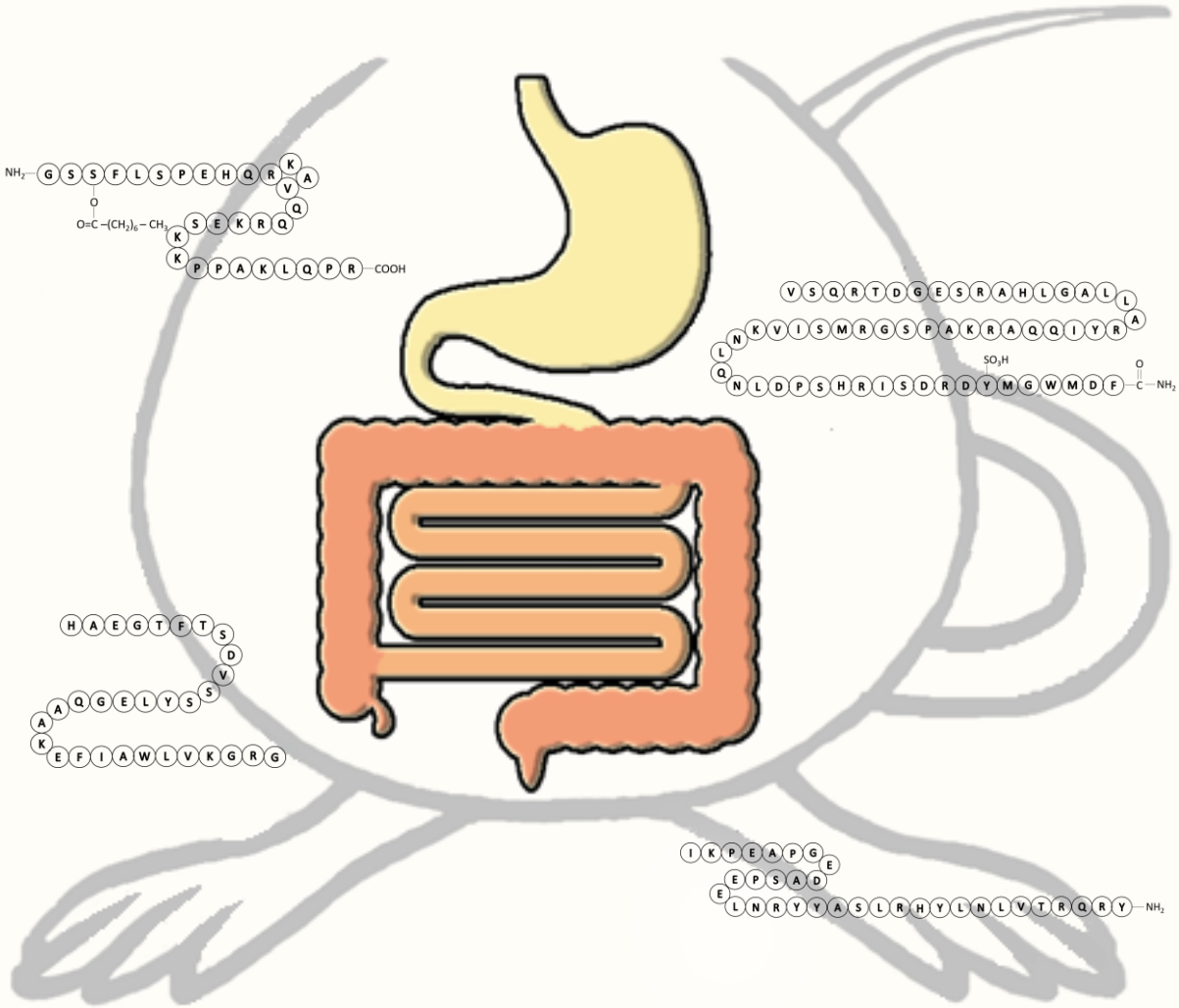

IRIS GINÉS MIR

UNIVERSITAT ROVIRA i VIRGILI

DOCTORAL THESIS
TARRAGONA 2019

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Bioactivity of flavanols on the mucosa of the intestinal wall: enteroendocrine effects for preventing diet-induced obesity and associated pathologies

DOCTORAL THESIS

Supervised by Dr. Anna Ardèvol Grau

and Dr. Montserrat Pinent Armengol

MoBioFood Research Group

Departament de Bioquímica i Biotecnologia

UNIVERSITAT ROVIRA i VIRGILI

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I STATE that the present study, entitled "**Bioactivity of flavanols on the mucosa of the intestinal wall: enteroendocrine effects for preventing diet-induced obesity and associated pathologies**", presented by **Iris Ginés Mir** for the award of the degree of Doctor, has been carried out under my supervision at the **Department of Biochemistry and Biotechnology** of this university and that this thesis is eligible to apply for the International Doctorate Mention.

FAIG CONSTAR que aquest treball, titulat "**Bioactivity of flavanols on the mucosa of the intestinal wall: enteroendocrine effects for preventing diet-induced obesity and associated pathologies**", que presenta la **Iris Ginés Mir** per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al **Departament de Bioquímica i Biotecnologia** d'aquesta universitat i que compleix els requisits per poder optar a la Menció Internacional de Doctorat.

HAGO CONSTAR que el presente trabajo, titulado "**Bioactivity of flavanols on the mucosa of the intestinal wall: enteroendocrine effects for preventing diet-induced obesity and associated pathologies**", que presenta **Iris Ginés Mir** 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.

Tarragona, 8th January 2019/Tarragona, 8 de gener del 2019/Tarragona, 8 de enero del 2019

Doctoral Thesis Supervisors/Els directors de la tesi doctoral/Los directores de la tesis doctoral

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A mi familia,

UNIVERSITAT ROVIRA I VIRGILI
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"Reserve your right to think,

for even to think wrongly

is better than not to think at all"

Hypathia of Alexandria

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SUMMARIES

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Summary

Obesity is one of the most prevalent diseases affecting the global population. It entails metabolic disruptions that affect the whole organism, including the correct functionality of the gastrointestinal (GI) tract. Proanthocyanidins have already proved to be effective at stimulating the enteroendocrine system in healthy conditions, but their effects under an obesogenic challenge have still to be determined. For this reason, this thesis was designed to study the effects of a grape seed proanthocyanidin extract (GSPE) on the enteroendocrine system in rats fed with a long-term cafeteria diet.

Our results showed that a preventive treatment, a synchronic intermittent treatment and a corrective treatment were all capable of modulating the enteroendocrine system differently. Furthermore, each GSPE treatment showed different enteroendocrine profiles associated with changes in body weight and/or food intake. However, we had certain difficulties regarding the quantification of enterohormone secretions, which led us to develop a new *ex vivo* methodology that stimulated different segments of the GI tract and quantified their enterohormone secretion response, thus keeping their vectoriality.

We also found that a 10-day pre-treatment with GSPE induced a long-term upregulation of GLP-1 gene expression in the ileum that was partly mediated by the hypomethylation of its GLP-1 promoter. Moreover, these effects were maintained when GSPE was administered every other week during the seventeen weeks of cafeteria diet. In addition, since this preventive GSPE treatment presented a decreased respiratory quotient and tended to reduce the body weight gain, we evaluated if there were also long-lasting GSPE effects on lipid management in the peripheric tissues. The results showed a limitation on adipose storage and an

increase in lipid oxidation in the liver and skeletal muscle that lasted seven weeks after the last GSPE dose.

To sum up, this thesis revealed that grape seed proanthocyanidins are capable of modulating the enteroendocrine system and improving the energetic state altered by a cafeteria diet, thus demonstrating that they are good agents for treating metabolic alterations induced by obesity.

Resum

L'obesitat és una de les malalties més freqüents que afecta la població de tot el món. Inclou una sèrie de trastorns metabòlics que afecten tot l'organisme, incloent la correcta funcionalitat del tracte gastrointestinal (GI). Les proantocianidines han demostrat ser efectives estimulant el sistema enteroendocrí en condicions saludables, però els seus efectes sota un context d'obesitat encara estan per determinar. Per aquest motiu, aquesta tesi va ser dissenyada per estudiar els efectes d'un extracte de proantocianidines de llavor de raïm (GSPE) sobre el sistema enteroendocrí en rates alimentades amb una dieta de cafeteria a llarg termini.

Els nostres resultats van mostrar que uns tractaments preventiu, intermitent sincrònic i correctiu son capaços de modular el sistema enteroendocrí de diferent manera depenent del tractament. A més, cada tractament amb GSPE va mostrar diferents perfils enteroendocrins associats a canvis de pes corporal i/o d'ingesta. No obstant això, vam trobar algunes dificultats en la quantificació de les secrecions d'enterohormones que ens van conduir al desenvolupament d'una nova metodologia *ex vivo*, que ens va permetre estimular diferents segments del tracte gastrointestinal i quantificar la seva resposta de secreció d'enterohormones, mantenint la seva vectorialitat.

També vam trobar una regulació de llarg termini sobre l'expressió gènica de GLP-1 a ili, induïda per un pre-tractament de 10 dies amb GSPE, mediada parcialment per una hipometilació sobre el promotor de GLP-1. Aquests efectes es van mantenir quan el GSPE es va administrar cada dues setmanes durant les disset setmanes de la dieta de la cafeteria. A més, atès que els animals que van rebre el tractament preventiu de GSPE presentaven un quocient respiratori disminuït i tendien a reduir l'augment de pes corporal, es va avaluar si el GSPE induïa també efectes de llarg termini sobre la gestió de lípids en els teixits perifèrics. Els resultats van mostrar una

limitació d'emmagatzematge al teixit adipós i un augment de l'oxidació lipídica al fetge i múscul esquelètic que va durar set setmanes després de l'última dosi de GSPE.

En resum, aquesta tesi ha mostrat que les proantocianidines de llavors de raïm són capaces de modular el sistema enteroendocrí i millorar l'estat energètic, alterat per una dieta de cafeteria, demostrant ser uns bons agents per tractar les alteracions metabòliques induïdes per l'obesitat.

Resumen

La obesidad es una de las enfermedades más frecuentes que afecta a la población de todo el mundo. Incluye una serie de trastornos metabólicos que afectan a todo el organismo, incluyendo la correcta funcionalidad del tracto gastrointestinal (GI). Las proantocianidinas han demostrado ser efectivas estimulando el sistema enteroendocrino en condiciones saludables, pero sus efectos bajo un contexto de obesidad aún están por determinar. Por este motivo, esta tesis fue diseñada para estudiar los efectos de un extracto de proantocianidinas de semilla de uva (GSPE) sobre el sistema enteroendocrino en ratas alimentadas con una dieta de cafetería a largo plazo.

Nuestros resultados mostraron que unos tratamientos preventivo, intermitente sincrónico y correctivo son capaces de modular el sistema enteroendocrino de diferente forma dependiendo del tratamiento. Además, cada tratamiento con GSPE mostró diferentes perfiles enteroendocrinos asociados a cambios de peso corporal y/o de ingesta. Sin embargo, encontramos algunas dificultades en la cuantificación de las secreciones de enterohormonas que nos condujeron al desarrollo de una nueva metodología *ex vivo*, que nos permitió estimular diferentes segmentos del tracto gastrointestinal y cuantificar su respuesta de secreción de enterohormones, manteniendo su vectorialidad.

También encontramos una regulación de largo plazo sobre la expresión génica de GLP-1 en íleon, inducida por un pre-tratamiento de 10 días con GSPE, mediada parcialmente por una hipometilación sobre el promotor de GLP-1. Estos efectos se mantuvieron cuando el GSPE se administró cada dos semanas durante las diecisiete semanas de la dieta de la cafetería. Además, dado que los animales que recibieron el tratamiento preventivo de GSPE presentaban un cociente respiratorio disminuido y tendían a reducir el aumento de peso corporal, se evaluó si el GSPE inducía también

efectos de largo plazo sobre la gestión de lípidos en los tejidos periféricos. Los resultados mostraron una limitación de almacenamiento en el tejido adiposo y un aumento de la oxidación lipídica en el hígado y músculo esquelético que duró siete semanas después de la última dosis de GSPE.

En resumen, esta tesis ha mostrado que las proantocianidinas de semillas de uva son capaces de modular el sistema enteroendocrino y mejorar el estado energético, alterado por una dieta de cafetería, demostrando ser unos buenos agentes para tratar las alteraciones metabólicas inducidas por la obesidad.

LIST OF ABBREVIATIONS

UNIVERSITAT ROVIRA I VIRGILI
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LIST OF ABBREVIATIONS

Ac-CoA	Acetyl-CoA
AKT	Serine threonine kinase
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BW	Body weight
CAF	Cafeteria
CCK	Cholecystokinin
ChGA	Chromogranin A
COMTs	Catechol-O methyltransferases
COX-2	Cyclo-oxygenase-2
DAG	Diacyl glycerol
DNA	Deoxyribonucleic acid
DNL	*De novo* hepatic lipogenesis
DNMTs	DNA methyltransferases
DPP-4	Dipeptidyl peptidase-4
EE	Energy expenditure
EECs	Enteroendocrine cells
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EI	Energy intake
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
FA	Fatty acids
FFAs	Free fatty acids
FI	Food intake
Flavanols	Flavan-3-ols
GC	Gallocatechin
GHS-R	Growth hormone secretagogue receptor
GI	Gastrointestinal
GIP	Gastric inhibitory polypeptide

GLP-1	Glucagon like peptide 1
GLP-1R	GLP-1 receptor
GLUT-4	Glucose transporter 4
GOAT	O-acyltransferase
GSPE	Grape seed proanthocyanidin extract
GTD	Green tea decoction
HAT	Histone acetyltransferases
HDAC	Histone deacetylase
HFD	High fat diet
HMT	Histone methyltransferases
HOMA-IR	Homeostatic model assessment-insulin resistance
IAAP	Islet amyloid polypeptide
IGFBP-1	Insulin like growth factor binding protein-1
IKKβ	IκBα kinase beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IMLC	Intramyocellular lipid content
iNOS	Inducible nitric oxide synthase
IR	Insulin resistance
IRS	Insulin receptor substrate
JNK-1	C-Jun N-terminal kinase-1
LCFA	Long chain fatty acid
LPL	Lipoprotein lipase
LPS	Lipopolysaccharides
MAPK	Mitogenic activated protein kinase
miRNA	Micro RNA
MPO	Myeloperoxidase
NF-κβ	Nuclear factor-κβ

NPY	Neuropeptide Y
PACs	Proanthocyanidins
PC	Pyruvate carboxylase
Pdx1	Insulin promoter factor-1
PGC1-α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
POMC	Proopiomelanocortin
PPARγ	*Proliferator-activated receptor* γ
PUFA	Polyunsaturated fatty acids
PYY	Polypeptide YY
RNA	Ribonucleic acids
RQ	Respiratory quotient
SCFA	Short chain fatty acids
SCFAs	Short chain fatty acids
SGLT-1	Sodium-dependent glucose transporter-1
SULTs	Sulfotransferases
TAG	Triacyclglycerols
T2DM	Type 2 diabetes mellitus
TEER	Trans electrical epithelial resistance
TNF-α	Tumour necrosis factor-α
TRP	Transient receptor potential channels
UCPs	Uncoupling proteins
UGTs	Glucuronosyl-transferases
VLDL	Very low-density lipoprotein
WAT	White adipose tissue

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INTRODUCTION

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Introduction

1. Obesity: metabolic disorders and therapeutic management

Obesity is one of the most prevalent diseases affecting the population all around the world. The last upload from the OMS indicates that in 2016, there were more than 1.9 billion of adults considered overweighed and 650 million of them were obese [1]. It increases the risk factor of developing hypertension, hyperglycaemia, pro-thrombotic states and a pro-inflammatory states alterations which can lead to the appearance of pathologies such as cardiovascular disease, type 2 diabetes mellitus (T2DM) and chronic inflammation [2]. These metabolic alterations are, in part, related to excessive accumulation of triacyclglycerols (TAG), particularly if fat accumulation occurs in the abdominal region, increasing the risk for metabolic alterations, cardiovascular diseases and insulin resistance (IR).

It is believed that the increase in adipose tissue mass is linked to alterations of the endocrine and metabolic functions of adipose tissue that lead to worsening the systemic physiology. For example, adiposity is negatively correlated with production of adiponectin, a hormone produced by the adipocytes that it is thought to induce insulin sensitivity [3–5]. Moreover, other authors add that obesity and insulin resistance are related to a state of chronic, low-grade inflammation in adipose tissue, characterized by infiltration of adaptive and innate immune cells and an altered production of proinflammatory molecules (also called adipokines) such as tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, inducible nitric oxide synthase (iNOS), among others, that have direct effects on cellular metabolism [6–8]. Finally, due to the impaired capacity to rapidly store dietary fat of hypertrophic adipocytes, lipids are released into the circulation as free fatty acids (FFA) and ectopically stored in non-adipose tissues, such as the liver, skeletal muscle, heart, pancreas and intra-

abdominally [9]. This ectopic fat accumulation is generally higher in obesity and T2DM and leads to interference with insulin signalling [10, 11].

1.1 Metabolic disorders associated to ectopic fat accumulation

The **adipose tissue** is involved in energy storage and has an endocrine function, and plays an important role in maintaining body energy balance, thermogenesis and the production of various cytokines [12, 13]. In normal conditions adipose tissue responds to insulin, an anabolic hormone secreted by pancreatic β cells in response to food intake, inducing glucose and fat transport in adipose tissue, skeletal muscle and liver, and glycogen synthesis in skeletal muscle and liver [14]. Insulin interacts with the insulin receptor substrate (IRS), and the IRS/ phosphatidylinositol 3-kinase (PI3K)/ serine threonine kinase (AKT) pathway promoting the cellular translocation of glucose transporter 4 (GLUT-4) to the cell membrane, thus leading to cellular glucose uptake [15]. In obesity, according to the lypotoxicity hypothesis, the excess of fat induces IR by blocking the IRS/PI3K/AKT pathway [16]. The alteration of this pathway blocks the entrance of glucose to the cell, which enhances its plasma levels and aggravates the IR [15]. Furthermore, once the adipose tissue has developed IR, it increases its lipolytic character and weakens the promotion of lipid synthesis [16, 17] that is accompanied an impairment of its storage capacity. This forces other peripheral tissues as the liver, the skeletal muscle and the pancreas, among others, to become fatty acids (FA) storage tissues [9].

In normal conditions the **liver** responds to fasting conditions due to the raised glucagon, which induce the production of glucose through gluconeogenesis and glycogenolysis, whereas in fed conditions insulin reduces hepatic glucose production and glycogenolysis, increases glycogen synthesis and the synthesis of fatty acids for storage with the subsequent utilization by other tissues [18, 19]. Fatty acids in the liver come from several different sources: derived from dietary fat, released from

adipocytes via lipolysis, and from *de novo* hepatic lipogenesis (DNL) [20]. Part of the FFAs of the liver are converted into TAG through esterification and are either stored in lipid droplets within hepatocytes, or are packaged and released as very low-density lipoprotein (VLDL) particles into the blood [21]. In obesity, the increased FFAs that reach the liver, increase the hepatic Acetyl-CoA (Ac-CoA) which activates pyruvate carboxylase (PC), that leads to an increase of hepatic gluconeogenesis [22]. Furthermore, the impairment of peripheric glucose uptake induces a state of hyperglycaemia that forces the pancreas to produce more insulin which guides to a hyperinsulinemic state [23]. Hence, hyperinsulinemia leads to an upregulation of transcription factors regulating DNL and an inhibition of FFA β-oxidation, further promoting hepatic fat accumulation [23, 24].

Fig. 1. Mechanisms of insulin resistance in the adipose tissue, the liver and the skeletal muscle. Extracted from [25].

Furthermore, the accumulation of FA as diacyl glycerol (DAG) and ceramides, induce a pro-inflammatory response mediated by several serine kinases such as protein kinase (PKC) Cε, c-Jun N-terminal kinase-1 (JNK-1) and IκBα kinase beta (IKKβ), that leads to an impairment of hepatic insulin signalling, interfering with the tyrosine phosphorylation of IRS and thus, constraining insulin-stimulated hepatic glycogen synthesis [26–28].

Additionally the increased adipose lipolysis, leads to an increment of FA and glycerol turnover to the liver, that further increases hepatic conversion of glycerol to glucose through a substrate push mechanism [22, 29]. Furthermore, insulin resistance is also enhanced by chronic metabolic inflammation and endoplasmic reticulum (ER) stress in the liver [30, 31].

The **skeletal muscle** plays an important role in glucose regulation and energy homeostasis, being the responsible of most of insulin-stimulated glucose utilization [32]. Under normal conditions, FFAs enter the skeletal muscle through fatty acid translocase and fatty acid-binding protein, and then form long chain fatty acids (LCFA)-CoAs, which are partitioned to the synthesis of lipids (TAG) or toward the mitochondria for oxidation [33]. In obesity, the increased deliver of FFA acids by the adipose tissue, leads to an excessive intramyocellular lipid content (IMLC), which reduce the mitochondrial oxidative and phosphorylation activity and leads to skeletal muscle IR [34, 35]. Furthermore, the DAG-mediated activation of PKCθ impairs muscle insulin signalling limiting the phosphorylation of IRS-1, blocking insulin-stimulated muscle glucose uptake through GLUT-4 receptors, thus increasing glucose delivery to the liver [36]. This will further drive hepatic lipid synthesis and activate hepatic gluconeogenesis via ac-CoA–mediated activation of PC and glycerol, increasing glucose production via substrate push [25]. In figure 1 it can be observed a schematic draw of the integration of the metabolic pathways that have been commented in this section.

The endocrine **pancreas** is mainly composed of different types of cells, of which pancreatic β cells are vitally important in maintaining glucose homeostasis by producing and secreting insulin in response to the blood glucose concentration [37]. In obese subjects, excess levels of circulating FFAs, impair the function of β cells [38]. A long-term cafeteria (CAF) diet has been shown to induce a pre-diabetic state with ectopic lipid accumulation in the pancreas, just as to increase the insulin content and gene expression in the pancreas, a condition that leads to hyperinsulinemia. In addition, it has also been shown initial signs of apoptosis in the pancreas [39].

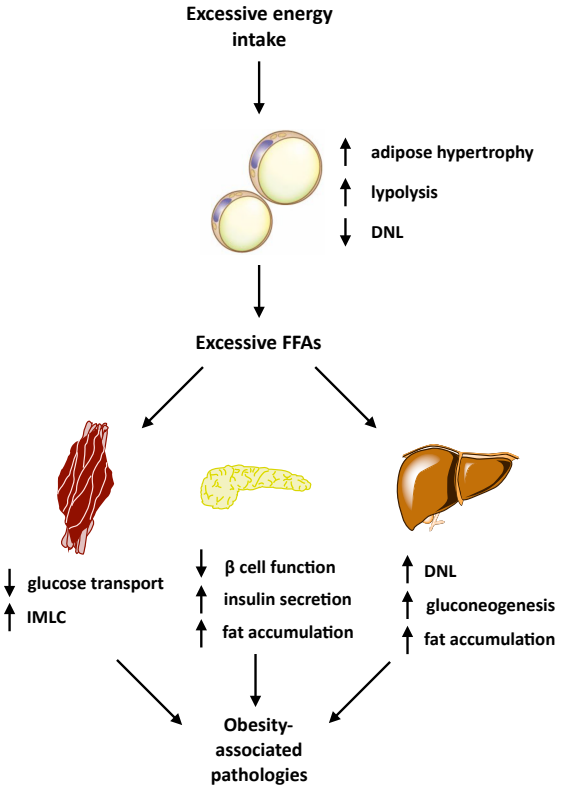

Fig. 2. Representation of the main metabolic disorders induced by obesity in the peripheral tissues that contribute to the development of obesity-related pathologies.

The second most abundant peptide secreted by pancreatic β-cells is amylin, or also known as islet amyloid polypeptide (IAPP). It is stored and secreted with insulin in a ratio 1:100 [40–42]. Like insulin, plasma amylin levels are low during fasting and increase during meals. It also increases following glucose administration, and the levels are all directly proportional to body fat [43]. The biological actions of IAPP are still not fully understood, but appear to involve central nervous system-mediated induction of satiety, slowing of gastric emptying and potentiation of leptin signalling [40]. At the brainstem, amylin interacts with other neuropeptides involved in food

intake including cholecystokinin (CCK), glucagon like peptide 1 G(LP-1), and polypeptide YY (PYY) [44]. After long-term access to high fat diet, it has been observed a reduction of amylin's ability to inhibit food intake [45]. Moreover, it has been detected that in obesity, it occurs an inefficient proteolytic conversion of proIAPP to IAPP, occurs and an increase in proIAPP levels may contribute to islet amyloid deposition and β-cell disfunction, subsequently contributing to the development of type-2 diabetes [40, 46]. Chronically elevated glucose and free fatty acids have been demonstrated to enhance amyloid fibril formation [47–49] that in turn, promotes the auto stimulation of pro-inflammatory cytokines that can initiate islet inflammation [50].In addition, IAAP aggregates have been shown to acts as a potent stimulus for inflammatory cytokines thus aggravating the inflammatory process [51].

Finally, the endocrine pancreas is also composed by α-Cells, which compose approximately 25% of the human islet and are classically associated with their role in producing glucagon for counter regulation of the actions of insulin in blood glucose homeostasis [52]. Studies of the effects of fatty acids on α-cells have shown that they enhance glucagon secretion by means of fatty acid oxidation and TAG accumulation in a time- and dose-dependent manner, but decrease cell proliferation [53]. Piro et al. suggested that fatty acids augment glucagon release and enhance glucagon expression and protein content, probably by activating the mitogenic activated protein kinase (MAPK) pathway [54]. In contrast, they observed that the inhibitory action of insulin on glucagon release was impaired in a long-term incubation with fatty acids, probably due to palmitate-induced insulin resistance because of the defects in the IRS-1/PI3K/AKT pathway [54].

1.2 The role of the gastrointestinal tract on obesity

One of the main functions of the gastrointestinal (GI) tract is **nutrient digestion and absorption**. Hence, on the treatment of obesity, the development of both, nutrient digestion and absorption inhibitors is one of the main strategies to reduce energy intake through GI mechanisms [55, 56]. In addition, the GI tract is one of bigger hormonal tissues since it is a source of various regulatory peptide hormones, secreted along all the GI tract by different **enteroendocrine** cells (EECs), which are involved in the coordination of digestive processes within the gastrointestinal system via autocrine and paracrine effects. But, they also exert endocrine effects on other organ systems particularly in the brain, where some of them have also been found to exist as neurotransmitters [57]. Together the brain and the GI tract interweave to induce a proper satiety signalling and their interaction is known as the gut-brain axis. It has the control of food intake through the action of gut peptides, acting as both, hormones and neurotransmitters, allowing signalling between the periphery and central nervous system to coordinate systemic changes in our physiology [58]. Moreover, the parasympathetic nerves innervating the stomach play an important role in digestion and absorption and the sympathetic excitation and parasympathetic inhibition affect energy homeostasis, feeding behaviour and also reduce food intake and body weight [59].

Another important role of the intestine is being the largest and principal **barrier protecting tissue** from the external environment [60]. It allows the exchange of molecules between the host and the environment and nutrient absorption from the diet [61–64], while preventing the entry of antigens and microorganisms into the body [61, 65]. Moreover, the **immune cells** present in the intestinal barrier provide a measured inflammatory and defensive response to threats from pathogens [66, 67]. Obesity is associated with inflammatory processes that contribute to IR. Apart

from adipocytes, the the GI tract has been described as another potential source of inflammation that is associated with diet- and/or obesity-related pathologies [68].

The **colonic microbiota** is also considered to play a role on the influence of gut homeostasis. The microbiota is able to influence gut homeostasis. Bacterial metabolic products or bacterial factors are able to directly interact with the intestinal epithelium and activate EECs to secrete gut peptides and regulate GI motility and hormone secretion [69]. Due to their position in the GI tract and their constant exposition to its contents, EECs are seen as intermediates in the communication between the gut microbiota and its host. The principal mechanism by which EECs sense microbiota is by their products, such as short chain fatty acids (SCFAs), which are produced through microbial fermentation and can directly stimulate enteroendocrine secretions through the activation of transmembrane G-protein coupled receptors [70–72]. Furthermore they can also act as epigenetic regulators of gene expression by the inhibition of histone deacetylase (HDAC) [73]. This communication is disrupted in various pathophysiological conditions and contributes to their complications, as shown for obesity and inflammatory bowel disorders [74].

In order to explain the interaction between central and peripheral signalling molecules affecting homeostasis regulation, food intake and/or satiety, it has been proposed a role for the hormones secreted by the enteroendocrine system, including the pancreatic hormones and the hormones secreted along all the GI tract [58, 75, 76], which main functions are reviewed in figure 3.

For 40 years it has been believed that each EEC only secreted specific hormones, for example, CCK secretory cells were called I cells and were located in the duodenum, or those containing GLP-1 and PYY which were located in the ileum and colon and called L cells [77, 78]. However, recent studies have revealed that these EECs are

more interrelated with more complex secretory patterns, being able to secret different types of enterohormones depending on whether the activated G-protein coupled receptors engages an inhibitory Gi protein or excitatory Gs/Gq protein [79]. For example, Glass et al found that a single gut sensor can express both satiety-inducing peptides (PYY, CCK, GLP-1) and the hunger-inducing hormone ghrelin [80].

Fig. 3. A schematic diagram of the gastrointestinal tract illustrating where particular gut hormones are concentrated and their major putative functions. Extracted from [75]**.**

Nevertheless, in this thesis it will be used the classic system to explain the role of the different enterohormones, since it will be easier to expose the different hormones one by one, and to show the effects of flavanols on every one of them afterwards. Furthermore, it should be mentioned that even though there are more enterohormones that the ones that will be described [81, 82], their roles and the mechanisms by which flavanols induce or inhibit the expression, synthesis and secretion of many of them have no so clearly been defined. For this reason, hereunder there will be described the main enterohormones to give a general look of their role on energy homeostasis.

Ghrelin is an orexigenic peptide composed of 28-amino acid produced mostly in X/A endocrine cells in the oxyntic mucosa of the stomach, although the highest content of ghrelin is found in the gastric fundus [83, 84]. However, it has been observed that ghrelin cells are also present in the duodenum, ileum, cecum and colon. Nevertheless, the greatest number of ghrelin cells are found in the stomach, and the number of the opened-type cells gradually increase in the direction from stomach to the lower gastrointestinal tract [85]. Its active form appears with an addition of a octanoyl group to the Ser3 residue by O-acyltransferase (GOAT) and binds to the growth hormone secretagogue receptor (GHS-R) which is highly expressed in the hypothalamus and brain stem [86]. When nutrient availability is low, levels of ghrelin increase, and, after consumption of a meal, ghrelin levels are decreased [87]. Apart from regulating energy balance in the short term via induction of appetite and in the long term via increased body weight and adiposity [88], ghrelin has also been found to act in distinct areas including learning and memory, gut motility and gastric acid secretion, sleep/wake rhythm, reward seeking behaviour, taste sensation and glucose metabolism [89]. Despite the large body of literature that documents that alterations of the orexigenic hormone ghrelin play an important role in appetite fluctuation following meals, there is controversy about its in role on obesity. Makris et al. review the main limitations in investigating ghrelin that make it difficult to get to a consensus about correlation between ghrelin and obesity [90]. One of the hypotheses that has gained force is the appearance of ghrelin resistance in obese subjects. In figure 4 there can be observed the different mechanisms reviewed by Cui et al. by which obesity-associated ghrelin resistance might be developed [87].

Fig. 4. Hypothalamic ghrelin resistance. Obesity-associated ghrelin resistance might develop via different mechanisms, such as decreased circulating levels of ghrelin (1); impaired transport of ghrelin through the blood–brain barrier (BBB) (2); reduced expression of growth hormone secretagogue receptor (GHSR) (3); and reduced expression of agouti-related protein (AgRP) and neuropeptide Y (NPY) (4), which reduces the orexigenic action of ghrelin. The molecular mechanisms leading to the reduction of neuropeptide expression are unclear, but possible candidates include hypothalamic inflammation, lipotoxicity, endoplasmic reticulum (ER) stress and impaired AMP-activated protein kinase (AMPK) or mechanistic target of rapamycin (mTOR) pathways. 3v, third ventricle; ARC, arcuate nucleus of the hypothalamus; DMH, dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; PVH, paraventricular nucleus of the hypothalamus; VMH, ventromedial nucleus of the hypothalamus. Extracted from [87].

CCK is a gut hormone generated from pro-CCK by post-translational modifications in the GI tract by I-cells, predominantly in the duodenum and jejunum but it is also widely distributed within the hypothalamus, generally in the median eminence and ventromedial nucleus [91–93]. EECs contain a mixture of the medium-sized CCK-58 (the most abundant molecular form), CCK-33, CCK-22, and CCK-8 (the most active form), whereas neurons mainly release CCK-8 and to some extent CCK-5 [94]. CCK

acts as an anorexigenic peptide, inducing a decrease in food intake and body weight and an increase in perception of fullness, as well as regulating gastric emptying, gall bladder contraction and pancreatic enzyme release [76, 94]. CCK has been shown to be secreted in response to the presence of some luminal nutrients such as protein hydrolystates, individual amino acids and long chain fatty acids [95–98]. It has been demonstrated that when rats are placed on a high-fat diet, there is a marked reduction in afferent sensitivity to satiety related stimuli, which leads to a development of CCK-resistance [99]. For now, the exact mechanisms of how CCK administers its glucoregulatory effects and how CCK resistance develops remain unclear [100].

PYY, a member of the PP-fold family, is released by L cells in response to the stimulation of nutrient intake in the terminal ileum, colon and rectum. It is often co-expressed and secreted with GLP-1 [101, 102]. There are 2 forms of PYY in plasma, the predominant form $PYY_{3–36,}$ and $PYY_{1–36}$, which is digested by the enzyme dipeptidyl peptidase-4 (DPP-4) and converted to the active form $PYY_{3–36}$ [103, 104]. Due to its PP-fold structure (as PP and NPY), it binds to the Y family of G protein-coupled receptors Y1, Y2 and Y5 [105]. PYY is secreted into the circulation in response to food intake and is reduced by fasting. Its stimulation is not only induced by fatty acids, but also in response to carbohydrates and proteins [71, 106–109]. It has been proposed that PYY be involved in energy homeostasis by regulating food intake and suppressing excessive consumption through the activation of proopiomelanocortin (POMC) neurons and inhibition of NPY within the melanocortin system [110]. However, the relationship between obesity and PYY secretion has been recently reviewed to remain unclear because of the controversy found within the publications available [104].

GLP-1, a peptide derived from the glucagon precursor pro-glucagon, is released by L cells from ileum and colon, as a full length GLP-1 (7-36) amide, having a plasma half-

life of few minutes, and rapidly degraded and inactivated by the endopeptidase dipeptidyl peptidase-4 (DPP-4), resulting in the formation of GLP-1 (9-36) amide [111, 112]. Its effects are mediated by GLP-1 receptor (GLP-1R), a member of the glucagon receptor family of G protein-coupled receptors [113]. The incretin effect of GLP-1 goes from increasing glucose-dependent insulin release, to reducing glucagon secretion, thereby contributing to limit postprandial glucose excursions, and decreasing gastric emptying [114, 115]. It also has an anorectic effect on appetite, inhibits gastrointestinal motility and promotes pancreatic β-cell growth [116–118]. In obesity, the GLP-1 response to meal has been described to be reduced and associated to an increase in body mass index [119–122]. Nonetheless, obese subjects remain sensitive to peripherally-administered GLP-1 and its anorexigenic effects [123]. Part of the feeding inhibitory actions of endogenous GLP-1 appear to occur peripherally and dependent on intact vagal afferent mediation [124]. This impairment in the incretin effect has been observed even in the absence of impaired glucose tolerance or diabetes mellitus [120].

Altogether, considering the importance of the intestine on energy homeostasis and how obesity alters it by disrupting different functions of the intestine, makes it an adequate organ to focus as a therapeutic target against obesity.

1.3 Therapeutic approaches against obesity

Obesity has become a public health issue with significant and profound impact on morbidity, mortality, and cost of health care. There is a rich evidence that presents obesity as a complicated chronic medical condition caused by the interplay of multiple genetic, environmental, metabolic, and behavioural factors [125]. The main approaches to treat it are based in energy balance equation: the body weight gain, which accompanies obesity, results from an energy imbalance between energy intake (EI) and energy expenditure (EE), both influenced by environmental and

genetic factors [126]. A negative energy balance is needed to produce weight loss and can be achieved by either decreasing EI or increasing EE [127, 128]. Nevertheless, from the point of view of the lypotoxocitic theory, an improvement of the metabolic flexibility, defined as the ability of an organism to respond or adapt according to changes in metabolic or energy demand as well as the prevailing conditions or activity [129], might be an important target in body weight regulation and related metabolic disorders [130].

Caloric restriction and physical activity are the main approaches prescribed to improve the overweight condition. However, normally this is difficult to maintain for a prolonged period, as it implicates important changes in someone's lifestyle [131] and therefore, it appears the need to change to pharmacological treatments and/or bariatric surgery if more aggressive interventions are required. Recently, there have been reviewed the current major FDA-approved anti-obesity medications, which comprises a wide variety of targets, and together with the new obesity drugs under investigation, they provide hope for increasing the medicinal armoire against obesity with more effective treatment strategies [132]. Among the most used medications for prescription, there can be found Orlistat, a gastrointestinal lipase inhibitor, Lorcaserin, a serotonin agonist that modulate midbrain dopaminergic tone to suppress binge-related food intake, naltrexone-bupropion, which are a combination of an opioid receptor agonist and a reuptake inhibitor of dopamine and norepinephrine and Liraglutide, a GLP-1 receptor agonist named Liraglutide [132–134]. Furthermore, new promising pharmacotherapies are currently under study, in example the use of enterohormone agonists (reviewed in [135]). Although pharmacological and surgical interventions are often the more efficient means to preventing obesity, there are still several negative effects, high costs and potentially hazardous side effects associated with these two therapies [58, 132], suggesting that other therapies, as could be natural products administration, may be a safest and a

most cost-effective option for those who are moderately obese [136]. The potential of natural products, such as flavonoids, for treating obesity is currently under investigation and some of them have been demonstrated to present anti-obesity properties [137–140], thus opening a wide range of possibilities in forthcoming studies addressing obesity.

2. Flavanols: structure, classification, metabolization and mechanism of action

2.1 Structure and classification

Flavanols, a subclass of flavonoids of the family of polyphenols and are becoming a subject of interests for their beneficial properties over human health. Most of them are secondary metabolites which are synthesised in most vegetables and fruits in response to stress conditions as could be herbivores, allelopathic agents, ultraviolet radiation, microbial invasion, among others [141]. Polyphenols represent a large family of secondary metabolites which can be divided into two major groups: flavonoids and non-flavonoids [142]. In particular, the study of flavonoids has become a topic of interest in human nutrition research. There have been identified more than 9000 plant-derived flavonoid compounds, all of them sharing the C6-C3-C6 ring structure [143]. The different subclasses of flavonoids are determined by the substitution of the functional group of the C3 ring, the oxidation state of this heterocyclic ring and its conjugation pattern, thus giving the flavan-3-ols (flavanols), flavones, flavonols, anthocyanidins, flavanones, isoflavones, isoflavans and pterocarpans [144, 145]. In plants flavonols, flavanones and anthocyanins exist as glycosides, where the predominant attached sugars are glucose and rhamnose.

Fig. 5. Schematic classification of flavonoids and flavanols.

Flavanols are the most structurally complex subclass of flavonoids and they are among the main phenolic compounds present in human diet. They are present mostly in their free forms catechin and epicatechin, but can be galloylated, or polymerised to form proanthocyanidins (PACs) [146], as shown in figure 5. Catechins are mostly found in green tea, chocolate and red wine, but also in fruits, which are the main source of epicatechins too. On the other hand, their gallate forms gallocatechin (GC), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) are found in some seeds of legumes, grapes and tea [147]. Dietary flavan-3-ols, unlike other flavonoids, exist in plants mainly as aglycones rather than glycosides.

2.2 Flavanols mechanisms of action

For years it has been widely accepted that the main role of flavanols was their anti-oxidant activity. However, these days, since it has been observed that

concentrations in blood are low compared with other antioxidants and extensive metabolism following ingestion lowers their antioxidant activity, the direct effect of polyphenols *in vivo* is questionable [148]. Nevertheless, apart from their *in vivo* antioxidant activity, most of the beneficial, chemopreventive and therapeutic properties associated with PACs appear to be due to specific interactions with proteins and enzymes. The interactions between PACs and proteins result in a biological effect determined by the function of the proteins involved, including the modification of enzymatic activities, binding of receptors and ligands, and transcription factors binding to their specific sites in deoxyribonucleic acid (DNA) [149]. For example, a grape seed proanthocyanidin extract (GSPE) has shown to modulate glucose homeostasis interacting with the insulin receptor insulin receptor in order to stimulate the uptake of glucose [150], or to regulate pro-inflammatory pathways through the modulation of the MAPK and nuclear factor-κβ (NF-κβ) activities [151]. Moreover proanthocyanidins have also shown to induce the transactivation of some nuclear receptors, such as the farnesoid X receptor [152] and the retinoic acid-related orphan receptor alpha [153] and to partially prevent cell apoptosis by attenuating ER stress via regulation of the caspase-12 pathway [154]. Moreover, Blade et al. reviewed that PACs may also interact with phospholipid membranes by forming hydrogen bonds and hydrophobic interactions between the phospholipid OH groups and phenolic rings of the PACs. These interactions may indirectly affect cell function by modifying cell membrane structure and physical characteristics such as fluidity, density, and electrical properties [149].

As it is shown in figure 6, other mechanisms by which flavonoids have demonstrated to exert their effects are through epigenetics modulation. On one hand, it has been described that flavonoids can prevent the hypermethylation of the DNA, through the inhibition of DNA methyltransferases DNMTs [155–158]. In addition, flavanols and other flavonoids influence on aberrant histone modifications leading to chromatin

changes, acting as histone deacetylases (HDAC), histone acetyltransferases (HAT), histone methyltransferases (HMT), or histone demethylases (HDM) inhibitors or inducers [159–164], which result in global and/or gene-specific changes in both, histone acetylation and methylation. Furthermore, it has been observed that flavanols can upregulate or downregulate expression levels of micro RNAs (miRNAs) [165–168], thus affecting their interaction with its target gene. Moreover, it has been suggested that proanthocyanidins might have the ability to bind to miRNAs, thus proposing a new mechanism by which flavanols modulate the metabolism [169].

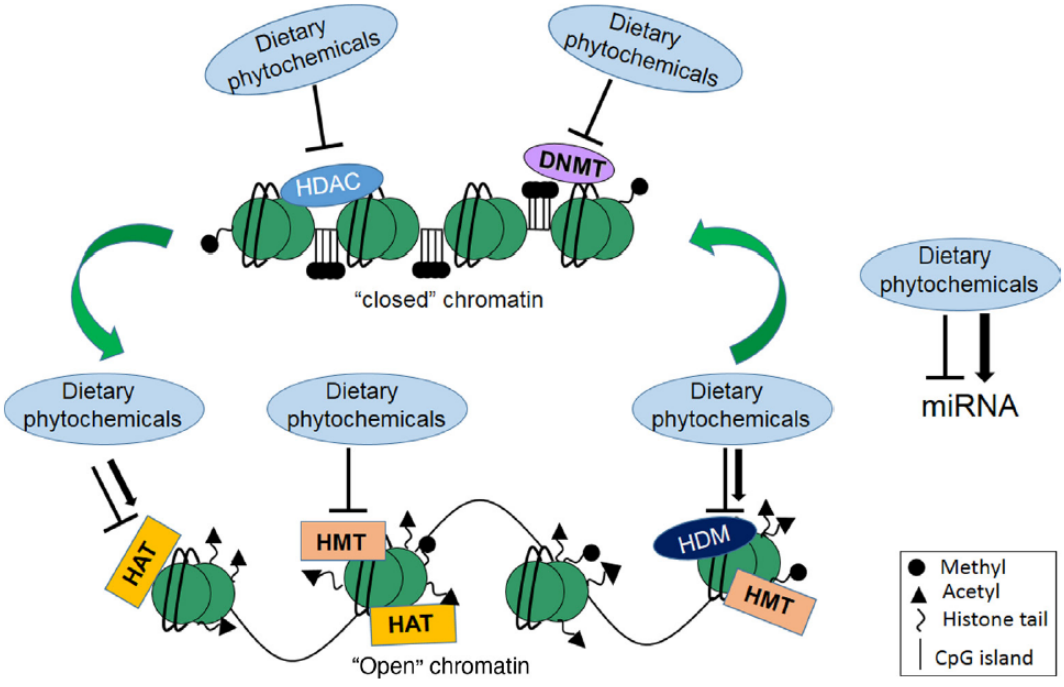

Fig. 6. Simplified depiction of epigenetic mechanisms of action by dietary flavanols. A variety of histone modifications with DNA methylation status define the dynamic chromatin conformation as either "closed" or "open." Dietary phytochemicals through modulation of the epigenetic machinery involved in both these chromatin conformations (inhibition of HDACs, DNMTs, HATs, HMTs, HDMs, miRNAs, and/or activation of HATs, HMTs, and miRNAs) may have potential health benefits. Extracted from [170].

All these mechanisms described above can be carried out directly by molecules in their original form as the low molecular weight ones, by microbial-metabolized molecules, which are then reabsorbed, or they can exert a local activity in the GI tract through non-metabolized flavanols or phenolic acids produced through microbial degradation. Indeed, they have shown to be important in the maintenance of the intestinal barrier, keeping it impermeable ant protecting it from oxidative stress and cytokine-induced inflammation [171]. Moreover, they also play an important role in the regulation of the enteroendocrine system through the modulation of gut hormone releases, as well as in the regulation of gut microbiota [172].

2.3 Flavanols metabolization

As mentioned before, depending on their structure, flavanols can exert their effects in the periphery in their original form, absorbed as low-molecular weight molecules or after being metabolised by the microbiota and/or the liver. The main products that have been used to study their absorption and metabolism in humans are green tea and some commercial cocoas because of their high concentration [141]. Most polyphenols are probably too hydrophilic to penetrate the gut wall by passive diffusion. However, flavanol monomers can enter directly by passive diffusion through the enterocytes and are immediately metabolized [173]. Nevertheless, the aglycones must be metabolized by sulfotransferases (SULTs), uridine-5'-diphosphate glucuronosyltransferases (UGTs) and catechol-O methyltransferases (COMTs), becoming sulfate, glucuronide, and methylated sulfate/glucuronide metabolites which can be absorbed by the small intestine[173, 174]. Once absorbed, the unmetabolized monomers are glucoronidated, sulfated or O-methylated in the liver prior to their renal excretion (see figure 7).

Procyanidins are oligomeric flavanols with a high molecular weight, which makes them poorly absorbed in the GI tract due to their inability of being degraded in the acidic conditions *in vivo*. Even so, it has been observed that procyanidin dimers and trimers present a minor absorption [175, 176]. A study with human volunteers showed that after an ingestion of a cocoa beverage, the maximal plasma concentration of procyanidin B2, reached 2 h after ingestion, was much lower than that reached after a roughly equivalent intake of epicatechin [177]. The majority of the procyanidin B2, and other oligomers with a degree of polymerization ≥ 2 reach the colon and are extensively metabolized by colonic microbiota. A study with rats, suggested that most molecular weight phenolic acids and other compounds detected in the blood, produced from procyanidin B2 after an oral administration, were produced by gut microorganisms before absorption [178]. Zhang et al. reviewed that the main phenolic acids found after colonic metabolization of PACs are phenylvaleric acids, phenylpropionic acids, phenylacetic acids, benzoic acids derivatives and phenyl valerolactone (one of the typical metabolites of catechins and gallocatechins). Just a small amount of PACs dimers are able to retain one intact molecular structure of catechin after colonic fermentation [179].

Fig. 7 Schematic representation of flavanols metabolization. Adapted from [176].

Regarding their excretion, Stoupi et al. observed that after intravenous administration of B2 procyanidin, approximately a 76% was excreted via urine, reflecting an extensive renal clearance, while approximately a 28% of the dose was excreted in the faeces, indicating biliary excretion and also suggesting an enterohepatic recycling [178].

3. Flavanols to ameliorate obesity-related pathologies

Flavanols have been proved to be effective meliorating several pathologies related to metabolic syndrome [180–182]. The most accepted diagnostic tool for metabolic syndrome is the global consensus described by the International Diabetes Federation, which include a set of pathologies that are frequently related to obesogenic situation [183]. In this sense, one way through which flavanols have demonstrated to act against obesity is increasing the energy expenditure and reducing body weight (BW) [184–186].Recently, our research group has studied whether the anti-obesity effect of a 500 mg/kg BW dose of GSPE treatment was better if administered as a pre-treatment, a simultaneous-intermittent treatment or a corrective treatment. Although all the assessed GSPE treatments were associated with reduced respiratory quotients (RQ) during the light period measured, the results showed that GSPE is effective under moderate obesogenic conditions, and it has a greater effect as a preventive agent when it is administered from the beginning of the obesogenic diet. Furthermore, the two pre-treatment studies showed, in the seventh week after a 10-day treatment, that there was a lasting effect on BW that remained until the 14th week. This is a very novel effect that has not been previously shown for this parameter. Even so, the administration of this dose every other week resulted in the most effective treatment of those assessed: it limited BW gain induced by the CAF diet by 50% and adipose accumulation by 60% [187]. Regarding the available information of human studies there is controversy, since there are

studies that affirm that dietary supplementation with flavanols do not enhance EE [188, 189], while there are other that do [185, 190], thus suggesting that there are needed more studies to clarify the role of flavanols on the EE of obese humans.

There are discrepancies regarding potential anti-hyperglycaemic effect of dietary flavanols, since there are studies that show that their intake is inversely associated with the risk of developing T2DM [191, 192] and meliorates the glycaemic profile in T2DM patients [193–195], whereas other do not show any improvement in glucose and/or insulin levels [196, 197]. The same controversy has been found regarding the anti-hyperglycaemic effect of flavanols in animal studies (reviewed in [198, 199]).

Apart from meliorating BW and EE, some studies have shown that flavanols may improve glucose and lipid homeostasis altered by obesity by interacting with the intestine and peripheric tissues [200–205]. In this sense, despite there are discrepancies about their anti-hyperglycaemic effect [193–197], an explanation of this variety of results might be that this anti-hyperglycaemic is effect is dependent on the quantity of procyanidins that the animals receive, including the daily dose, which in turn depends on the method and period of administration [199]. Focusing on GSPE treatments, while a dose of 50mg/kg BW has been shown to be ineffective improving insulin resistance in rats fed with a high fat diet (HFD), a lower dose of 25 mg/kg BW has demonstrated to be effective improving the glycaemic state and insulin resistance when is administered for 21 or 30 days, but not if given for a shorter period of time (10 days) [202, 206]. Dorenkott et al. evaluated the effect of monomeric, oligomeric and polymeric forms of cocoa flavanols in obese mice fed with HFD, and a 12 weeks supplementation showed that the oligomer-rich fraction proved to be most effective in preventing weight gain, fat mass, impaired glucose tolerance, and insulin resistance in this model [207].

Depending on their size, flavanols may induce their anti-obesogenic effects through their low-molecular weight compounds that are directly absorbed and may act by direct interaction with the molecules of the body, or as bigger metabolites, that are further metabolised by the colonic microbiota and absorbed. Besides, both low and high-molecular weight compounds may act locally along the GI tract in their original form, thus interacting with the intestine and exerting many anti-obesogenic effects [171, 172]. For this reason, the effects of flavanols will be described in 2 sub sections differentiating the effects observed in peripheral tissues induced by the absorbable low molecular weight forms and the metabolites derived from colonic metabolism, and the direct effects that they exert on the GI tract.

3.1 Anti-obesogenic effects of flavanols on the peripheral tissues

As mentioned before, in addition to their net effect on body weight and EE, flavanols modulate the functionality of skeletal muscle, adipose tissue, liver, and pancreas thereby improving obesity-related pathologies [200–205, 208], and contributing to meliorate their lipid and glucose management [198, 209, 210]. Our research group has previously reviewed that glucose uptake modulation is one important mechanism through which PACs induce their antihyperglycemic effect (reviewed in [199]). Furthermore, Gonzalez-Abuín et al. describe that PACs increase glucose uptake in hepatocytes, adipocytes, and myotubes, being the AMP-activated protein kinase (AMPK) pathway a common target in all of these insulin sensitive cell lines [198].

3.1.1 Anti-obesogenic effects of flavanols in the adipose tissue

In obesity, hypertrophic adipocytes lead to adipose tissue hyperplasia, and it is a result of a positive energy balance. Flavanols, have been shown to counteract hypertrophy through the regulation of rate of lipid synthesis and degradation [211].

Interestingly, GSPE supplementation has shown to present anti-hypertrophic and hyperplasic activities in rats with established obesity, mainly in visceral white adipose tissue (WAT) inducing a healthier expansion of WAT to match the surplus energy provided by the CAF diet [212]. Dietary supplementation of green tea catechins has been shown to reduce adipose tissue mass and ameliorate plasma lipid profiles in HFD-induced obese mice through the regulation of the expression of multiple genes involved in adipogenesis, lipolysis, β-oxidation and thermogenesis in white adipose tissue [213, 214]. Furthermore, flavanols have also been proposed to reduce fat depots in rats and/or mice fed with high fat diet by increasing the insulin like growth factor binding protein-1 (IGFBP-1) in adipose tissues [215], or by the regulation of Peroxisome proliferator-activated receptor γ (PPARγ) mediated via extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) pathway [216, 217]. GSPE has been proposed to decrease both the plasma free fatty acids (FFA) levels and lipid accumulation in adipose tissue via the activation of both β-oxidation and glycerolipid/ FFA cycle in hamsters fed with high fat diet [218]. Flavanols have also shown to improve glucose metabolism in hyperinsulinemic both *in vitro* [219, 220] and *in vivo* [219]. A daily dose 25 mg GSPE/kg BW administered for 30 days showed a reduction of plasma insulin as well as improvement of the homeostatic model assessment-insulin resistance (HOMA-IR) index. Despite the down-regulation of PPAR-γ2, GLUT-4 and IRS-1 observed in mesenteric white adipose tissue, the authors suggest a possible insulinomimetic effect of GSPE on the adipose tissue [202]. Nevertheless, other doses of procyanidins have shown to up-regulate the expression of GLUT-4 and to favour its translocation to the cell membrane *in vivo* [219, 221]. It is suggested that procyanidins induce this anti-hyperglicaemic effect through the activation of AMPK and AKT pathways [150, 221, 222].

The decrease in body weight gain due to consumption of dietary flavanols has also been associated to an increase in EE. Indeed, the consumption of black soybean seed

coat extract rich in flavanols has been associated to a decrease in body weight gain through the upregulation of uncoupling proteins (UCPs) in WAT and brown adipose tissue (BAT), respectively, thus enhancing EE [223]. Furthermore, a corrective treatment of GSPE to HFD-obese rats exhibited a protection against weight gain correcting the energy imbalance caused by obesity, improving the mitochondrial function and thermogenic capacity of the BAT [224]. In humans, it has been observed that a single orally ingested tea catechin with caffeine acutely increased EE associated with increased BAT activity and chronically elevates non shivering cold-induced thermogenesis, probably because of the recruitment of BAT [225].

3.1.2 Anti-obesogenic effects of flavanols in the liver

Dietary flavanols have been proved to ameliorate the metabolic disorders caused by obesity decreasing fat synthesis and increasing the energy expenditure in the liver [203, 226, 227]. Moreover, it has been reviewed that in high-fat diet-induced obese mice, rats, or chickens, green tea and catechins downregulate the expression of genes coding for fat synthesis, and upregulate the messenger ribonucleic acid (mRNA) levels of enzymes for fatty acid β-oxidation in the liver [137, 228, 229]. As with adipose tissue, it seems that flavanols improve fasting and postprandial hyperglycemia and reduce hepatic *the novo* lipogenesis, restoring the activation of the AMPK pathway in the liver of animals fed with HFD [230, 231].

Regarding the effect of flavanols on EE in the liver, Ikarashi et al. showed that the administration of the anti-obesity agent acacia polyphenol, rich in catechin-like flavanols, to mice fed with HFD, resulted to increase the expression of EE-related genes in skeletal muscle and liver, and decreased fatty acid synthesis and fat intake in the liver [226]. Besides, it has been reported that flavanols might induce their effect enhancing EE due to the effect of stimulation of mitochondrial complex chain and increased energy expenditure, particularly from the oxidation of lipid substrates,

thereby contributing to the prevention of hepatic steatosis and improving insulin sensitivity [227]. Furthermore, recent studies have revealed that a epicatechin supplementation to HFD-fed mice, resulted in a regulation of in the modulation of the insulin pathway in the liver and skeletal muscle, thus restoring the insulin sensitivity lost by the HFD [232].

3.1.3 Anti-obesogenic effects of flavanols in the muscle

Muscle is the major site of adenosine triphosphate (ATP) production and energy consumption; the uptake and oxidation of glucose and fatty acids are key molecular events controlled by the muscle which are impaired in obesity. An oligonol rich in catechins and procyanidins treatment to HFD fed mice was able to improve glucose intolerance and facilitate glucose uptake and insulin sensitivity in skeletal muscle by restoring IRS1 and AS160 phosphorylation. Moreover, oligonol lowered intramuscular lipids in parallel with enhanced SIRT1 expression and restored AMPK-α activity, indicating that reduced intramuscular lipid by oligonol could be via increasing fat oxidation [231]. A 16 weeks chronic treatment with pure EGCG was reported to reduce body weight gain and improve insulin sensitivity in HFD-fed mice. These effects were associated with increased expression of genes related to mitochondrial FA oxidation in skeletal muscle and by modulating fat absorption from the diet [233]. Furthermore, Gonzalez-Abuín et al. suggest that since AMPK is involved in the translocation of GLUT-4 to the plasma membrane, the mechanisms by which PACs up-regulate this glucose transporter in adipose tissue and muscle might involve the activation of AMPK [198].

The supplementation with a cocoa liquor procyanidin extract to C57BL/6 mice fed with a HFD, increased expression of UCP-1 in BAT, UCP-2 in WAT and UCP-3 in skeletal muscle was proposed as an underlying mechanism to the suppressed HFD-induced fat deposition, due to the involvement of UCPs on thermogenesis and

energy metabolism. The authors indicated that the cocoa beans prevented obesity by up-regulating the expression levels of UCPs and Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) through the action of AMPK, thus preventing obesity by increasing EE, which resulted in the reduction of the WAT weight [230]. By using a β2 AR blocker and a β3 AR blocker, Kamio et al. have confirmed that the enhancement of energy expenditure (increased mRNA expression of UCP-3, and phosphorylated AMPKα) induced by the ingestion of single dose of a flavan-3-ol fraction derived from cocoa in mice is mediated by sympathetic nerve stimulation [234]. Furthermore, the same group has revealed that flavan-3-ols decreased both, body weight and the RQ, enhanced lipolysis and promoted mitochondrial biogenesis in gastrocnemius and soleus muscles and brown adipose tissue [235]. Similarly, Casanova et al. found that a 21 days supplementation of GSPE to rats fed with CAF diet was able to decrease body weight gain and RQ, in part through the reduction insulin resistance and the improvement of the muscle status, through the promotion of fatty acid oxidation and the increase of UCP-2.The authors attribute such modifications to the activation of the AMPK signaling pathway [205].

Regarding the studies with humans, a randomized controlled trial in which obese subjects received for 12 weeks a combined epigallocatechin-3-gallate and resveratrol supplementation (EGCG+RES), showed to increase mitochondrial capacity and stimulates fat oxidation in the muscle, but it was not translated into increased tissue-specific insulin sensitivity in overweight and obese subjects [190].

3.1.4 Anti-obesogenic effects of flavanols in the pancreas

Pinent et al. and Salvadó et al. reviewed that the anti-hyperglycaemic effect of proanthocyanins seems to be mediated by mimicking insulin action on the liver and peripheral tissues and/or by affecting insulin secretion through the modulation of pancreatic β-cell functionality and the incretin system [180, 199]. Specifically, GSPE

has showed to modulate insulin secretion in both, isolated Wistar rat islets and pancreatic beta-cell lines [236]. In *in vivo* experiments, it has been shown that a corrective daily dose of 25 mg GSPE/kg BW for 30 days to female Wistar rats fed during 13 weeks with a CAF diet, was capable to reduce insulin production downregulating pancreatic insulin promoter factor 1 (Pdx1) and insulin gene expression. Furthermore, it also reduced the TAG content in the pancreas and down-regulated the expression of lipid synthesis-related genes. GSPE treatment did also counteracted the decrease of AMPK protein levels after cafeteria treatment. The authors concluded that lack of triglyceride accumulation induced by GSPE in β-cells, counteracts its negative effects on insulin production, allowing for healthy levels of insulin production under hyperlipidemic conditions [208]. Interestingly, it has also been found an anti-proliferative effect of GSPE in the pancreas. A 21 days of 25 mg GSPE/kg BW corrective administration to male rats previously fed wit CAF diet for 7 weeks, showed to improve insulin resistance and counteracted the cafeteria-induced effects on insulin synthesis [206], results that resemble those obtained by Castell-Auví et al. [208]. However, the administration of the extract enhanced the cafeteria-induced increase in Bax protein levels, suggesting increased apoptosis [206]. This result contradicts previous results from cafeteria-fed female rats, in which GSPE seemed to counteract the increased apoptosis induced by the cafeteria diet. Specifically, it was observed that 25 and 50mg/kg of GSPE seemed to counteract the deleterious effects of the cafeteria diet by inhibiting the down-regulation of Bcl-2 protein expression after 10 and 30 days of treatment. In addition, 50mg/kg BW of GSPE also counteracted the decrease in the Bcl-2/Bax ratio at the protein level after 10 days of administration [237]. The authors suggest that together, these results indicate that the effects of GSPE on apoptosis markers are dose, time, and/or gender dependent. Moreover, this hypothesis can be also extended to the effect of GSPE on the regulation of insulin secretion.

The beneficial effects of flavanols on amylin secretion in obesity are not clear. Nevertheless, as it was mentioned before, in T2DM amylin fibrils may induce death of pancreatic islet cells [40, 44]. In this sense, flavanols have shown to inhibit amylin fibrils formation in pancreatic cells, thus protecting from the cytotoxicity production in pancreatic cells [238–241].

In summary, the available data suggests that flavanol supplementation might meliorate glucose and lipid management, modulating lipolytic pathways, insulin sensitivity, and glucose uptake, and downregulating adipogenesis in the adipose tissue, liver and muscle. Furthermore, flavanols have also shown to play a role in EE regulation in the same tissues, by affecting pathways involved with thermogenesis. In the pancreas, flavanols might contribute to meliorate the obesogenic state through the reduction of fat accumulation, modulating insulin secretion and cell apoptosis in a dose, time, and/or gender-dependent manner and decrease pancreatic cell cytotoxicity by inhibiting the formation of amylin fibrils in pancreatic cells. However, the variability on the source of flavanols, the dosage and the duration and moment of the treatment makes it difficult to get into a clear conclusion, hence further studies should be carried out in order to get into a clear consensus.

3.2 Anti-obesogenic effects of flavanols on the GI tract

As mentioned before, flavanols are poor absorbed, fact that makes them great candidates to exert their effects over the GI tract. For this reason, over the last years there have been hypothesis suggesting that flavanols might limit energy absorption through their influence on the intestinal processes involved in the digestion and absorption of energy compounds [55, 56, 137, 242], they also induce a satiety effect and regulate energy homeostasis through the modulation of gut hormones [243, 244], they act as protective and anti-inflammatory agents of the intestinal barrier

[171, 245] and they are also a very powerful tool modifying microbiota along of gastrointestinal tract [172, 246].

3.2.1 Flavanols interaction with digestion and absorption processes

Trypsin, α-amylase and lipase, the main enzymes involved in the hydrolysis of dietary protein, starch and fat, are delivered into the intestinal lumen as constituents of the pancreatic juices. It has been reported that PACs present inhibitory effects on these enzymes *in vitro* [55, 247–250], being this, one of the mechanisms by which they exert their effect limiting the energy availability to the organism. However, there is controversy regarding the inhibitory effects of PACs *in vivo*, since Tebib et al. observed that these effects disappear, due to the fact that in the duodenal lumen, alkalinity and detergency from the pancreatic biliary secretion neutralized the ability of tannins to inactivate brush border hydrolase activities [251]. Furthermore, Serrano et al. supported Tebib's evidence observing that the ratio of energy absorbed between GSPE-treated rats and control group was quite similar, thus supporting the idea that the inhibitory effects observed *in vitro* differ from *in vivo* analysed effects [252]. Several authors also attribute the effects of flavanols lowering plasmatic glucose and TAG levels to an inhibition of digestive enzymes as α-glucosidase, alpha-amylase or pancreatic lipase [253–258], but these suppositions are made from results observed in *in vitro* experiments [249, 259–261] and therefore further *in vivo* experiments should be done to corroborate this hypothesis.

Regarding the effect of flavanols on nutrient absorption, it has been pointed that flavanols, especially those with a galloyl moiety, could significantly inhibit the GI absorption of dietary nutrients and increase faecal energy excretion [262, 263]. In mammals, once digested, intestinal glucose uptake is mainly performed by its specific transporters, such as sodium-dependent glucose transporter 1 (SGLT-1), and and GLUT-5, all expressed in the intestinal epithelial cells. Experiments using brush-

border membrane vesicles obtained from rabbit small intestine demonstrated that epicatechin-gallated inhibited SGLT-1 in a competitive manner, although it was not transported via SGLT-1 by itself [264]. In the study of glucose absorption on animal models, Snoussi et al. found that in fasted rats, an acute administration of 25mg/kg BW of a green tea decoction (GTD), rich in EGCG and EGC inhibited SGLT-1 activity, increased GLUT-2 activity and improved glucose tolerance. Similarly, to GTD, acute administration of synthetic phenolic compounds (2/3 EGCG+1/3 EGC) inhibited SGLT-1 activity. Moreover, GTD-treated rats for 6 weeks display significantly reduced SGLT-1 and increased GLUT-2 mRNA levels in the jejunum mucosa. These results indicate that GTD, a traditional beverage rich in EGCG and EGC reduces intestinal SGLT-1/GLUT-2 ratio, a hallmark of regulation of glucose absorption in enterocyte [265].

After fat digestion, lipids are emulsified, hydrolysed and absorbed through the intestinal tract. The absorption of dietary fats and cholesterol is largely dependent on the intestinal expression of several active transporters. The lipid transporters, which are highly expressed on the apical surface of the intestine, facilitate the fatty acid and cholesterol transfer/homeostasis in enterocytes which will be packaged into chylomicrons and secreted into the lymphatic system [266]. As for fat absorption, the bibliography available is mainly focused on the description of which are the lipids that are not absorbed after a treatment with flavanols [262, 263, 267], but there is not consensus about the specific mechanisms that explain this inhibition. Nevertheless, there are few studies that support the effect of flavanols on fat absorption. As an example, Quesada et al. have observed that GSPE presents an hypotriglyceridemic activity in the intestine modulating TAG secretion by repressing the expression of long chain acyl-CoA synthetases *in vitro* [268]. Furthermore, *in vivo* studies showed that GSPE was capable to reduce chylomicron-rich or VLDL-rich fractions in a time-dependent manner, thus contributing to a hypotriglyceridemic

action. GSPE also reported to repress lipoprotein secretion without increasing lipoprotein lipase (LPL) activity [269]. Furthermore, Sugiyama et al. performed a triglyceride tolerance test in mice and humans with a simultaneous ingestion of an apple polyphenol extract rich in procyanidins, and observed that procyanidins significantly inhibited the increase of plasma triglyceride levels in both models [249].

Flavanols have also shown to exhibit *in vitro* protease inhibitory properties, which in the case of porcine pancreatic elastase have been observed to be positive related to their degree of polymerisation. There were necessary procyanidins with a molecular weight of at least 1154 Da to observe a significant inhibitory ability, which also demonstrated to be reversible and competitive. Specifically, the tetramer structure presented a higher affinity to the enzyme due the establishment of more contact points with the amino acids present in its active site [270]. Procyanidins have also showed to interact with the digestive protease trypsin [271, 272]. Procyanidin B3 showed that depending on the concentration of procyanidins used, the interaction with trypsin was different, going from a specific interaction probably driven by hydrogen bonds between the protein backbone and the procyanidin with low concentrations, to a nonspecific interaction with high concentrations. Furthermore, carbohydrate pectin proved to induce a dissociation of the tannin- pancreatic trypsin complex [271].

Summarizing, despite there are well evidenced effects of flavanols inhibiting the digestion of carbohydrates, fat and proteins in vitro, there are discrepancies for their in vivo effect since alkalinity and detergency from the pancreatic biliary secretions might neutralize the ability of tannins to inactivate brush border hydrolase activities. Although there should be carried out more *in vivo* experiments, taking into consideration the available data, the studies suggest that flavanols are more prompted to inhibit GI absorption of dietary nutrients thus increasing faecal energy excretion.

3.2.2 Effect of flavanols on enterohormone secretions.

Hereunder there will be described the effects of flavanols on the main enterohormones to give a general look of their role on energy homeostasis.

3.2.2.1 Ghrelin

The available bibliography has demonstrated that flavanols present anti-obesogenic effects, in part through the modulation of ghrelin. Serrano et al. reveals that that monomeric flavanols stimulate ghrelin secretion by activating bitter taste receptors. In contrast, oligomeric flavanols inhibit ghrelin release. When they studied the effect of an acute high dose of GSPE on Wistar rats, it increased plasma ghrelin, but when administered chronically, they observed that GSPE decreased plasma ghrelin levels, ghrelin secretion in intestinal segments, and ghrelin mRNA expression in stomach. They concluded that GSPE stimulates ghrelin release due to the interaction between monomeric flavanols and bitter receptors, while a subchronic GSPE treatment reduces ghrelin production by acting on its secretion and/or synthesis [273]. Similarly, in a study made by Jambocus et al., a high fat diet decreased ghrelin levels and only the lower dose of a *Morinda citrifolia* L extract (150 mg/kg BW), rich in flavonoids as catechin among others, was able to restore them to basal levels in Male Sprague-Dawley rats [274]. Similarly, a 11 weeks treatment with a *Cosmos caudatus* Kunth leaf to rats fed with a CAF diet increased ghrelin levels [275]. Ramos-Romero et al observed that a 24-weeks supplementation with a grape seed extract (0.8 g/kg BW) plus ω-3 polyunsaturated fatty acids (PUFA) (16.6 g/BW) to HFD-fed rats resulted in an increase of plasma ghrelin, compared to the HFD group [276]. A recent meta-analysis, has reviewed the effect of green tea on plasma ghrelin levels, and shows that the consumption of green tea for long periods is associated with an increase of ghrelin levels. Nevertheless, the authors concluded that more randomized controlled trials with longer duration and more precise doses are

needed to assess green tea's effect on fat mass and obesity hormones [277]. Low fasting plasma ghrelin has been observed in obese rodents [278] and humans [279], and has been associated with insulin resistance [280, 281].

3.2.2.2 Cholecystokinin

Little is known about the effect of flavanols on CCK stimulation in a context of obesity, for this reason there will be also described the effect of other flavonoids in order to clarify a little bit more the effects of this hormone. *In vitro* assays with STC-1 cells have shown that flavonoids such as naringenin and hesperetin are able to stimulate CCK release through the activation of transient receptor potential channels (TRP channels) including TRP-1 and the increase of intracellular calcium levels [282, 283]. Some other flavonoids as quercetin, kaempferol and apigenin have also resulted to increase CCK levels *in vitro*, while others such as rutin and baicalein have not [284]. Under non-obesogenic conditions, it has been observed that in an *ex vivo* assay of murine intestines, 1 mM EGCG stimulates CCK secretions in duodenum [285]. On the contrary, a treatment of 0.2 mg/mL of GSPE in duodenal rats leads to a decrease of CCK secretion, which role is attributed to the presence of gallic acid since it is a compound found in the extract mixture. Moreover, this inhibition seems to not be caused by an increase of CCK basal plasma levels in fasted animals, since CCK release is not stimulated [286]. In animal studies, it has been observed that a 4 weeks treatment with isoflavones (flavonoid) to ovariectomized rats under a high-fat diet results in a decrease in plasma ghrelin, and an increase in CCK levels, and a tendency to increase PYY levels, which the authors associate to a reduction of body weight and food intake [287].

3.2.2.3 Peptide YY

Like with the previous enterohormones, there is not much information available about the effect of flavanols on PYY secretion, thus, the effects of other flavonoids

there will be described too. In animal studies, a recent study has revealed that a 2 months daily dose of 100 μg/BW of EGCG to mice fed with a high-fat diet was able to increase both PYY and GLP-1 plasma levels [288]. Furthermore, as it was mentioned before, isoflavones have been also been capable to reduce body weight, total abdominal fat, food intake in ovariectomized rats fed with a high-fat diet, partly due to a slightly increase in PYY plasma levels [287]. Furthermore, in studies with humans, the consumption of 50 grams of white, red, or brown sorghum biscuits (containing phenolic acids, flavonoids and condensed tannins) resulted in an area under the plasma concentration-time curve of postprandial PYY significantly higher in sorghum biscuits groups as compared to the control, despite the energy intake at a subsequent meal did not differ between treatments [289]. Moreover, isoflavones supplementation has also shown to increase PYY plasma levels in healthy postmenopausal women after 8 weeks of treatment, despite it could not significantly reduce energy intake or body weight [290].

3.2.2.4 Glucagon-like peptide 1

There are some *ex vivo* and *in vivo* studies that show the effect of flavanols on GLP-1 secretion. While Song et al. found that 1mM EGCG enhance GLP-1 secretions in murine ileum [285], Casanova et al. showed that GSPE directly promotes GLP-1 secretion in the ileum, and its metabolites do so in the colon. Such direct stimulation required activation of glucose-induced GLP-1-releasing pathways. They also suggested that *in vivo* GLP-1 secretion may also be mediated by indirect pathways involving modulation of other enterohormones that, in turn, regulate GLP-1 release, such as enhancing the gastric inhibitory polypeptide (GIP) and reducing CCK secretion in the duodenum [286]. Yamashita et al. performed a study where after 18 hours of fasting, a single oral ingestion of 10 mg/kg BW cinnamtannin A2 (tetrameric procyanidin) to male ICR mice, increased the GLP-1 and insulin levels in plasma without an oral glucose load [291]. Similarly, our research group found that an acute

dose of 1g/kg BW GSPE extract increased GLP-1 plasma levels after an oral glucose load in rats [292]. Working also with GSPE, Casanova et al. found that a 8-days sub-chronic treatment of 500 mg/kg BW GSPE, previously shown to reduce food intake, increased plasma GLP-1 in healthy female rats. They suggested that the changes in the microbiota may be linked to the modulation of enterohormone secretion induced by GSPE [172]. Regarding human interventions, the chronic consumption of 1.5 g/day of green tea extract rich in EGCG for 16 weeks, significantly increased GLP-1 plasma levels T2DM subjects [293].

As for the study on long-term high fat diets, González-Abuín et al. developed a 12-week CAF diet-fed rat model that caused a decrease in GLP-1 plasma levels and production in the intestine and clearly revealed an induction of insulin resistance. When the animals were treated with 25 mg of GSPE/kg simultaneously to the CAF diet, rats exhibited an increased amount of GLP-1 in colonic cells, with a simultaneous increase in GLP-1 mRNA. Moreover, they also observed that GSPE upregulated the colonic expression of both PYY, which has been reported to be co-expressed with GLP-1 in enteroendocrine L cells, and chromogranin A (GhGA), a marker of endocrine cells. This suggested them that GSPE might increase GLP-1 levels by preventing the loss of enteroendocrine cells induced by a CAF diet. GSPE treatment also increased hypothalamic GLP-1 production and downregulated GLP-1R, opposing the effects of the CAF diet. This preventive action also impacted intestinal DPP4, predominantly by preventing the decrease in its activity and protein levels [294]. Controversy, in another study with genetically obese rats, a similar dose of GSPE downregulated the gene expression of DPP4 [295]. This suggests that GSPE acts differently depending on if it is given as a preventive or a corrective treatment.

In summary, the currently available data shows that despite the variability in the extracts' composition as well as the duration of the treatments, in a situation of obesity, flavanols seem to increase ghrelin plasma levels, in order to return them to the basal ones; they also increase PYY and GLP-1 levels, and although the lack of studies with flavanols, the studies with flavonoids suggest that they are able to increase CCK levels in healthy conditions. More *in vivo* studies should be carried out to study the effect of flavanols on CCK secretions under obesogenic conditions, and to elucidate the effects and mechanisms by which the flavanol-modulation of enterohormones counteract the effects of a HFD, in the whole organism. Furthermore, it is unclear how much time do the flavanol effects last, and if these effects can be extrapolated when they are given as a corrective treatment. Finally, more investigations should be done to come to an agreement of which is the best mode (plant, extract or pure compounds), duration and dose of flavanol administration in order to achieve greater results.

3.2.2.5 Methodological approaches to study enteroendocrine system

The study of enterohormone secretions entail some methodological difficulties associated to the low number of enteroendocrine cells and their dispersion along the GI tract, which makes it laborious to obtain clear and reproducible values of enteroendocrine secretions. Nevertheless, depending on the requirement for the study and the equipment available, the study of the enteroendocrine system can be accomplished through different methodologies, going from the simplest one, as would be working with intestinal cells [296–299], to the most complex, as would be working *in vivo* with animals [300, 301]. When the objective is to perform a mechanistic study thought, another alternative to the use of cell lines that permit to overcome the culture limitations, would be the utilization of *ex vivo* strategies using natural intact tissues structures in different controlled situations [302]. Some *ex vivo* approaches would be the use of isolated intestinal perfusions [303], everted sacs

[304], ligated intestinal loops [305], precision-cut intestinal slices [306], organoids [286] among others. Many of these methods require sophisticated surgical procedures and instrumentations and/or the tissue loses gradually viability and integrity. The use of Ussing Chambers, overcomes some of these problems and it allows to test vectorial enteroendocrine secretions [307] in response to drug stimulus. The main drawback is that due to the low availability of chambers, it is difficult to have enough samples to minimise variability [308–310]. To overcome these limitations some authors work with *ex vivo* tissue fragments from animal intestines [273, 311]. These crude explants from animal intestines make it possible to produce numerous replicates, depending on the animal's size, although it does not mimic the effect of the apical stimulation that takes place in the *in vivo* GI tract.

3.2.3 Effects of flavanols on the inflammation and permeability of the intestinal barrier

We have recently reviewed the effect of flavonoids on intestinal inflammation and barrier integrity during diet-induced obesity [312], which are summarized in figure 8, and among them, flavanols have shown to exert some particular beneficial effects.

On one hand, regarding the effect of flavanols on intestinal inflammation, in vivo studies have shown that Marie Ménard lyophilized apples, which are rich in flavonols and flavan-3-ols, reduced myeloperoxidase (MPO) activity and the gene expression of the inflammatory markers (cyclo-oxygenase-2) COX-2 and iNOS. MPO is considered a marker of disease activity in patients with intestinal inflammation. Furthermore, low doses of GSPE (5, 25 and 50 mg/kg BW) have shown to present corrective effects against a long-term CAF diet. The three doses showed to attenuate MPO increased activity, it was found an ameliorative effect of the low dose decreasing IL-1β and a beneficial effect of the doses 25 and 50 mg GSPE/kg BW in ROS levels and iNOS gene expression in the ileum [171]. Furthermore, in a study with

17-weeks CAF diet-fed rats, the cafeteria diet increased ovalbumin, lipopolysaccharides (LPS), MPO and TNF-α levels, and a dose of 500 mg GSPE/kg BW given as a preventive treatment (before or together with the CAF diet) showed to prevent this metabolic endotoxemia induced by the CAF diet, being the second treatment the most effective [245].

On the other hand, regarding the effects of flavanols ameliorating the integrity and the permeability of the intestinal barrier, *in vitro* studies have shown that EGCG restores the trans electrical epithelial resistance (TEER) and reduces the isothiocyanate-labelled dextran (FD-4) transport across the cell monolayer after an induced-disruption of the intestinal barrier [313, 314].

Fig 8. Schematic view of the anti-inflammatory mechanisms of flavonoids on intestinal inflammation. Extracted from [312]

In another study, in HT-29 cells, a treatment with pomegranate juice, rich in anthocyanidins and catechins, reduced TNF-α-induced COX-2 expression [315]. The authors suggest that these findings may be related to the modulation of PI3K, AKT and/or MAPK pathways. Other authors have found that a pre-treatment with a wine extract rich in flavanols, flavonols and anthocyanidins, prevents IL-6 and IL-8 expression and synthesis after being challenged with an oxysterol mixture in Caco-2

cells [316]. With regard to the *in vivo* effects of flavanols, 500 mg/kg BW of GSPE (before or together with the CAF diet) have also shown to prevent the decrease of the permeability in ileum and colon induced by a CAF diet being the second treatment, the most effective one against barrier dysfunctions [245].

Summarizing, flavanols have demonstrated to prevent and improve the intestinal inflammation and barrier-function alterations induced by high fat diets.

3.2.4 Effects of flavanols on gut microbiota

Although little is known about the interaction between flavanols and gut microbiota under an obesity context, there has been observed that dietary polyphenols, including flavanols, have the ability to induce oscillations in the composition of the microbiota populations, thus becoming potential gut microbial modulators [317–319]. Strat et al. highlights that the dosing method may impact the mechanisms by which flavanols act *in vivo.* When they are given *ad libitum* by adding it into the chow or the drinking water, flavanols were co-consumed with macronutrients, thereby facilitating flavanol-mediated alteration of nutrient digestion. On the contrary, when flavanols are supplemented by an oral gavage, it is often done during the fasted state, in which case flavanols would not be co-consumed with macronutrients, thereby precluding the opportunity for flavanol-mediated alteration of nutrient digestion [318]. Moreover, dietary components might modulate the composition and metabolic and immunological activity of the gut microbiota acting as probiotics, microorganisms whose intake confers health benefits to the host, or in the case of polyphenols, as prebiotics, whose fermentation results in changes in the composition and/or activity of gut microbiota [320].

It has been reported that flavanols can act as both, as antimicrobials against pathogenic microorganisms and as promoters of health-beneficial gut microbiota

strands. For example, *in vitro* assays have demonstrated catechins inhibit the growth of *Helicobacter pylori* [321], *Staphylococcus aureus, E. coli* [322], Pseudomonas aeruginosa [323], among others. There are other studies with flavanols that have revealed their influence on the composition of the non-pathogenic gut microbial community, thus contributing to the health of the gut microbiota and its host. As an example, a study with cocoa-derived flavanols, which have shown to modulate the human gut microbiota towards a more 'health-promoting profile' by increasing the relative abundance of *Bifidobacteria* and *Lactobacilli* [324]. Casanova et al. revealed the short-term effectiveness of GSPE at modifying microbiota, increasing the amount of Bacteroidetes and reducing that of Firmicutes, and altering specific genera within these phyla. The authors link the modifications in the microbiota with changes in the short chain fatty acids (SCFA) profile from the caecal content, which in turn, these changes in the microbiota correlate with a modulation of plasma TAG, adiposity, and enterohormone secretion induced by GSPE [172]. In a recent study, the co-administration of HFD and tea polyphenols to a human flora-associated C57BL/6J mice model showed that tea polyphenols meliorate glucidic and lipidic metabolism and it also increases acetic acid and butyric acid levels. Furthermore, tea polyphenols increased the richness and diversity of colonic microbiota compared to the HFD group [325].

All in all, this data shows that the modulation of bacteria composition through the administration of dietary flavonoids might be a useful tool to control or treat obesity and other related metabolic diseases.

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HYPOTHESIS AND OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
BIOACTIVITY OF FLAVANOLS ON THE MUCOSA OF THE INTESTINAL WALL: ENTEROENDOCRINE EFFECTS FOR PREVENTING DIET-INDUCED OBESITY AND ASSOCIATED PATHOLOGIES
Iris Ginés Mir

Hypothesis and objectives

Obesity and its associated pathologies have become a focus of attention for researchers during the last decade due to their increasing prevalence worldwide. Phenolic compounds such as grape-derived flavanols have been shown to exert beneficial effects on the gastrointestinal (GI) tract under healthy conditions, though as yet there is no consensus on their effectiveness against an obesogenic challenge such as the cafeteria diet.

We have recently proved the effectiveness of a 500 mg/kg BW dose of grape seed proanthocyanidin (GSPE) extract to prevent or correct cafeteria-diet-induced damage. The above study showed that GSPE when administered intermittently (every other week), throughout the period of cafeteria treatment, limited body weight gain and adiposity and meliorated certain metabolic disruptions induced by the cafeteria diet. The study also demonstrated that the GSPE effect lasted for several weeks after the final dose was administered.

The above GSPE dosage has also demonstrated satiating properties and lipolytic activity in subcutaneous adipose tissue under healthy conditions in rats. Some satiating properties were due to their activity in the enteroendocrine system in the GI tract. However, it is still unclear whether modification of the enteroendocrine cells is also exerted under a cafeteria diet. The compounds found in GSPE have been shown to interact with the luminal surface of the GI tract, thus modifying enteroendocrine cell function. However, the molecular interaction between the phenolic compounds and the enteroendocrine cells is not clearly defined since the scattered distribution of the compounds along the GI tract renders studying them difficult.

Based on these results we postulate that **the beneficial effects of GSPE on cafeteria-diet-induced obesity can be partly mediated by modulation of the enteroendocrine system and, more specifically, that the long-lasting effects of GSPE can be explained by epigenetic mechanisms.**

Thus, the main objectives of this thesis were:

1. To determine the role played by the enteroendocrine system in GSPE treatments against the cafeteria diet.

2. To verify the role of epigenetics in the long-lasting effects induced by GSPE.

3. To describe the metabolic readjustments by which pre-treatment with GSPE acts to prevent dysfunctions caused by the cafeteria diet.

4. To design a new method for studying the impact of food-derived molecules on enterohormone secretions in the various segments of the gastrointestinal tract while maintaining the vectoriality of the tissue.

RESULTS

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Manuscript 1

Objective

Grape seed proanthocyanidins target the enteroendocrine system in cafeteria diet-fed rats.

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Keywords: enteroendocrine system, intestine, flavonoids, phenolic compounds, GLP-1.

Abstract

We analysed the interaction on enteroendocrine system of three different grape seed proanthocyanidin extract (GSPE) treatments in rats on a cafeteria diet for 17 weeks and analzed its relationship with their effects on body weight gain and food intake.

Most of GSPE treatments led to ghrelin accumulation in the stomach, limited CCK secretion in the duodenum and increased GLP-1 and PYY mRNA in colon. It also increased caecal hypertrophy and reduced butyrate content. When the treatment was administered one week every fortnight during 17 weeks, there was too an increase in colon size. 15 days with 500mg GSPE/kg reduced food intake. Multivariate regression analysis revealed a different pattern of relationship between food intake or body weight and plasma hormones between the controls and the GSPE-treated animals, pointing out at GLP-1 and ghrelin's involvement in the body weight-reducing effects of GSPE.

1. Introduction

Obesity is a primary risk factor for the most prevalent diseases affecting the worldwide population, including cardiovascular disease, type 2 diabetes mellitus (T2DM) and inflammation [1]. It is regulated by a complex biochemical process and its pathological mechanisms have been widely studied in adipose tissue, the liver and muscle [2, 3]. The GI tract plays a role in controlling the metabolism through peptide hormones secreted by enteroendocrine cells. These hormones from the gut play a central role in nutrient intake signalling, and regulating appetite and energy expenditure. There is evidence that specific enterohormones administered at physiological concentrations can influence the appetite of rodents and humans (reviewed in [4]). Likewise, the effects of gut hormones on food intake and body weight have been observed in bariatric surgery (such as Roux-en-Y gastric bypass), which induces a huge increase in GLP-1 and PYY secretion and is used to treat obesity. Therefore the modulation of enterhormone signalling may represent an important target for preventing obesity and related/associated pathologies.

Natural compounds could be used to prevent the development of overweight and obesity-related problems from early preclinical stages [5]. Of these, grape-derived proanthocyanidins have been described as potential bioactive compounds that exhibit a wide array of beneficial effects on health. They have been reported to improve lipid [6] and glucose [7] metabolism, and although there are discrepancies in the literature, several studies show reduction in body weight gain and increase in energy expenditure (reviewed in [8]). Over the years *in vitro*, *ex vivo* and *in vivo* studies as well as clinical trials have provided ever-increasing evidence of the role polyphenols play as potential health compounds [9, 10]. However, the scientific evidence shows that the beneficial effects of polyphenols on health are directly linked to their absorption, distribution, metabolism and excretion. Some mechanisms used by flavonoids are exerted in the intestine, such as the inhibition of

the enzymes that participate in carbohydrate digestion and the reduction of glucose absorption through the inhibition of the transporters involved in glucose uptake (reviewed in [7]). Moreover, flavonoids have also been reported as having effects on the incretin system, which could be related to an improvement in glucose homeostasis (reviewed in [11]). Grape seed proanthocyanidins have been shown to modulate the enteroendocrine system. Acute grape seed proanthocyanidin extract (GSPE) treatment promotes the secretion of GLP-1 *in vivo* [12], while *ex vivo* studies also show modulation of PYY secretion [13]. GSPE and the specific pure compounds it contains modulate ghrelin production and secretion in cell lines, and *in vivo* studies also show acute and subchronic effects of GSPE on this hormone [14]. However, these effects have been shown in animals fed a standard diet or as acute effects on a palatable diet, but there is a lack of knowledge regarding the effects on the enteroendocrine system on an obesogenic diet.

We have recently shown that 500 mg GSPE/kg bw administered every other week for a period of 17 weeks in animals fed a cafeteria diet reduces body weight gain and respiratoy quotient (RQ) [15]. In this study, we analyse the effects on the enteroendocrine system of this GSPE treatment in the context of an obesogenic diet intervention, and compare it with the effects of GSPE administered in a corrective manner (for only 15 days). We then relate the GSPE effects on this system to its effect on body weight gain and food intake.

2. Materials & Methods

2.1 Proanthocyanidin extract

The grape seed extract enriched in proanthocyanidins (GSPE) was kindly provided by *Les Dérivés Résiniques et Terpéniques* (Dax, France). According to the manufacturer, the GSPE composition used in this study (Batch number: 124029) contains monomers of flavan-3-ols (21.3%), dimers (17.4%), trimers (16.3%), tetramers

(13.3%) and oligomers (5-13 units; 31.7%) of proanthocyanidins. A detailed analysis of the monomeric to trimeric structures can be found in Margalef et al. [16].

2.2 Animal experiments

Female rats weighing 240-270g were purchased from Charles River Laboratories (Barcelona, Spain). After one week of adaptation, the rats were individually caged in animal quarters at 22°C with a 12-hour light/12-hour dark cycle and were fed *ad libitum* with a standard chow diet (Panlab 04, Barcelona, Spain) and tap water. After a period of acclimation, the animals were randomly distributed into 5 experimental groups (n=7-10/group) and fed *ad libitum* a standard chow diet for the whole duration of the experiment. The control group (**STD**) received only the standard chow diet. All the other groups, in addition to the standard chow, received a cafeteria diet as a model of a high fat/high sucrose diet. The cafeteria diet consisted of bacon, sausages, biscuits with pâté, carrots, muffins and sugared milk, which induces voluntary hyperphagia [17]. This diet was provided fresh *ad libitum* every day to the animals for 17 weeks. One of these groups was used as a control for cafeteria (**CAF**), while the three remaining groups received the cafeteria diet and also received an oral GSPE supplementation (see 2.2 Dosage information).

Body weight was monitored weekly. 20-hour food intake was measured right before the corrective treatments (week 15) and in the last week of the experiment (week 17).

All the procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili (code: 0152S/4655/2015).

2.3 Dosage information

Study intervention is summaryzed in Figure 1. The treatments performed to the animals fed a cafeteria diet, as previously described [15], were the following: a) a dose of 500 mg GSPE/kg administered from the beginning of the cafeteria diet feeding until the end of the experiment,daily every other week (Simultaneous-Intermittent-Treatment-CAF; **SIT-CAF**), b) a dose of 500 mg GSPE/kg daily administered during the last 15 days of the cafeteria intervention as a corrective treatment (**CORR500-CAF**), or c) a dose of 100 mg GSPE/kg daily administered during the last 15 days of the cafeteria intervention as a corrective treatment (**CORR100-CAF**).

Figure 1. Schematic diagram of the experimental design.

For the administration, at 17:00h food was removed and at 18:00h GSPE dissolved in water was orally gavaged in a volume of 500 µL. The animals not supplemented with GSPE received water as a vehicle. At 19:00 food was replaced.

The dose of 500 mg GSPE/kg was chosen due to its effects at modulation of enteroendocrine system observed after acute treatments and in standard-fed

rats[18, 19]. This dose corresponds to 81 mg/kg bw in adult humans, when considering the body surface area according to Reagan-Shaw et al. [20]. This is a dose achievable through supplements. The dose 100 mg GSPE/kg corresponds to 81 mg/kg bw in adult humans [20]. The GSPE administration in the SIT-CAF group, was performed every other week so animals could recover from the daily oral gavage, as well as to reduce the total administered dose.

2.4 Blood and tissue collection

At the end of the study, the animals were fasted for 1-4 hours, anaesthetized with sodic pentobarbital (70 mg/kg body weight; Fagron Iberica, Barcelona, Spain) and exsanguinated from the abdominal aorta. The blood was collected using lithium heparin (Deltalab, Barcelona, Spain) as an anticoagulant. The blood was collected and aliquoted. Samples to measure GLP-1 were treated with a commercial DPP4 inhibitor (DPPIV, Millipore, Madrid, Spain) and a serine protease inhibitor (Pefabloc SC, Roche, Barcelona, Spain). The samples to be analysed for active ghrelin were treated with the serine protease inhibitor and 0.1 M HCl. All the samples were stored at −80ºC. Plasma was obtained by centrifugation (1500g, 15 minutes, 4°C) and stored at −80°C until analysis. The caecum was quickly weighed before and after caecal content removal. Intestinal segments were measured. The caecal content together with stomach and intestinal segments from the duodenum, jejunum, ileum and proximal colon were immediately frozen in liquid nitrogen and then stored at -80º C for further analysis.

2.5 Ussing chamber methodology

Intestinal segments of 0.28 cm^2 were mounted in Ussing chamber apparatus (Dipl.-Ing. K. Mussler Scientific Instruments, Aachen, Germany). Up to 6 segments from each animal were used. Mucosal compartments were filled with 1.5 ml KRB buffer (with D-Mannitol 10 mM) and the serosal compartments filled with KRB buffer (with

D-Glucose 10 mM) [21]. The chambers were kept at 37 °C and continuously oxygenated, 95% O_2 /5% CO_2, with a circular gas flow. Before starting the measurements, tissues were equilibrated for 15 min in the chambers to achieve steady-state conditions in transepithelial potential differences.

The transmucosal potential difference was continuously monitored under open circuit conditions and recorded through 0.8 mm Ag/AgCl glass electrodes. The basal transepithelial electrical resistance (RT) was calculated according to Ohm's law from the voltage deflections induced by bipolar constant current pulses of 50 mA (every 60 s) with a duration of 200 ms applied through platinum wires (Mussler Scientific Instruments, Aachen, Germany).

After the the equilibration period, measurements of the basal secretion of enterohormones of intestinal segments obtained from the treated animals were performed.

2.6 Plasma and tissue hormone analysis

Enterohormones were analysed using commercial ELISA kits for insulin, glucagon (Mercodia, Uppsala, Sweden), GLP-1 7-37 amide (Millipore, Billerica, MA, USA), desulfated CCK8 (Peninsula Laboratories, San Carlos, CA, USA), PYY (Phoenix Pharmaceuticals, Burlingame, CA, USA), amylin (islet amyloid polypeptide, Cloud-Clone, Katy, TX, USA) and specific octanoyl ghrelin (Phoenix Pharmaceuticals, Burlingame, CA, USA).

2.7 mRNA analysis

Total RNA was extracted using Trizol (Ambion, USA) and trichloromethane-ethanol (Panreac, Barcelona, Spain), and purified using a Qiagen RNAeasy kit (Qiagen, Hilden, Germany). The cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA). Real time PCR amplification

was performed using specific TaqMan probes (Applied Biosystems, Waltham, USA): Rn01460420_g1 for PYY, Rn00563215_m1 for CCK, Rn00562293_m1 for proglucagon and Rn00572319_m1 for ghrelin. The relative amount of mRNA transcripts was calculated against the control group using the $2^{-\Delta\Delta Ct}$ method, with cyclophilin A, PPIA (Rn00690933_m1), as reference.

2.8 Short chain fatty acid quantification

The concentration of short chain fatty acid (SCFA) (formic, acetic, propionic, butyric, isovaleric, valeric, lactic and succinic) were assayed in cecal content thawed at 4ºC. Briefly, approximately 1 g of sample was added to a screw cap glass tube containing 1 ml ultrapure deionized water (1:1, w/w), and was vortexed vigorously. 100 microliters of the internal standard 4-methyl-valeric acid, 2 ml of ethylic ether and 0.5 ml of HCl 37% were added to this samples, and in parallel to 1 ml a reference solution containing different concentrations of the standard SCFA. The tube was vortex mixed for 1 min and centrifuged for 15 minutes at 3500 g. 65 µl of supernatant was used for the derivatization step, performed with 10 µl of MTBSTFA ((N-methyl-N (tert-butyldimethylsilyl) -trifluoroacetamide), Aldrich 375934) at 80ºC for 30 minutes. Derivatized SCFAs were analysed using gas chromatography (Agilent 6890-NT, Santa Clara, USA,) coupled with a 30 m × 0.25 mm i.d., column, with a film thickness of 0.25 micras (Agilent DB-23, Barcelona, Spain) and a flame ionization detector (FID) to determine SCFA concentrations [22]. The carrier gas used was helium. A constant flow mode was used (split 25:1; 30 ml/min split flow). The column temperature was programmed to gradually increase from 60°C to 220°C during the analysis. In addition, injector port and FID temperatures were fixed at 250°C. Injection volume was set to 1 µl, and analyses were performed in duplicate [23].

2.9 Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). A Student's T-test was used to compare the treatments with the cafeteria control. P-values < 0.05 were considered to be statistically significant. These calculations were performed using XL-Stat 2017 software.

The role of plasma enterohormones in food intake and body weight was assessed by multivariate linear regression analysis for each treatment group. The analyses were performed with all the available data, including the most scattered, to preserve the original correlation of variables in each animal. Multiple linear regression models were constructed with up to 4 predictors and followed a stepwise backwards elimination of the nonsignificant variables ($p>0.05$). The most significant model is presented for each dependent variable, regardless of its R2 value. The statistical analyses were performed using XL-Stat 2017. P-values < 0.05 were considered statistically significant.

3. Results

3.1 GSPE limits ghrelin secretion in the stomach

The effects of the different treatments with GSPE on the enterohormone profile was studied throughout the gastrointestinal tract. As a first site of action, ghrelin was measured in the stomach. 500 mg/kg bw of GSPE administered simultaneously and intermittently with the cafeteria diet every other week (SIT-CAF) significantly increased the amount of ghrelin in tissue (Table1). This seemed not to be due to an increased production or increased activation (mRNA of ghrelin O-acyltransferase (GOAT) was significantly reduced in SIT-CAF) (Table1). A corrective treatment led to a higher increase in ghrelin (doubling the effect of SIT-CAF, $p\leq0.05$), either with 500 mg/kg (CORR500-CAF) but also at a lower dose of 100 mg/kg bw (CORR100-CAF) (Table 1). In this case the ghrelin-activating gene (GOAT) mRNA was not reduced as

in SIT-CAF. This accumulation in the cells was accompanied by a reduction in plasma levels (30-40%), although this was not statistically significant (Table 1). The effects of GSPE in the duodenum might not contribute to action on ghrelin since it was below detection limits in both amount and mRNA.

Table 1. **GSPE effects on ghrelin**.

	GHRELIN MRNA	GOAT MRNA	PG GHRELIN/µG TISSUE (STOMACH)	PG GHRELIN/ ML (PLASMA)
STD	0.74 ± 0.1 #	1.30 ± 0.17	1.90 ± 0.1	181.19± 21.10
CAF	1.09 ± 0.2	1.01 ± 0.06	2.01 ± 0.2	240.30 ± 72.94
SIT-CAF	1.44 ± 0.3	0.65 ± 0.06*	3.51 ± 0.5 *	162.38 ± 46.34
CORR500-CAF	1.38 ± 0.2	1.05 ± 0.08	6.76 ± 0.7 *	138.55 ± 31.75
CORR100-CAF	1.62 ± 0.5	0.96 ± 0.1	6.82 ± 1.3 *	NA

mRNA levels are relative to cafeteria controls (CAF). Values represent mean ± SEM of 5-7 animals per group. *$p \leq 0.05$ versus CAF; # $p \leq 0.1$ versus CAF. NA: not analysed.

3.2 In the duodenum GSPE limits basal CCK secretion

Duodenal sections of treated animals were mounted in Ussing chambers in order to analyse basal CCK secretion. Table 2 shows that the CAF group has an increased CCK secretion compared to standard-fed animals, and a reduced CCK tissue content. The SIT-CAF group had a reduced basolateral CCK levels compared to the CAF group, thus avoiding the stimulation of basal secretion exerted by the cafeteria diet. This reduced secreted CCK levels did not seem to reflect defects in CCK production, since mRNA levels or tissue content were not modified (Table 2). The same dose applied as a corrective treatment showed no significant effects on basolateral CCK secretion although tissue content was significantly increased compared to the cafeteria. A lower dose (CORR100-CAF) did reduce basolateral CCK levels compared to CAF (0.334 ± 0.01, $p \leq 0.05$). The effects were not translated to CCK plasma levels, where CCK values were not significantly modified by any of the GSPE treatments (Table 2),

while the cafeteria controls showed a tendency to have increased plasma CCK levels compared to the STD group.

Table 2. **Effects of GSPE treatments on CCK.**

First column, mRNA levels relative to cafeteria controls (CAF), in the duodenum. Second column, CCK levels found in the basolateral media of duodenum segments mounted in Ussing chambers. Third column, CCK content in duodenum samples obtained at sacrifice. Last column, plasma CCK concentration at sacrifice. Values represent mean ± SEM of 5-7 animals per group. *p≤0.05 versus CAF; # p≤0.1 versus CAF.

3.3 Modulation of GLP-1 and PYY by GSPE treatments

Total GLP-1 and PYY were measured in the ileum and the colon in all the treatments. A corrective treatment with 500 mgGSPE /kg bw significantly decreased PYY gene mRNA in the ileum, opposite to the effects of the cafeteria diet that tended to increase it (Table 3). However, this treatment increased the mRNA of PYY and GLP-1 in the colon, while no stimulation was observed in PYY secretion of samples mounted in Ussing chambers or plasma levels at sacrifice. When the same dose of GSPE was administered in a simultaneous intermittent way for 17 weeks, the effects in the colon were somehow maintained, but they were different in the ileum, where PYY was not changed and GLP-1 tended to increase. When the corrective treatments were performed with a lower dose, a different profile was observed, since the mRNA increase in PYY and GLP-1 was found in the ileum, but GLP-1 in the colon was maintained at control levels. In this case, however, total plasma GLP-1 was significantly increased and PYY decreased by the CORR100-CAF treatment (Table 3). Also, the corrective treatments showed a tendency to oppose the cafeteria diet PYY

secretion profile in ex vivo samples, although only tendencies were observed (Table 3).

Table 3. Effects of GSPE treatments on GLP-1 and PYY.

mRNA results are relative to cafeteria controls (CAF). PYY secretion in basal medium of intestinal samples mounted in Ussing chambers, in the ileum and the colon. GLP-1 in basolateral medium was not detected. *p≤0.05 versus CAF, # p≤0.1 versus CAF, T-test, mean ± SEM of 5-7 animals per group.

3.4 GSPE effects on microbiota metabolites

A simultaneous intermittent GSPE treatment increased colon size compared to the cafeteria-fed animals (15.42 ± 0.4 and 17.00 ± 0.6 cm in CAF and SIT-CAF groups respectively, $p<0.05$), while the corrective treatments did not modify it. No differences in small intestine size were observed in any group. Despite the fact that the cafeteria-fed animals showed no statistically significant differences in the small or large intestine when compared to the standard-fed animals, the small intestine/colon length ratio was significantly increased by the cafeteria diet and normalized by simultaneous intermittent GSPE treatment (Figure 2.a).

The caecum was weighed and Figure 2.b shows that the empty caecum weight was significantly increased by GSPE when this was administered concomitantly with the cafeteria diet, every two weeks for the 17 weeks' duration of the experiment. Interestingly, a corrective treatment of 10 days with GSPE at the same dose at the end of the experiment induced a significant increase in caecum weight, not different from that of the simultaneous intermittent treatment. The corrective treatment with a lower dose, however, did not modify caecum weight.

Figure 2. Effects of the different GSPE treatments on a) the length of the intestine (ratio between small intestine and colon length, measured in cm, and b) the weight of the empty caecum. STD: standard chow-fed controls, CAF: cafeteria-fed controls, SIT-CAF: rats fed cafeteria diet plus GSPE (500 mg/kg bw) every other week during 17 weeks, CORR500-CAF: 500 mg GSPE/kg daily administered during the last 15 days of cafeteria, CORR100-CAF: 100 mg GSPE/kg daily administered during the last 15 days of cafeteria. Values represent mean ± SEM of 6-10 animals per group. *p≤0.05 versus CAF, T-test.

Short chain fatty acids of caecal content were measured. As can be seen in Table 1 supplementary materials, the cafeteria diet significantly reduced butyric acid content by 73% and tended to increase propionic and isobutyric acid content. The CORR500-GSPE treatment significantly reduced the butyric acid content compared to cafeteria-fed rats (10.5 ± 1.4 and 19.07 ± 3.6 in CORR500-GSPE and CAF, respectively, $p<0.05$). The simultaneous intermittent treatment led to butyric acid levels not different from the corrective treatment (12.21 ± 3.0), despite values that were not statistically different from the cafeteria values. However, if considering the % of butyric acid in the caecal content (% of the sum of all other measured SCFA),

then simultaneous intermittent and corrective treatments led to a reduction in its % (30.15 ± 0.9, 12.22 ± 1.5, 7.81 ± 1.2, 4.80 ± 0.2, 7.61± 0.9 in STD, CAF, SIT, CORR500, and CORR 100 respectively, all of them p≤0.05 versus CAF).

3.5 GSPE corrective treatment reduces energy intake. Relationship between food intake and hormones

We finally tried to define the relationship between the enterohormones and body weight and food intake. The effects on body weight and adiposity of different treatments with GSPE 500 mg/kg have been previously published [15]. We observed no significant effect on body weight or food intake of the 100 mg /kg BW dose (CORR100-CAF), and therefore this treatment was not included in the further analysis. 20 hours' energy intake was measured at different time points. When administered as corrective treatment, i.e. after 15 weeks of cafereria feeding, 500 mg GSPE/kg bw reduced energy intake. No other effects on food intake were found in the other treatments (Figure 3).

Figure 3. **Effects of the different GSPE treatments on relative food intake between week 15 (before the corrective treatment) and week 17 (after the corrective treatment) in animals fed a cafeteria diet for 17 weeks.** STD: standard chow-fed controls, CAF: cafeteria-fed controls, CORR500-CAF: 500 mg GSPE/kg daily administered during the last 15 days of cafeteria, CORR100-CAF: 100 mg GSPE/kg daily administered during the last 15 days of cafeteria. Values represent mean ± SEM of 5-9 animals per group. *p ≤0.05 versus CAF, T-test.

In order to evaluate whether the effect of GSPE on food intake and/or body weight could be related to modulation of plasma hormones, we analysed the multivariate linear regression between hormones (Tables 1-3 and supplementary materials Table 2) and food intake or body weight gain. Table 4a shows that 95 % of body weight gain in the standard-fed animals was statistically explained by glucagon and insulin. Administration of a cafeteria diet modulated this relationship, since ghrelin, in addition to glucagon and insulin, was contributing towards explaining the body weight gain. GSPE totally changed this assotiation in different ways depending on the treatment: the SIT-CAF administration led to GLP-1 and CCK explaining the body weight gain, while in the CORR500-CAF none of the assayed hormones were significantly related to the body weight changes (Table 4a and Figure 4).

A

B

Figure 4. Multivariate linear regressions after a simultaneous-intermittent treatment and a corrective treatment of 500 mg/bw grape seed proanthocyanidin extract (GSPE). Table a) Plasma

levels of amylin, glucagon, insulin, GLP-1, CCK and ghrelin were measured using ELISA kits to determine their fit with the multivariate linear regression of $Y = aX1 + bX2 + cX3 + d$, where a) Y is the animal's body weight increase, and b) Y is the animal's relative food intake between week 15 (before the corrective treatment) and week 17 (after the corrective treatment) in animals fed a cafeteria diet for 17 weeks. p-value ≤0.05 indicates statistically significant regression. Non-significant regressions are expressed as NS. Adjusted R2 identifies the degree of explanation between related parameters. **Figure b)** Diagram of modeled relationships between hormones and body weight or food intake. Solid lines represent a positive contribution to the dependent variable, while dashed lines represent a negative contribution. Numbers within brackets correspond to beta coefficient.

As regards food intake (Table 4b and Figure 4), in cafeteria-fed animals glucagon and amylin were the most significant variables included in the model that toghether were strongly related to food intake, while in the standard-fed rats, PYY, in addition to amylin and glucagon, contributed to explaining 97% of food intake variability. GSPE again changed this relationship, but this time it was the in the CORR500-CAF treatment were 99% of the food intake variability could be explained by plasma hormones (i.e. total GLP-1 and amilyn). In the SIT-CAF treatment no significant relationship was found.

4.Discussion

In the present paper we describe that an intermittent GSPE treatment alters the intestinal enteroendocrine system in cafeteria-fed rats. Interestingly, several parameters that are modified by the simultaneous intermittent treatment are also altered in a corrective manner, suggesting that the effects on the gastrointestinal tract do not require long-term treatments, although their possible translation to body weight homeostasis modulation does require a more prolonged treatment.

We find changes in stomach acylated ghrelin content, suggesting an accumulation of ghrelin not due to increased production. Our resuts show that only 15 days are needed to exert such an effect and that, when a simultaneous intermittent treatment is performed, the effects are still found but to a lower extent. Ghrelin has previously been shown to be a target for GSPE [24]. In fact, in standard diet-fed animals, an 8-day treatment with the same GSPE dose (500 mg/kg bw) led to a lower

plasma ghrelin due to a reduced production in the stomach and secretion in the intestine, in fasted animals. Our present results suggest that a reduction in basal secretion by subchronic treatment with GSPE in the stomach also takes place in animals on an obesogenic diet. Furthermore, it is not a dose-dependent effect, since 100 mg/kg bw exerts the same effect as 500 mg/kg. This effect is mitigated when the treatment is prolonged intermittently for 17 weeks, possibly due to a long-term effect of GSPE on the regulation of the ghrelin-activating enzyme (GOAT). In the present sacrifice conditions, with only 4 hours of fasting, we found no significant change in ghrelin plasma levels, possibly because it usually peaks right before meal initiation. However, our multivariate linear regression analysis shows that plasma ghrelin levels contribute significantly to explaining the body weight increase in cafeteria-fed control animals, while it does not in GSPE-treated animals. This supports the idea that GSPE-modulation of the ghrelin system is involved in its metabolic effect controlling body weight gain. In fact ghrelin has been shown to act centrally, modulating not only food intake but also energy homeostasis, since when administered to different parts of the brain it increases food intake but also reduces energy expenditure and increases RQ [25, 26]. Thus the modulation of ghrelin levels might be related to the previously observed effects of GSPE in promoting the use of lipids as substrate [15].

Multivariate linear regression analysis considering body weight gain and hormones revealed two more enterohormones with distinct patterns between the cafeteria diet and the SIT-CAF GSPE treatment, namely CCK and GLP-1. Our results show that in the duodenum, a simultaneous intermittent treatment with GSPE leads to lower basal secretion of CCK compared to the cafeteria-fed animals, which might not be linked to a lower production, counteracting the effects of the cafeteria diet. A role for GSPE acutely inhibiting CCK secretion in explants (Serrano, Casanova-Martí, et al., 2016) and *in vivo* [19] has been previously shown. However, it is unlikely that the

present reduction in secretion is related to acute effects, since the last GSPE dose was administered 36 h previous to sacrifice and the tissues were thoroughly washed. CCK is released after stimulus such as aminoacids, protein hydrolyzates or long chain fatty acids, although in our system there was no such stimulus. Instead we measured basal CCK secretion, although for the moment we do not know which mechanism is involved in it. Thus a better understanding of the basal secretory mechanisms and the pathways by which cafeteria diet modifies them will help in elucidating the role of GSPE in this.

As mentioned earlier, in addition to CCK, plasma total GLP-1 contributed to explaining the effects on body weight of an intermittent treatment with GSPE, while it did not in any other condition. GLP-1 was also found among the hormones that were related to the food intake of CORR500-CAF, but not in the other conditions including the controls. The role of GLP-1 as a mediator of GSPE's acute inhibition of food intake has previously been demonstrated [27], and we have previously shown that GSPE stimulates GLP-1 secretion in a glucose dependent manner [28]. We found that, as previously shown for animals on a standard diet sub-chronically (8 days) treated with GSPE [19], a sub-chronic GSPE treatment also has satiating properties in rats that have been subjected to an obesogenic (cafeteria) diet. Furthermore, we found that when a prolonged treatment is performed, the effects on food intake are lost, and GLP-1 is no longer involved in regulating the food intake in the SIT-CAF treatment. However, GLP-1 contributes to explaining the body weight gain of these animals. GLP-1 has a well-described role as an anorexigenic and insulin-stimulating hormone, but it has recently been shown that GLP-1R signalling modulates energy expenditure rather than eating behaviour during HFD feeding [29]. Our results suggest that, similarly to what happened with ghrelin, the prolonged effects of GSPE involve the metabolic action of the enterohormone, regardless of its orexigenic/anorexigenic role.

Concerning the mode of modulation of GLP-1, our results point to GSPE acting through regulation of its gene expression. It has previously been shown that GSPE acutely modulates GLP-1 mRNA depending on the feeding state [28], and studies have reported an increase in intestinal GLP-1 gene expression after a chronic GSPE treatment with a lower (25 mg/kg bw) dose in rats fed a cafeteria diet [12]. Here we confirm that GSPE modulates intestinal GLP-1 mRNA depending on the treatment and tissue. The differential effects found on GLP-1 mRNA between the ileum and the colon might also derive from the different molecules that reach these tissues, since the microbiota in the caecum and the colon contributes greatly to the metabolism of ingested structures. We found that 500 mg/kg bw increased the caecum empty weight, and this was maintained when the treatment was prolonged intermittently. Caecum enlargement has previously been shown by treatment with prebiotic fibres, and it parallelled a reduction in adiposity in high-fat diet-fed [30] and standard-fed animals [31]. Gut hypertrophy and hyperplasia have been shown as an effect of fibre after only 8 days' treatment, attributed to the fermentation products of dietary fibre. Consumption of nopal (a prickly pear cactus with high fibre and polyphenol content) on an HF diet for 6 weeks results in an enlarged caecum and increased caecal SCFA (propionate, acetate, isobutyrate, isovalerate and valerate, but not butyrate) while at the same time it counteracts high-fat induced adiposity [32]. Modulation of caecum weight was accompanied by small changes in SCFA composition, where the GSPE treatments led to a reduced % of butyric acid. Butyric acid has been claimed beneficial effects on intestinal homeostasis and energy metabolism, although its role in obesity remains controversial (reviewed in [33]). Our results do not support that modulation of SCFA are directly involved in the antiobesity effects of GSPE,but these reflect changes in the microbiota that take place after only 15 days of GSPE treatment, as previously observed in standard-fed animals after 8 days of GSPE treatment [34], and that might be maintained after a simultaneous intermittent

treatment. These changes may contribute to the increased colon length that we found in the simultaneous intermittent treatment.

Finally we also found that the mRNA of PYY partially parallelled that of GLP-1, since it was up-regulated in colon or ileum depending on the treatment. However regardless of whether there is more PYY available in the cells, our results also show that its secretion is dependent on stimulus, since basal secretion from ex-vivo explants was not significantly altered.

To conclude, we show here that GSPE treatment in the context of an obesity-inducing cafeteria diet modulates the enterohormone system throughout the gastrointestinal tract. Our results suggest that 15 days with GSPE treatment modulates ghrelin accumulation in the stomach, CCK secretion in the duodenum and GLP-1 and PYY mRNA in the ileum or the colon (depending on the dose). It also increases caecal hypertrophy and reduces butyrate content. These effects are maintained if the treatment is performed every other fortnight during 17 weeks, and then lead to an increase in colon size. These intestinal effects might be related to reduced adiposity, likely through mechanisms other than only modulating food intake.

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Authors declare no conflict of interest.

Author contributions

I.G. Formal analysis, Writing – original draft, Methodology; K.G-C. Methodology; X.T. Assistance with statistical analysis and experimental design; MT. B. Funding acquisition; AM. P.-V. Methodology; M. P. Supervision, Writing – original draft; A. A. Supervision, Funding acquisition, Writing – review & editing.

Supplementary materials

Table 1. Caecal short chain fatty acids.

Values represent mean ± SEM of 6-10 animals per group. *p≤0.05 versus CAF; # p≤0.1 versus CAF. Lactic acid was also measured but not found.

Table 2. Plasma hormone levels at sacrifice.

	GLUCAGON (PM)	INSULIN (PG/ML)	AMYLIN (PG/ML)
ST	7.24 ± 2.28	3.91 ± 0.63	8.79 ± 1.10 #
CAF	9.81 ± 1.43	4.48 ± 0.33	12.62 ± 2.08
SIT-CAF	5.52 ± 1.03 *	3.71 ± 0.90	9.28 ± 1.05
CORR500-CAF	8.73 ± 1.54	6.18 ± 0.51 *	12.74 ± 2.25
CORR 100	15.63 ± 2.29 #	6.36 ± 1.10	12.21 ± 0.22

Values represent mean ± SEM. * p<0.05 vs standard, # p≤0.1 vs cafeteria, T-Test.

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UNIVERSITAT ROVIRA I VIRGILI
BIOACTIVITY OF FLAVANOLS ON THE MUCOSA OF THE INTESTINAL WALL: ENTEROENDOCRINE EFFECTS FOR PREVENTING DIET-INDUCED OBESITY AND ASSOCIATED PATHOLOGIES
Iris Ginés Mir

Manuscript 2

Objective

Long-lasting effects of GSPE on ileal GLP-1 gene expression are associated to a hypomethylation of the GLP-1 promoter

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Long-lasting effects of GSPE on ileal GLP-1 gene expression are associated to a hypomethylation of the GLP-1 promoter

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Keywords: flavanols; proanthocyanidins; enterohormone; epigenetics; intestine; GLP-1

Abstract

A grape seed proanthocyanidin extract (GSPE) presents long-lasting effects, reducing body weight gain and increasing lipid oxidation in cafeteria-diet-fed animals. It has also shown to modulate the enteroendocrine system. In this paper we determine the role of several GSPE treatments, previously shown to alter body weight gain and/or respiratory quotient in cafeteria-diet-fed rats, in the gene expression of several enterohormones and to ascertain whether they are modulated by epigenetic mechanisms. We found that 10-day GSPE administration prior to administration of the cafeteria diet (pre-treatment) led to upregulation of GLP-1 mRNA in the ileum 17 weeks after the GSPE treatment that was associated with hypomethylation on the GLP-1 promoter. These effects were also found when GSPE treatment was maintained as simultaneous-intermittent treatment (administered every other week) during the 17 weeks of cafeteria-diet treatment. Also, the hypomethylation of the GLP-1 promoter correlated positively with body weight, respiratory quotient and plasma insulin. In the colon, GSPE had no effect on gene expression after pre-treatment. On the other hand, GSPE administered at the end of the cafeteria diet upregulated PYY and GLP-1 mRNA, though it was not regulated by either the hypomethylation of the promoters or the acetylation of H3K9 and tri-methylation of H3K27 histones on the GLP-1 promoter. In conclusion, we have identified long-lasting effects of GSPE on GLP-1 gene expression in the ileum. These were partly mediated by a reduction in methylation at the gene promoter, which in turn was associated with changes in body weight, energy expenditure and plasma insulin.

1. Introduction

Obesity is one of the most prevalent diseases that affects people all over the world. The development of obesity is affected by many factors, including alterations on the energy balance, genetic predisposition, gut microbiota disorders, imbalances in the oxidative stress-antioxidant defence, environmental factors, endocrine imbalances, etc., all of which can lead to metabolic and epigenetic alterations [1,2]. One widely studied approach to treating the metabolic disorders caused by obesity is to use natural bioactive compounds. Flavonoids in particular have been reported to act against obesity by modulating many metabolic pathways [3,4]. They have been proved to act as lipolytic agents, to limit white adipose formation, to activate energy consuming pathways [5], and to act in the gastrointestinal (GI) tract [6].

Since flavonoids are poorly absorbed, they have plenty of opportunity to exert their effects on the GI tract. It has been shown that flavanols limit energy absorption by influencing the intestinal processes involved in the digestion and absorption of energy compounds [5,7–10], they modulate inflammation and barrier properties [11], they can alter the bacterial populations in the gut [12], and they can also induce a satiety effect by modulating gut hormones [13,14]. Serrano et al. showed that a subchronic dose of 500 mg/kg bw of a grape-seed proanthocyanidin extract (GSPE) was able to decrease food intake [15], modify enteroendocrine hormone secretions [16] and decrease gastric emptying in female rats, thus inducing a satiating effect [13]. All these effects can be explained by a variety of mechanisms; the antioxidant antioxidant properties of flavanols [17–19], their interaction with proteins [20,21], the fact that they modify mRNA expression [16,22,23] and the epigenetic mechanisms modulated by flavonoids [24–27].

DNA methylation, the most studied epigenetic mechanism, takes place when a methyl group is added to the C5 position of cytosine (5mC), predominantly at CpG

sites [28]. The enzymes responsible are the DNA methyltransferases (DNMTs) [29]. It has been suggested that flavonoids reverse DNA hypermethylation, and that this is mediated by the inhibition of DNMT [24]. Flavonoids are known to inhibit histone acetyltransferase and histone deacetylate so, therefore, they can also interfere in histone remodelling [25]. Histone modifications are associated with changes in the structure of chromatin. Acetylation/deacetylation and methylation/demethylation correlate with chromatin accessibility, and the effect of transcription and methylation depends on the number of methyl groups and the position of the residues [30]. Some studies have focused on the study of the role that flavonoids play in modulating epigenetic processes (for example, DNA methylation and histone modification) to control obesity [26,27]. Boqué et al. showed that an apple polyphenol extract can induce epigenetic changes in the adipose tissue, which could explain some of the anti-obesogenic effects observed [27].

Recent studies by our research group have shown that GSPE presents long-lasting effects. Several weeks after the last dose of GSPE, body weight, adiposity and RQ remain lower [31]. Here we analyse the long-lasting effects on intestinal gene expression and whether the epigenetic mechanism is part of the explanation and compare the different GSPE treatments.

2. Materials & Methods

2.1 Proanthocyanidin extract

The grape seed proanthocyanidin extract (GSPE) was kindly provided by Les Dérivés Résiniques et Terpéniques (Dax, France). According to the manufacturer, the GSPE composition used in this study contains (Batch number: 124029): monomers of flavan-3-ols (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5–13 units; 31.7%) of proanthocyanidins. A detailed analysis of the monomeric to trimeric structures can be found in the study by Margalef and col [32].

2.2 Animal experiments

Female rats weighing 240–270g were purchased from Charles River Laboratories (Barcelona, Spain). After one week of adaptation, they 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 (Panlab 04, Barcelona, Spain) and tap water. The rats were randomly distributed into the experimental groups (n=7–10/group) and were fed *ad libitum* a standard chow diet until the end of the experiment. The control group (STD) received only the standard chow diet. The other groups, in addition to the standard chow, received a cafeteria diet as a model of a high fat/high sucrose diet and/or a GSPE supplement at different times. The cafeteria group (CAF) received tap water as vehicle together with the cafeteria diet. The preventive treatment group (PRE) received an oral dose of 500 mg GSPE/Kg for 10 days before they started the cafeteria diet. The simultaneous intermittent treatment-CAF (SIT) group received a dose of 500 mg GSPE/Kg together with the cafeteria diet every other week, and the corrective treatment (CORR) group received a dose of 500 mg GSPE/Kg during the last 15 days of the long-term cafeteria intervention.

The cafeteria diet consisted of bacon, sausages, biscuits with paté, carrots, muffins, and sugared milk, which induced voluntary hyperphagia [31]. This diet was offered *ad libitum* every day to the animals for 17 weeks. GSPE was dissolved in water and was orally gavaged to the animals at 18:00 for each treatment in a volume of 500 µL, one hour after all the available food had been removed. The animals not supplemented with GSPE received water as a vehicle.

At the end of the study, animals were fasted for 1–4 hours, anesthetized with sodic pentobarbital (70 mg/kg body weight; Fagron Iberica, Barcelona, Spain) and exsanguinated from the abdominal aorta. Intestinal segments from the duodenum,

jejunum, ileum and proximal colon were immediately frozen in liquid nitrogen and then stored at -80º C for further analysis.

All the procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili (code: 0152S/4655/2015).

2.3 Quantitative real-time RT-PCR analysis

Total RNA was extracted using Trizol (Ambion, USA) and trichloromethane-ethanol (Panreac, Barcelona, Spain), and purified using a Qiagen RNAeasy kit (Qiagen, Hilden, Germany). The cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA). Quantitative PCR amplification was performed using specific TaqMan probes (Applied Biosystems, Waltham, USA): Rn00572200_m1 for ChGA, Rn01460420_g1 for PYY and Rn00562293_m1 for proglucagon. The relative expression of each gene was compared with the control group using the $2^{-\Delta\Delta Ct}$ method, with cyclophilin A, PPIA (Rn00690933_m1), as reference.

2.4 DNA methylation analysis by pyrosequencing

Genomic DNA was extracted from the ileum using TRIzol Reagent (Life Technologies) and from the colon using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). It underwent bisulfite modifications using a commercially available modification kit (Zymo Research, Irvine, CA, USA).

DNA methylation was assessed by pyrosequencing. Bisulfite-treated DNA was amplified by PyroMark PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The polymerase chain reaction conditions were as follows: 95°C for 15 min, followed by 45 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s and, finally, 72°C for 10 min. Polymerase chain reaction products were verified by agarose electrophoresis. Pyrosequencing methylation analysis was conducted using

the PyroMark Q24 (Qiagen). The level of methylation was analysed using PyroMark Q24 Software (Qiagen), which calculates the methylation percentage mC/(mC+C) (where mC is methylated cytosine and C is unmethylated cytosine) for each CpG site, and allows quantitative comparisons. The primer set sequences used for pyrosequencing were the ones that presented most CpG islands in the maximum number of pair-bases permitted by the PyroMark Q24 machine (see Table 1).

Table 1. Primer sets used for pyrosequencing.

rat GLP-1	Forward	5'-GTTGAGGGGGAGTTTGGA-3'
	Reverse	5'-ACCCCAAAAATAAAACCTCCAACTCTA-3'
	Sequencing	5'-GGGAGGAGGGTTTTAATG-3'
rat PYY	Forward	5'-GGAATGATTTGGTATTGTGATGT-3'
	Reverse	5'-TCACCTCAAATAAACCCTACCC-3'
	Sequencing	5'-GATGTTTTGTGGGGA-3'
rat ChGA	Forward	5'-GGGATTTAGAAGGTGGGGAAAGG-3'
	Reverse	5'-CAACAACCCCCAAACAATACTATACCCTC-3'
	Sequencing	5'-AAGGTGGGGAAAGGG-3'

2.5 Chromatin immunoprecipitation (ChIP)

The ileum and colon tissue samples used for chromatin preparation were dissected on a frozen surface. The samples were then immediately fixed for 12 min in cold 1% formaldehyde/PBS, followed by glycine incubation to stop further cross-linking. The fixed tissue was washed five times with cold PBS containing Na-Butyrate 20 μM and frozen for later use. ChIP experiments were performed as previously described [33]

with some modifications; tissue samples were homogenized in a nuclear extraction buffer (10 mM Tris; pH 8.0, 100 mM NaCl, 2 mM MgCl2, 0.3 M Sucrose, 0.25% IGEPAL CA-630) containing protease inhibitors (1 mM PMSF, 0.1 mM aprotinin, 1:100 Protease Inhibitor Cocktail (Sigma-Aldarich, Madrid, Spain)), by douncing 25 times using a 2 ml loose grind pestle followed by a 5-minute incubation on ice. The homogenate was dounced another 25 times using a 2 ml loose grind pestle for nuclear release, followed by 10 min centrifugation at 2400 g to pellet nuclei. The nuclei were then lysed in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% (wt/vol) SDS and protease/phosphatase inhibitors, diluted in RIPA buffer [10 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% (vol/vol) Triton-X-100, 0.1% (wt/vol) SDS, 0.1% (wt/vol) sodium deoxycholate] and the DNA was sonicated to an average size of 300e500 bp using a sonicator (Vibra Cell; 3 cycles 30 s ON, 30 s OFF, 40 Hz). A total of 3 μg of anti-H3K27me3 (Cell Signaling) and 3 μg of anti-H3K9ac (Cell Signalling, Massachusetts, USA) were incubated at 4C with 25 ml of washed Dynabeads protein A (Invitrogen, California, USA) and RIPA in a total volume of 100 ml. The bead-antibody complexes were then incubated at 4ºC for 2 h with 42 μl of chromatin in a total volume of 250 μl. Beads were washed three times in RIPA, once in high salt wash buffer [20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 2 mM EDTA, 0.1% Triton-X-100, 0.1% SDS] and once in TE buffer. After washes, DNA was eluted from beads and de-crosslinked in 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM NaCl, 1% (wt/vol) SDS and 50 mg/ml protease K at 68ºC overnight. For input, 42 μl chromatin was de-crosslinked in 20 mM TriseHCl, pH 7.5, 5 mM EDTA, 50 mM NaCl at 68ºC overnight. ChIP and input DNA were then purified and eluted using NucleoSpin Gen and PCR Clean-up kit (MACHEREY-NAGEL, Düren, Germany). Enrichments on selected loci were measured by qPCR in triplicates relative to input chromatin. The primer sequences used were the ones shown in Table 1.

2.6 Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). A Student's T-test was used to compare the treatments with the cafeteria group. *P*-values < 0.05 were considered to be statistically significant. These calculations were performed using XL-Stat 2017 software.

Spearman's correlation coefficient was used to test for correlations between the methylation of the GLP-1 promoter and the respiratory quotient (RQ), the final weight, the adiposity index and the concentration of plasma insulin of the animals. *P*-values < 0.05 were considered to be statistically significant. These calculations were performed using XL-Stat 2017 software.

3 Results

3.1 GSPE has long-term effects on GLP-1 gene expression in the ileum but not in the colon

First, we checked whether a 10-day pre-treatment of 500 mg/bw GSPE followed by a 17 week-cafeteria diet (PRE) was able to induce changes in gut hormone gene expression. Figure 1 shows that in the ileum, GLP-1 gene expression was increased 17 weeks after the GSPE pre-treatment. This seems to be GLP-1 specific, since we checked PYY, a hormone usually co-expressed with GLP-1, and found no increase in its gene expression. Furthermore, ChGA, a marker of endocrine cells, was not increased but decreased by all GSPE treatments in the ileum.

In the colon, it seems that the effect of GSPE increasing GLP-1 gene expression is linked to the treatments in which the last dose was administered recently, as is the case of the SIT and CORR groups. It can be observed that GSPE has similar effects on both colonic PYY and GLP-1 gene expression, while ChGa showed no differences in any of the treatments.

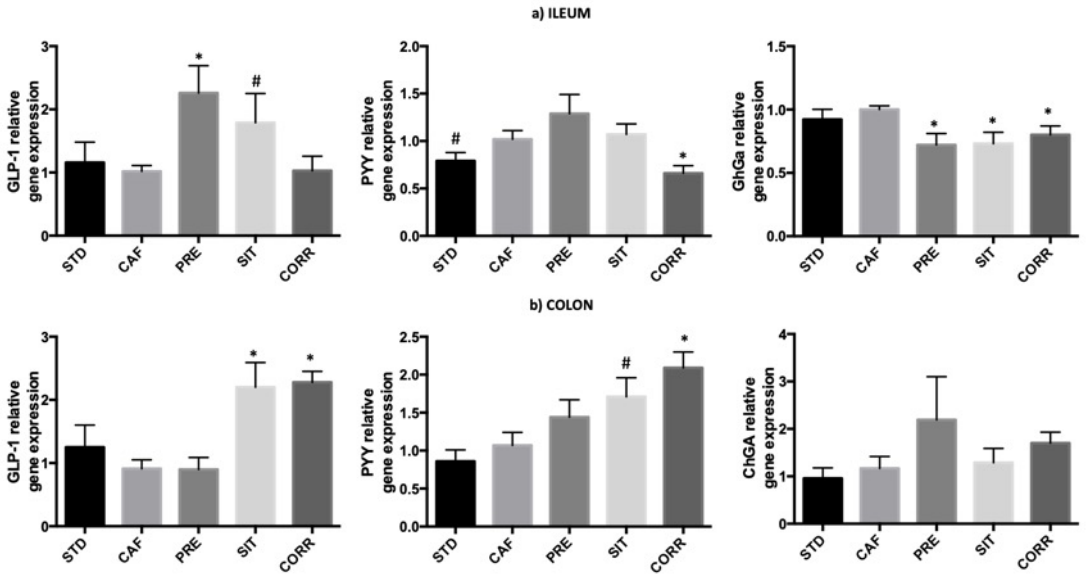

Fig 1. Effects of GSPE on GLP-1, PYY and ChGA gene expression in a) ileum and/or b) colon. * p<0.05, # p<0.1 vs CAF using T-test.

3.2 GSPE decreases the methylation of the GLP-1 promoter in the ileum

In order to see whether the long-lasting effects of GSPE on gene expression in the ileum were related to epigenetic mechanisms, the methylation of the CpG islands present in the GLP-1 promoter was studied by pyrosequencing analysis. In agreement with increased GLP-1 gene expression, figure 2a shows a decrease in the methylation of the promoter 17 weeks after GSPE treatment (PRE). Furthermore, methylation is also decreased by a 10-day GSPE treatment (CORR) and maintained when the treatment is performed synchronically during the 17 weeks of the cafeteria period (SIT) (Figure 2a). To determine whether the results of GLP-1 gene expression and the methylations of its promoter were strongly associated, we used Spearman's correlation test. As expected, the test showed a negative correlation between gene expression and positions 2 and 4, and the average of all the positions of CpG islands found (p=0.039, 0.046 and 0.038, respectively).

As happened with gene expression, these effects were GLP-1 specific, since PYY and ChGA promoters were also studied and no effects on average methylation of CpG sites were found in the various GSPE treatments (Figures 2b and 2c).

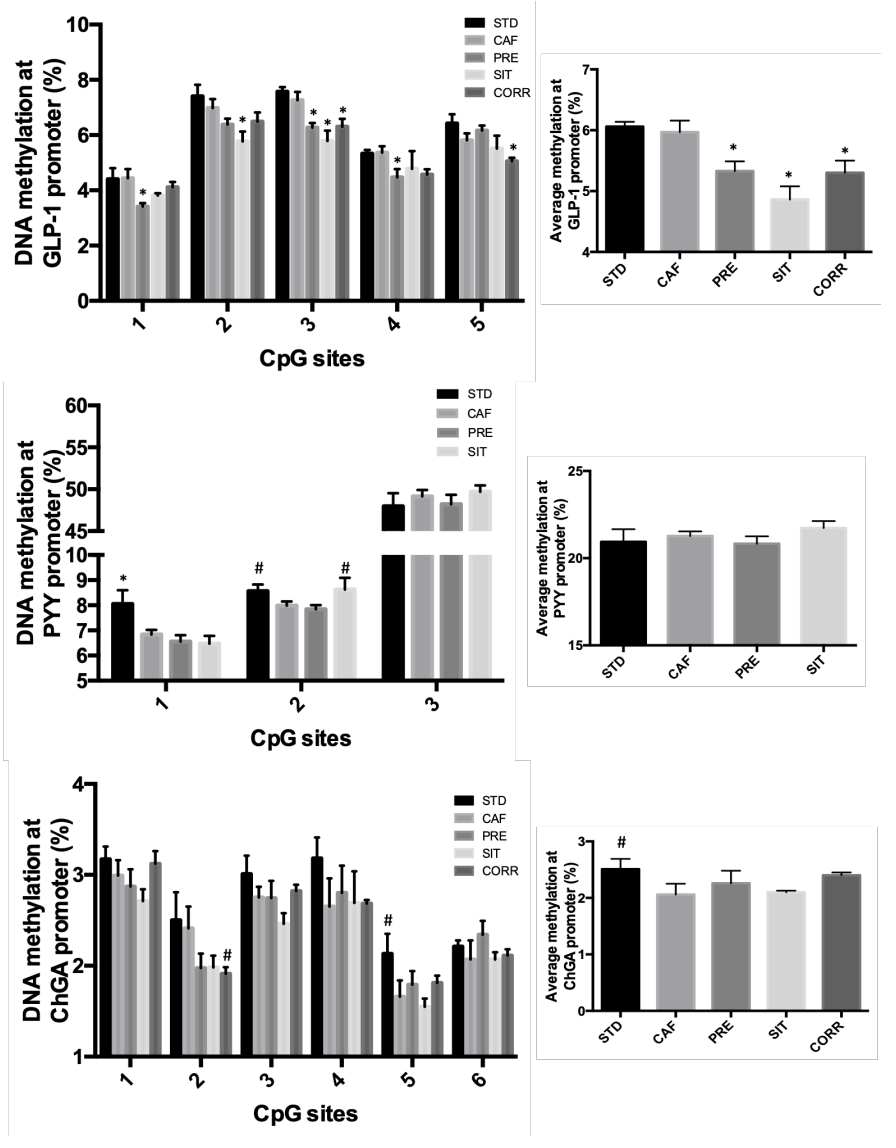

Fig 2. Effects of GSPE on DNA methylation of GLP-1, PYY and ChGA promoter in ileum. *p<0.05, # p<0.1 vs CAF using T-test.

Finally, we observed a tendency towards a lower average methylation in ChGA because of the cafeteria treatment. This was not accompanied by changes in the ChGA gene expression.

The methylation of the same hormone promoters was also analysed in the colon. In this case, the increased gene expression observed in SIT treatment was not related to the methylation pattern of the promoters, which was not modified (figures 3a and 3b). Neither did we find that the promoters had any long-lasting effects on methylation, in agreement with the profile of gene expression that was not modified in the PRE animals (figure 1).

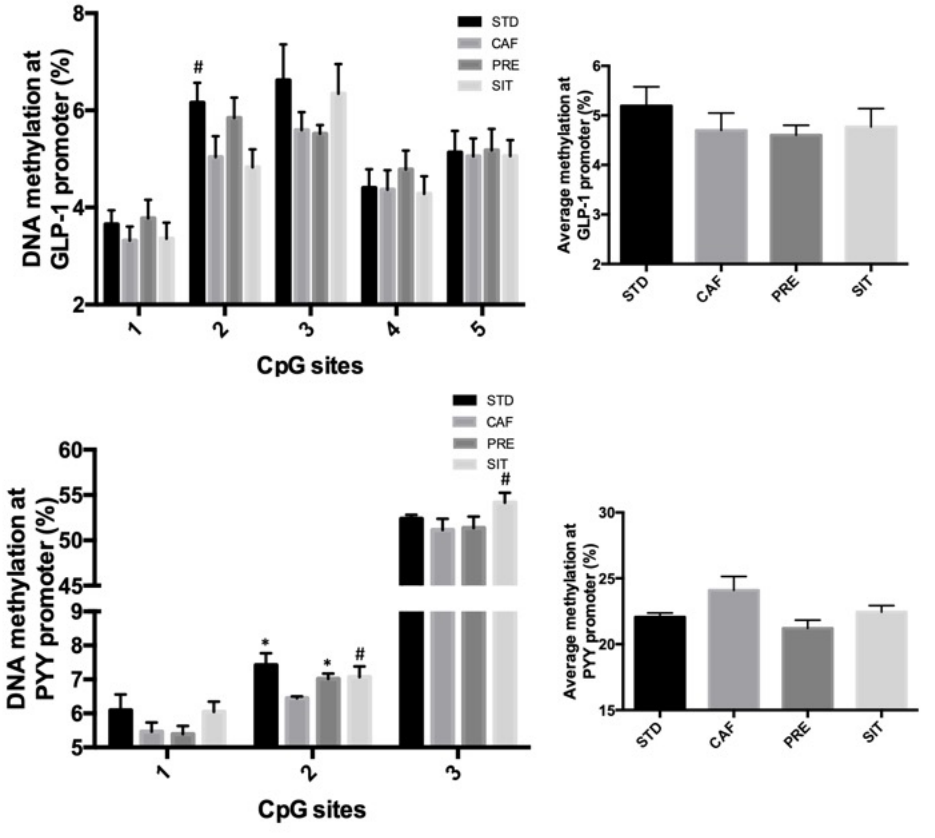

Fig. 3. Effects of GSPE on DNA methylation of GLP-1 and PYY promoter in colon. * p<0.05, # p<0.1 vs CAF using T-test.

3.3 GSPE does not modify the acetylation of H3K9 and tri-methylation of H3K27 at the GLP-1 promoter site in the colon

As the effects of GSPE in the colon were significantly different from their effects in the ileum, we tested whether the changes in GLP-1 gene expression observed in the SIT and CORR groups were mediated by another epigenetic mechanism: the modification of histones. We studied the acetylation of lysine 9 (AcH3K9) and the tri-methylation of lysine 27 (3meH3K27), both in histone number 3. Figure 4a shows that there are no differences in the H3K9 acetylation either due to GSPE or to the cafeteria diet. As far as the H3K27 tri-methylation is concerned, the only tendency observed is an increase in the methylation of the CORR group.

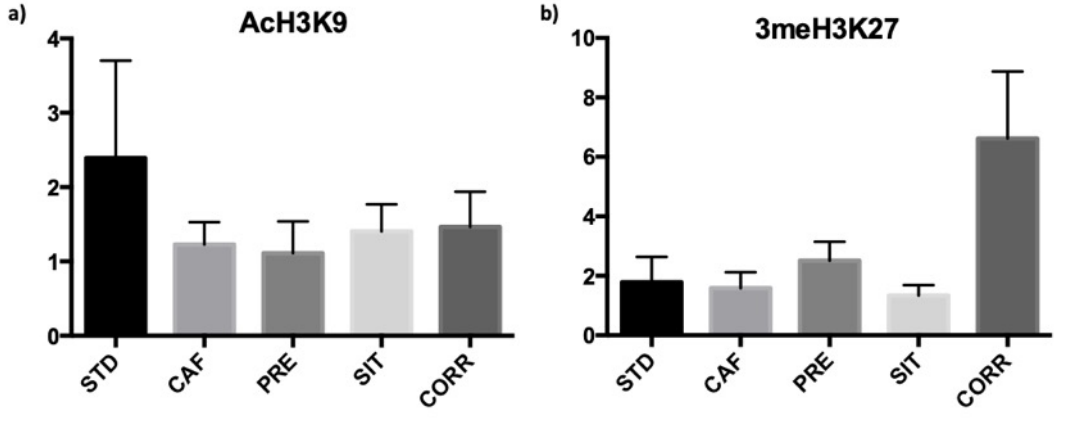

Fig. 4. GSPE-mediated acetylation of H3K9 and tri-methylation of H3K27 at the promoter site of GLP in colon. Signals from histone marks were normalized to the input using the same primers. The signals of all the groups were normalized to the STD group. * $p<0.05$, # $p<0.1$ vs CAF using T-test.

3.4 A reduction in GLP-1 promoter methylation by GSPE is related to decreased RQ, body weight and plasma insulin

To identify whether the methylations found in the GLP-1 promoter in the ileum were associated with metabolic and morphometric variables, previously shown to be modified by GSPE, we used Spearman's correlation test to evaluate all the groups. Figure 5 shows that the RQ of the animals was positively associated with the average of the methylated CpG sites found in the GLP-1 promoter. Moreover, final weight and plasma insulin are associated with positions 3 and 1 of the CpG sites methylated in the GLP-1 promoter, respectively.

Fig. 5. Plot of the significant associations between the methylation of GLP-1 promoter and the respiratory quotient (RQ), the final weight and the plasmatic concentration of insulin from the STD, CAF, PRE, SIT and CORR. Each plot presents each Spearman's rho correlation value and the corresponding p-value. *p*-values < 0.05 were considered statistically significant.

4. Discussion

GSPE has been shown to interact with the enteroendocrine system by reducing food intake in a GLP-1-dependent manner, and enhancing enterohormone secretion in rats [13,16]. In rats fed a cafeteria diet, an intermittent GSPE treatment every other week for 17 weeks leads to modifications in enterohormone gene expression (submitted results). This treatment has also been reported to counteract the effects

of the cafeteria diet on body weight, adiposity and RQ [31]. Interestingly, when GSPE was administered preventively, for only 10 days, before the cafeteria diet, some of its anti-obesogenic effects were maintained [31]. Here, we have evaluated the role of epigenetic mechanisms in modulating the enteroendocrine system by a dose of 500 mg/kg bw GSPE, depending on the moment of the dose and under a long-term cafeteria diet challenge.

First, we studied whether a 10-day pre-treatment with GSPE, followed by a 17–weeks cafeteria diet, modulated the intestinal gene expression of GLP-1, PYY and ChGa, and compared it with the effects of an intermittent treatment (every other week) in conjunction with the cafeteria diet and a 15-days corrective treatment, administered during the last two weeks of the cafeteria diet.

By pre-treating with GSPE, the expression of GLP-1 in the ileum was up-regulated 17 weeks after the last dose. This up-regulation was also observed when the treatment was given every other week during these 17 weeks. GSPE therefore increased GLP-1 gene expression through mechanisms that had long-lasting effects. As we did not find this effect in the corrective treatment, we hypothesize that it can only be noticed after the long-term period, at least under our study conditions. Since EECs can sense SCFA and stimulate the expression of GLP-1 and PYY in the distal small intestine and proximal colon [34,35], and GSPE pre-treatment did not induce any significant changes in SCFA composition (supplementary data), we discount microbiota changes induced by GSPE as an explanation for this effect. Nevertheless, the epigenetic modulation of DNA might be possible [36,37]. In the present study we show that a 10-days pre-treatment with GSPE modulates the DNA methylation of the GLP-1 promoter, which persists several weeks after GSPE treatment. This is consistent with the increased gene expression observed in PRE and SIT groups. However, the down-regulation observed in the CORR-CAF group is not consistent with epigenetic modulation, which suggests that different metabolic pathways might be involved

[38–41]. It has been reported that flavonoids modulate DNA methylation by attenuating the effect of DNMTs, thus inducing a reduction in overall DNA methylation [36,42–44]. Hence, we propose that GSPE exerts its long-lasting effect on GLP-1 gene expression at the beginning of the treatment and leaves a print that persists for 17 weeks. The exact mechanism of DNMT1 inhibition by flavonoids is still under study, but it may take place by direct enzyme inhibition, indirect enzyme inhibition, reduced DNMT1 expression and translation, interaction with methyl-CpG binding domain proteins, among others [42]. Bladè et al. report that proanthocyanins and their metabolites can induce different epigenetic modifications [45], which suggests that GSPE might be modifying other epigenetic marks that together could induce changes in chromatin organization and make some zones of the DNA more accessible to changes in methylation patterns. This might also explain the fact that only GLP-1 seems to be affected by epigenetics even though PYY and ChGa are also expressed in L-cells. Actually, the rapid replacement of the intestinal epithelium [46,47] complicates the presence of long-term changes, which suggests that these epigenetic changes take place in the stem cells located in the crypts. Subsequently, these will be differentiated into enteroendocrine L-cells and reach the brush border of the villus, where they exert their activity.

Our study of the methylation of the GLP-1 promoter also showed that the ileum and colon were affected differently by GSPE. While in the ileum a clear reduction in the methylation of the GLP-1 promoter can be observed, none of the treatments induced any change in the colon. This pattern has also been observed in other studies, where the terminal ileum presented different methylation patterns in the ascending and sigmoid colon [48,49]. The molecular mechanisms underlying the regional variations in methylation patterns along the GI tract are not understood but the molecules that reach the colon have frequently been metabolised by gut microbiota and, consequently, are different to those that reach the ileum [50,51]. Thus, our results

suggest that the GSPE molecules that induce the modulation of methylation patterns are not reaching the colon and, consequently, they cannot induce epigenetic changes. Nevertheless, we did find changes in PYY gene expression in the colon, similar to the ones found in GLP-1. However, in this case, it seems that, since the differences in gene expression are only observed in SIT and CORR treatments, the GSPE molecules together with the microbiota-metabolized metabolites that induce these changes act through other metabolic pathways that are not explained by changes in the methylation of the PYY promoter (submitted results).

Actually, our results also discounted some other epigenetic changes in the colon. Since GLP-1 mRNA was up-regulated in this segment, we checked if it could be mediated by epigenetic mechanisms other than DNA methylation. Dietary polyphenols have been reported to modulate transcription by altering the posttranslational modifications of histones. In this context, GSPE can increase the levels of histone acetyl-H3-Lys 9 (AcH3K9), by repressing histone deacetylases (HDAC) activity [36,37]. EGCG has also been found to decrease class I HDAC levels, thus increasing the acetylation of H3K9, and decreasing the repressive chromatin mark 3meH3K27 [52]. Other studies have also shown that EGCG inhibits 3meH3K27 [53]. However, the lack of changes in chromatin immunoprecipitation, with the exception of a tendency to increase in the CORR, does not correlate with the increase in gene expression observed in the SIT and CORR, since 3meH3K27 is known to be an epigenetic mark of repression of DNA transcription [54]. Thus, the present data shows that the expression of GLP-1 was not modulated by the chromatin remodelling markers AcH3K9 and 3meH3K27.

The effect of GLP-1 on energy expenditure has been widely studied, and a recent meta-analysis concludes that more studies are needed if GLP-1 is to be attributed a direct role in the reduction of energy expenditure [55]. Furthermore, if not directly, GLP-1 might exert its effect by increasing glucose-dependent insulin release in the

brain which, in turn, produces a net catabolic effect by reducing food intake and increasing energy expenditure [56]. In our case, it can be observed that the hypomethylation of the GLP-1 promoter is correlated with low insulin levels. If it is taken into account that the animals were sacrificed after 4 hours of fasting, high levels of fasting insulin would indicate insulin resistance [57], but this is not the case. Even though, insulin concentration was evaluated under fasting conditions, its effects on energy expenditure and body weight are not limited to this specific moment, but to the general insulin signalling that takes place throughout the day. Following the hypothesis that GLP-1 increases glucose-dependent insulin release, insulin might reach the brain where it triggers catabolic pathways that increase energy expenditure and reduce body weight [58]. This is reflected with the positive correlation between the methylation of the GLP-1 promoter and both RQ and body weight, in which the hypomethylation of the promoter is associated with low RQ values or, in other words, a higher oxidation of lipids, which in turn reduces body weight [59].

In summary, a 10-day pre-treatment with GSPE (500 mg/Kg bw) induces long-lasting effects on GLP-1 gene expression possibly driven by a reduction in DNA methylation at the gene promoter in the ileum. Moreover, the DNA methylation pattern of this promoter positively correlates with plasma insulin, energy expenditure and body weight.

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Competing financial interests.

The investigators have no conflict of interest relating to this study

Supplementary materials

Table 1 supplementary materials. Caecal short chain fatty acids.

Values represent mean ± SEM of 6-10 animals per group. *p≤0.05 versus CAF; # p≤0.1 versus CAF. Lactic acid was also measured but not found.

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Manuscript 3

Objective

Long-lasting effects of proanthocyanidins in rat prevent cafeteria diet effects by limiting adipose tissue accrual

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UNIVERSITAT ROVIRA I VIRGILI
BIOACTIVITY OF FLAVANOLS ON THE MUCOSA OF THE INTESTINAL WALL: ENTEROENDOCRINE EFFECTS FOR PREVENTING DIET-INDUCED OBESITY AND ASSOCIATED PATHOLOGIES
Iris Ginés Mir

Long-lasting effects of proanthocyanidins in rat prevent cafeteria diet effects by limiting adipose tissue accrual

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Keywords

Long-lasting effect; proanthocyanidin; rat; adiposity; LPL

Abstract

A dose of proanthocyanidins with satiating properties proved to be able to limit body weight increase several weeks after administration under exposure to a cafeteria diet. Here we describe the molecular targets and the duration of the effects. We treated rats with 500 mg GSPE/kg BW for ten days. Seven or seventeen weeks after the last GSPE dose, while animals were on cafeteria diet, we used RT-PCR to measure the mRNA of the key energy metabolism enzymes from liver, adipose depots and muscle. We found that a reduction in the expression of adipose LPL might explain the lower amount of adipose tissue in rats seven weeks after the last GSPE dose. Liver showed increased expression of CPT1a and HMGCS2 together with a reduction in FASn and DGAT2. In addition, fatty oxidation (OXCT1 and CPT1b mRNA) was increased in muscle. However, after seventeen weeks, there was a completely different gene expression pattern. As conclusion, seven weeks after the last GSPE administration there was a limitation in adipose accrual that might be mediated by an inhibition of the gene expression of the adipose tissue LPL. Concomitantly there was an increase in fatty acid oxidation in liver and muscle.

1. Introduction

Excessive adipose tissue significantly increases the risk and prognosis of metabolic syndrome (diabetes mellitus type 2, cardiovascular disease, hyperlipidemia, nonalcoholic fatty liver disease) and several types of cancer.[1] The causes of excessive body weight are diverse, one of the most prevalent in developing and developed countries being excessive or bad quality food intake[2] .

Proanthocyanidins (PACs) are a group of polyphenols that are widespread in nature (in fruits, vegetables and their beverage products). They have been described as bioactive agents against several unhealthy situations. More specifically, they have the well-documented effect of limiting lipid accumulation and favouring lipid oxidation in the organism[3] Their effect as specific inhibitors of fat digestion[4] and absorption.[5] Furthermore, PACs favour lower RQ [6],[7], due to a higher fat oxidation in liver and muscle[6]. As they are a group of different compounds, some of the effects could be explained by their interaction with molecules or structures located in the gastrointestinal lumen[8],[6]. They protect against cafeteria diet-induced damage to the intestinal barrier and are anti-inflammatory agents.[9] However, the absorbable low-molecular weight flavanols reach intracellular targets inside the body, where they act on different molecular targets to induce increased energy expenditure,[3] and prevent cholesterol increase in the organism[10], acting as antihipertensives[10], antioxidants[11]) and maintaining glucose homeostasis[12].

The diversity of structures in proanthocyanidin-rich extracts and their interactions are the reason why some of these effects are highly dependent on the dose used for the studies and the physiological state of the animal [7]. Most studies prove that PACs correct cafeteria diet-induced damage[13],[14]. Some studies focus on their possible preventive role in obesity-related pathologies (that is, when they are administered from the beginning of obesogenic diets) [15]. However, only very few studies have analysed their long-term effects after sub-chronic treatment[16], [17]. We have recently

attempted to determine the best moment to administer GSPE (500 mg/kg) so that it acts most effectively against the damaging effects of an obesogenic diet such as the cafeteria diet[17]. The results showed that PACs had a surprisingly long-lasting effect on body weight that needed a more in-depth analysis. In the present study, we have further analysed the long-lasting effects of sub-chronic GSPE treatment. We compare the duration of their effects, mainly on the energy metabolism and adipose depots, 7 weeks or 17 weeks after the last GSPE dose.

2. Materials & Methods

2.1 Proanthocyanidin extract

The grape seed extract enriched in proanthocyanidins (GSPE) was kindly provided by *Les Dérivés Résiniques et Terpéniques* (Dax, France). According to the manufacturer, the GSPE used in this study (Batch number: 124029) contains monomers of flavan-3-ols (21.3%), and dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5-13 units; 31.7%) of proanthocyanidins. A detailed analysis of the monomeric to trimeric structures can be found in the work by Margalef and col.[18]

2.2 Animal treatments

Female rats (Harlan Rcc:Han), each weighing 240-270 g, were purchased from Charles River Laboratories (Barcelona, Spain). After one week of adaptation, they were individually caged in the animal quarters at 22°C with a 12-hour light/12-hour dark cycle and fed *ad libitum* with a standard chow diet (Panlab 04, Barcelona, Spain) and tap water. Experiments were performed after a period of acclimation.

Short cafeteria (SC) experiment

The animals were randomly distributed into two experimental groups (n=7) and fed a standard chow diet *ad libitum* (figure 1, supplementary materials). One group of

animals received 500 mg GSPE/Kg bw together with a simplified high-fat-high-sucrose diet for 10 days. The diet consisted of a palatable hypercaloric emulsion presented in an independent bottle, containing (by weight) 10% powdered skimmed milk, 40% sucrose, 4% lard and 0.35% xanthan gum as a stabilizer.[19] The GSPE dissolved in tap water was orally gavaged to the animals at 18:00 in a volume of 500 µL, one hour after all the available food had been removed. The animals not supplemented with GSPE received water as a vehicle. After 10 days of treatment, all the animals were kept for 18 days on a standard chow diet. Afterwards, the rats started with **the cafeteria diet challenge for 35 days (SC)**. The cafeteria diet consisted of standard chow, bacon, carrots, and sugared milk, which induces voluntary hyperphagia.[20] This diet was offered *ad libitum* every day.

Long cafeteria (LC) experiment

A second group of thirty female Wistar rats was challenged with a long-term cafeteria treatment (LC), which was initially similar to the treatment described above. They were organized in three experimental groups (n=10), (figure 1, supplementary materials). One group was given the same amount of GSPE every day for 10 days at the same time, and the control group received the same amount of tap water. During the GSPE treatment period, all the rats were fed standard chow diet. On day eleven, a standard group (STD) stayed on the chow diet, and the two remaining groups started a cafeteria challenge, which in this case was maintained for 17 weeks.

In both experiments, the GSPE treatments were intragastrically (i.g.) administered 1 h before the onset of the dark cycle. Food intake was measured 20 hours after the daily chow replacement with an accuracy of 0.01 g.

2.3 Blood and Tissue Collection

At the end of the study, the rats were fasted for 1-4 hours, anesthetized with sodium pentobarbital (70 mg/kg body weight; Fagron Iberica, Barcelona, Spain) and exsanguinated from the abdominal aorta. The blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Plasma was obtained by centrifugation (1500g, 15 minutes, 4°C) and stored at −80°C until analysis. The different white adipose tissue depots (retroperitoneal (rWAT), mesenteric (mWAT) and periovaric (oWAT)), brown adipose tissue (BAT), liver and pancreas were rapidly removed and weighed.

All the procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili (code: 0152S/4655/2015).

2.4 Plasma metabolites and hormones

Plasma β-hydroxybutyrate was analysed by colorimetry (BEN, Mainz, Alemania). Total ghrelin from plasma samples was analysed with an extraction-free total ghrelin enzyme immunoassay (Phoenix Pharmaceuticals, Burlingame, USA). [21]. Plasma insulin and glucagon levels were analysed with rat ELISA kits (Mercodia, Sweeden). Plasma leptin levels were analysed with an ELISA kit (Millipore, Billerica, MA, USA).

2.5 Tissue triglycerides and mRNA quantification

Pancreatic triglycerides were assayed according to Castell et al.[22] Total RNA was extracted using Trizol (Ambion, USA) and trichloromethane-ethanol (Panreac, Barcelona, Spain) and purified using an RNA extraction kit (Qiagen, Hilden, Germany). Complementary DNA was obtained using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain), and the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) amplification was performed using TaqMan Universal PCR Master Mix and the respective specific TaqMan probes

(Applied Biosystems, Madrid, Spain). The relative expression of each mRNA was calculated against the control group using the $2^{-\Delta\Delta Ct}$ method, with cyclophilin A as reference.

2.6 Statistical analysis

The data are represented as the mean ± standard error of the mean (SEM). Statistical comparisons between groups were assessed by the T test. Analyses were performed with XLStat 2017.01 (Addinsoft, Spain). P-values <0.05 were considered statistically significant.

3. Results

3.1 Sub-chronic treatment with GSPE reduces food intake in rats on a palatable diet

In previous studies we used a GSPE dose that has satiating properties in animals on a chow diet.[7,23] Here, we reproduce this effect in animals with an enhanced appetite because they were offered a tasty diet. **Figure 1a** shows a 10% reduction in the total food intake of the healthy females while they were treated with GSPE. **Table 1** shows that this reduction was due to the amount of hypercaloric emulsion ingested.

Table 1. Characteristics of food intake during the short cafeteria study

		Cafeteria	GSPE pre-treated rats
Initial treatment: 10 days, tasty diet			
Chow ingested (g)		8.34 ± 0.38	9.10 ± 0.53
Hypercaloric emulsion ingested (g)		14.01 ± 0.82	10.54 ±1.07 *
Carbohydrates (Kcal)		43.04 ± 0.78	39.15 ± 0.60*
Lipids (Kcal)		6.84 ± 0.19	5.90 ± 0.21*
Protein (Kcal)		7.07 ± 0.17	7.12 ± 0.22
Final treatment: 35-day cafeteria diet			

Carbohydrates (Kcal)		43 ± 4	38 ± 2.0
Lipids (Kcal)		28 ± 1	28 ± 0.6
Protein (Kcal)		8.4 ± 0.3	8.0 ± 0.2

GSPE was administered for 10 days together with a tasty diet. After the GSPE treatment stopped, the rats were put on an 18-day chow diet and then a 35-day cafeteria diet. *: (P<0.05 vs C; T test)

The effects on food intake disappeared when GSPE administration finished, as previously shown.[7] **Figure 1b** shows that there was no difference between groups in the kilocalories (Kcal) ingested over the eighteen days after GSPE treatment, when animals received a standard chow diet. During this period, the rats obtained 68% of energy from CH, 12% from lipids and 20% from protein. When the animals were subsequently subjected to a short (35 days) cafeteria diet, the amount of Kcal ingested was not different between the groups either (f**igure 1c)**. During this last period of the study, animals obtained **5**4% ± 0.020 of energy from carbohydrates (CH) (mainly from the sucrose included in the milk), **36**% ± 0.02 from lipids and **11** % ± 0.003 from protein. As mentioned, these percentages were not statistically different for GSPE treated animals (51% ± 0.01; **38**% ± 0.009; **11**% ± 0.001, from CH, lipids and protein, respectively).

A

B

C

Figure 1. Food intake in the short-challenge group in different diet periods

Food intake was measured 20 hours after the daily food had been replaced for each diet administered. The black column indicates animals not treated with GSPE. The white column indicates animals treated with 0.5 g/Kg BW of GSPE for the first 10 days of treatment. The results showed the mean data obtained from each measurement throughout the period. **a)** Mean food intake from the daily measurement for the first ten days of treatment with a tasty diet. **b)** Mean food intake from measurements taken during the eighteen days of treatment with a chow diet. **c)** Mean food intake from measurements during the thirty-five days with a cafeteria diet. Statistical differences identified by T-test are defined by * when $p<0.05$ between treatments

3.2 A reduction in the expression of adipose LPL might explain the lower amount of adipose tissue in GSPE pre-treated rats

We have shown that a 10-day pre-treatment with GSPE followed by a cafeteria diet led to a reduction in adiposity and RQ[17] after 53 days. **Table 2** shows that in the GSPE pre-treated group there is a statistically significant reduction of around 35% in the size of subcutaneous depots (estimated by the difference between total adipose contents measured by RMN and the weighed intraabdominal depots). Although the mRNA levels of lipid metabolism genes did not help to explain it, there was a

statistically significant effect on the ratio between CPT1b vs FASn, suggesting a trend towards a higher oxidative profile in the subcutaneous depot. The next most abundant WAT depot is the periovaric WAT, which did not show any differences between the control and GSPE groups in either weight or gene expression. Instead, the retroperitoneal and mesenteric depots were of statistically different sizes due to the GSPE treatment (reductions of 23 and 35%, respectively). However, there were no significant changes in the gene expression profile, only a tendency to present lower FASn mRNA levels in mesenteric WAT (**table 2**).

Table 2. GSPE effects on white adipose depots in the short cafeteria treatment

	Cafeteria	GSPE pre-treated rats
Subcutaneous WAT		
Size of depot (g) (estimated)	*37.23 ± 3.78*	*24.05 ± 3.07**
CPT1b	1.03 ± 0.09	1.09 ± 0.12
LIPE	0.94 ± 0.09	0.70 ± 0.05$^{\#}$
FASn	1.03 ± 0.09	0.74 ± 0.18
DGAT2	0.98 ± 0.08	0.90 ± 0.08
CPT1b/FASn	0.93 ± 0.06	1.40 ± 0.15*
Periovaric WAT		
Size of depot (g)	*17.25 ± 1.05*	*15.21 ± 0.64*
CPT1b	1.01 ± 0.07	1.17 ± 0.05
Retroperitoneal WAT		
Size of depot (g)	*12.74 ± 0.90*	*9.81 ± 0.63**
CPT1b	1.15 ± 0.20	1.27 ± 0.29
FASn	1.00 ± 0.08	0.95 ± 0.12
DGAT2	1.00 ± 0.05	0.99 ± 0.09
CPT1b/FASn	1.26 ± 0.24	1.56 ± 0.41
Mesenteric WAT		

Size of depot (g)	*9.28 ± 0.98*	*5.96 ± 0.62**
CPT1b	1.05 ± 0.08	0.97 ± 0.1
FASn	0.95 ± 0.13	0.62 ± 0.1 #
DGAT2	1.01 ± 0.09	0.78 ± 0.16
CPT1b/FASn	1.22 ± 0.27	1.50 ± 0.18

Adipose depots were obtained at the end of the treatment and each depot was weighed. RT-PCR was used for each gene (results are presented as arbitrary units versus cafeteria group). The T test was used to determine statistical differences highlighted as *p<0.05 vs cafeteria treatment; #p<0.1 vs cafeteria group.

Brown adipose tissue was also analysed but there were no changes due to GSPE either in weight (0.91 ± 0.07 for the cafeteria group; 0.77 ± 0.04 for the GSPE-pre-treated group) or in CPT1b gene expression (1.02 ± 0.0 for the cafeteria group; 1.15 ± 0.2 for the GSPE-pre-treated group) suggesting a lack of effect on oxidative activity in this tissue.

Next, to identify the effects of GSPE on triglyceride entry into adipose tissue, we measured the gene expression of the genes related to this process: LPL, the enzyme that hydrolyses triglycerides into fatty acids and glycerol, before their uptake into the cell; CD36, involved in free fatty acid uptake into the cell; and AQP7, which facilitates the efflux of glycerol from the cell. **Figure 2** shows that the amount of LPL in the mesenteric depot was highly reduced, as was the amount of Aquaporin 7. There were no statistically significant differences for the fatty acid transporter (CD36).

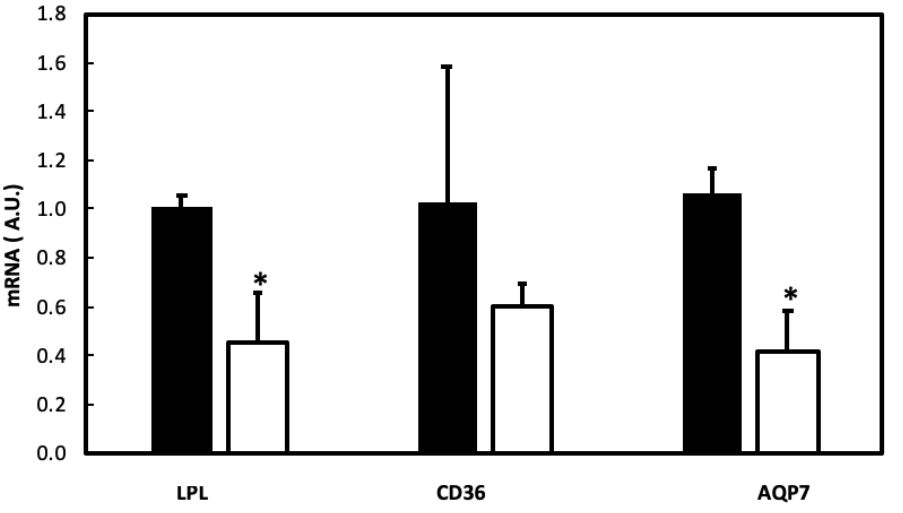

Figure 2. Effect of GSPE pre-treatment on mesenteric adipose gene expression in the short-challenge group at the end of the study. Rats were treated with 0.5 g/Kg BW for the first 10 days, and then they were put on a chow diet for 18 days and a cafeteria diet for 35 days. The black column indicates animals not treated with GSPE. The white column indicates animals treated with 0.5 g/Kg BW of GSPE for the first 10 days of treatment. The mRNA extracted from mesenteric adipose was quantified by RT-PCR and the relative gene expression of LPL, CD36 and AQP7 was obtained by the DDCt method in each gene. The data are the mean ± SEM (n=7). Statistical differences identified by T-test are defined by * when $p<0.05$ between treatments.

3.3 Lipid oxidation in liver and muscle is higher

In our search for an explanation for the lower RQ observed in GSPE pre-treated rats,[17] we analysed liver and muscle gene expressions. **Figure 3a** shows a significant increase in CPT1a and HMGCS2, suggesting the increased oxidation of fatty acids and the active synthesis of ketone bodies in the liver of GSPE-pre-treated animals. In addition, decreased FASn and DGAT 2 expression suggested decreased synthesis and esterification of fatty acids.

Plasma ketone bodies were not significantly modified (control: **4.14** ± 0.35; GSPE: **3.89** ± 0.56; mM), so we measured the extent to which they could be oxidized by muscle**.** **Figure 3b** shows a strong significant increase in OXCT1 due to GSPE treatment, concomitantly with a tendency to increased Cpt1b, which suggests that ketone bodies and fatty acids are the energy source in the muscle.

A

B

Figure 3. Effect of GSPE pre-treatment on gene expression in the short-challenge group at the end of the study. Rats were treated with 0.5 g/Kg BW for the first 10 days, and then they were put on a chow diet for 18 days and a cafeteria diet for 35 days. The black column indicates animals not treated with GSPE. The white column indicates animals treated with 0.5 g/Kg BW of GSPE for the first 10 days of treatment. The mRNA extracted from liver was quantified by RT-PCR and the relative gene expression detailed gens was obtained by the DDCt method in each gene. Figure 3a resumes liver results. Figure 3b summarizes muscle gene expression. The data are the mean ± SEM (n=7). Statistical differences identified by T-test are defined by * when $p<0.05$ between treatments.

3.4 Hormonal status of GSPE-treated rats after the short-cafeteria study

We next analysed the effects of GSPE on key hormones for the regulation of energetic homeostasis 7 weeks after finishing the GSPE treatment.

In pancreas, GSPE pre-treatment did not change insulin mRNA levels (**1.16** ± 0.25 in controls; **0.66** ± 0.11 in the GSPE pre-treated group; A.U.) but showed a tendency towards a lower glucagon mRNA (**1.16** ± 0.23; **0.60** ± 0.14; A. U.; p= 0.06). The amount of triglycerides in pancreas was not modified in GSPE pre-treated rats (**29.66** ± 2.21 in the control group and **27.10** ± 4.21 in the GSPE pre-treated group- μg TG/ mg tissue).

GSPE pre-treatment statistically increased the plasma levels of total ghrelin (ng/mL; control: **4.09** ± 0.15; GSPE: **6.10** ± 0.42; p<0.05), although stomach mRNA levels of this hormone were not modified by GSPE pre-treatment (control: **1.04** ± 0.16; GSPE: **1.05** ± 0.12). In addition, GSPE pre-treatment led to a trend towards lower leptinemia (ng/mL; control: **28.0** ± 4.04; GSPE: **18.02** ± 2.15; p=0.07).

3.5 Duration of GSPE effects

Finally, to estimate the duration of some of the effects described above, we analysed several parameters after a longer (17 weeks) cafeteria challenge.

The gene expression profile in liver differed considerably from that found in the short-challenge study (**table 3**). GSPE pre-treatment led to an increase in FASn and DGAT2 and a decrease in CPT1a compared to the cafeteria treatment. This profile resembled that of the standard group of animals.

The insulin/glucagon ratio of plasma levels in the GSPE pre-treated animals differed significantly from that of the cafeteria-fed animals, and produced a relationship closer to that of the standard-fed group (Chow: 0.86 ± 0.14*; cafeteria: 0.39 ± 0.10; GSPE: 0.95 ± 0.19*; *: p<0.05 vs cafeteria group).

Table 3. Effects of GSPE on liver seventeen weeks after treatment (long cafeteria study)

	Chow diet	Cafeteria diet	GSPE pre-treated rats
CPT1a (A.U.)	0.34 ± 0.12*	1.01 ± 0.08	0.35 ± 0.09*
FASn (A.U.)	4.24 ± 0.80*	0.92 ± 0.18	2.69 ± 0.87#
DGAT2 (A.U.)	1.23 ± 0.10	1.04 ± 0.12	1.36 ± 0.07*

Liver samples were obtained at the end of the treatment. RT-PCR was used for each gene. The T test was used to determine statistical differences highlighted as *p<0.05 vs Cafeteria treatment; #: p<0.1 vs cafeteria group.

4. Discussion

Grape-seed derived proanthocyanidins have been extensively studied, but few studies use doses that are higher than can be provided by standard food ingestion but which may be interesting for a potential functional food. We showed that a dose of 0.5 g GSPE/kg bw has satiating properties in healthy rats[7] and limits adipose accumulation induced by a cafeteria diet.[17] We have also shown that GSPE maintains some of its antiobesogenic effects for a long period after GSPE administration finishes. In the present study, we analysed the mechanisms that explain this. We show that GSPE limits adipose fat pad accumulation until seven weeks after the last GSPE administration due to an inhibition in the adipose tissue LPL. An increase in fatty acid oxidation in liver and muscle compensates for the inability of fatty acids to accumulate in WAT.

First, we show that GSPE also inhibits food intake if the diet is a tasty one (energy dense). During the ten-day GSPE treatment, animals reduced energy intake by 10% in comparison to the control group. Furthermore, these rats gained 30% less weight than the control (cafeteria) group.[17] These results confirm that GSPE effects on food intake are additive to slimming effects because the lipid oxidation of GSPE is

activated.[7] In our short-term experiment, after GSPE intake, animals changed to a chow diet, and showed no changes in body weight accrual. Unexpectedly, when the rats were again fed a cafeteria diet (still without GSPE), the differences in body weight reappeared. These differences were around 40% between GSPE pre-treated animals and the control group, which correlate with the lower adiposity (79%),[17] and there were no differences in either the quantity or quality of food intake. Our results, therefore, show that GSPE only has long-lasting anti-obesity effects under exposure to a high fat and/or a high sucrose diet.

A key element in triglyceride food-derived storage is the adipose lipoprotein lipase (LPL). GSPE pre-treatment limited the amount of LPL mRNA in mesenteric adipose tissue, which suggested an impairment of triglyceride storage in this tissue. LPL has been shown to be a target for GSPE by Del Bas and col.[24] They showed that five hours after an acute dose of 250 mg GSPE/kg bw there was a reduction in adipose LPL mRNA. The results of the study by Yoon and col are more similar to ours.[25] They showed that *Allomyrina dichotoma* larvae treatment had a considerable effect on LPL mRNA because it limits adipose tissue growth in mice fed a high-fat diet. However, adipose LPL limitation by itself is not sufficient to explain all GSPE effects. Weinstock and col, working with LPL knockout mice that maintain LPL expression only in muscle, showed no changes in the various adipose depots or in total lipid content.[26] This suggests that in our study, GSPE might also act on other targets in the body.

If triglycerides cannot be stored in WAT after GSPE treatment, they might be derived to other organs. One of these organs is the liver, where GSPE pre-treatment directed fatty acids towards β-oxidation, as we found increased expression of CPT1a concomitantly with lower esterification (DGAT2). CPT1a was also found to be up-regulated after two acute doses of 250 mg GSPE/kg bw[27] in chow-fed rats but not after a subchronic treatment for 10 days with 25 mg/kg bw[5] in 13-week cafeteria-fed

rats. Baselga and col[28] found a similar trend to ours in FAS and CPT1a mRNA levels after three weeks with a dose of 25 mg/kg bw in rats that had previously been on a cafeteria diet for 10 weeks. The main difference between our results and Baselga's is that that they found that GSPE decreased liver triglycerides but we did not. On the contrary, our results show that triglycerides increased in the liver of the GSPE pre-treated rats.[29] However, when we analyse the long cafeteria challenge (17 weeks) the triglyceride content in the GSPE pre-treated group did not differ from content in the vehicle-treated group, which suggests a limited trend toward their accumulation in liver. Yang-Xue and col,[30] working with partially KO mice for LPL, showed a strong LPL mRNA inhibition in the youngest animals that was partially reduced with aging. These animals also showed an increased deposition of triglycerides in the liver in adulthood due to the KO gene that reverted the aging period. We worked with different treatments, different species and different durations, but we noticed a changing relationship between GSPE pre-treated rats and the amount of liver triglycerides, which suggests that time affects the accrual of triglycerides in the liver.

In the short-cafeteria study, GSPE pre-treated rats sent more triglycerides to the liver, oxidized fatty acids and produced ketone bodies, which were then removed by muscle. In fact, del Bas and col also showed an increase in the mRNA of muscle LPL,[24] which suggests a derivation of fatty acids from adipose tissue to muscle that we cannot ignore. Similarly, fatty-acid uptake and oxidation were also found to be activated in muscle (higher mRNA CPT1b, LPL and CD36) in males on a 10-week cafeteria diet that subsequently received 21 days of 25 mg GSPE/kg BW.[6] Besides, the dose of GSPE does not seem to have a critical effect on muscle, as Crescenti and col found an overexpression of genes related to fatty acid uptake (Fatp1 and CD36) and b-oxidation in the skeletal muscle of STD-GSPE offsprings.[16] So the higher oxidation of fatty acids in liver and muscle explain the lower RQ found in the GSPE pre-treated rats, a common trait of several GSPE treatments.[3]

It is important to point out that the metabolic changes remain for some considerable time after GSPE treatment. There may be several explanations for this. On the one hand, GSPE is an extract composed of absorbable compounds and non-absorbable structures. Absorbable compounds can reach the various tissues assayed [31],[32] and non-absorbable structures can interact with intestinal sensors[33]. Through their interaction with microbiota, they can produce new compounds that can reach different targets in the body[34],[35]. Thus, we cannot discount that there might be flavonoids remaining in the tissues. However, previous studies with a lower dose (100 mg GSPE/kg bw) administered for a longer time (12 weeks) suggested that flavonoids do not accumulate in liver or mesenteric adipose tissue.[36] Another explanation might be that some epigenetic activity is taking place in the target tissues. GSPE modified liver miR-33a and miR-122[37] at doses as low as 5 mg/kg BW for 3 weeks after a 15-week cafeteria diet. Similarly, Milenkovinc and col[38] found changes in hepatic miRNA working with doses of proanthocyanidins closer to 5 mg/kg BW for two weeks. GSPE activity on histone deacetylases was proved by Downing and col[39], who showed that GSPE regulates liver HDAC and Pparα activities to modulate lipid catabolism and reduce serum triglycerides *in vivo*. Similarly, Bladé and col proved that PACs modulate hepatic class III HDACs, which are often called sirtuins (SIRT1-7), in a dose-dependent manner. This was associated with significant protection against hepatic triglyceride and cholesterol accumulation in healthy rats.[10] All this evidence, in conjunction with our findings on the regulation of gene expression in liver 7 weeks after GSPE treatment, suggests an epigenetic modelling of hepatic functioning by GSPE. The duration of these effects after GSPE administration is not clear. Crescenti and col showed effects in offspring 24 weeks after GSPE had been administered to their mothers during pregnancy.[40] We observe that seventeen weeks after GSPE treatment CTP1a, FASn and DGAT2 expression in the liver changed profile compared to 7 weeks after GSPE, which suggests that hepatic epigenetic regulation had come to an end. Instead, at this time point, the

changes in liver clearly agree with the insulin/glucagon ratio modulation by GSPE. In fact, we showed that GSPE (45 days with 25 mg GSPE per kg of body weight) modulates pancreatic miRNAs.[41] miR-483, which we showed is down-regulated by GSPE treatment in rat pancreatic islets, has been related to the optimum equilibrium between β-cells and α-cells (that is, its upregulation leads to increased insulin production and decreased glucagon synthesis).[42] Therefore, our results also point to epigenetic changes in pancreas, which will need to be addressed in future work.

In conclusion, a short-term pre-treatment with GSPE repressed adipose LPL and activated fatty oxidation in the liver. In conjunction with a greater utilization of ketone bodies in muscle, this would help to prevent an increase in body weight caused by a long-term high-fat diet after the end of treatment.

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Competing financial interests

The investigators have no conflict of interest relating to this study

Supplementary material

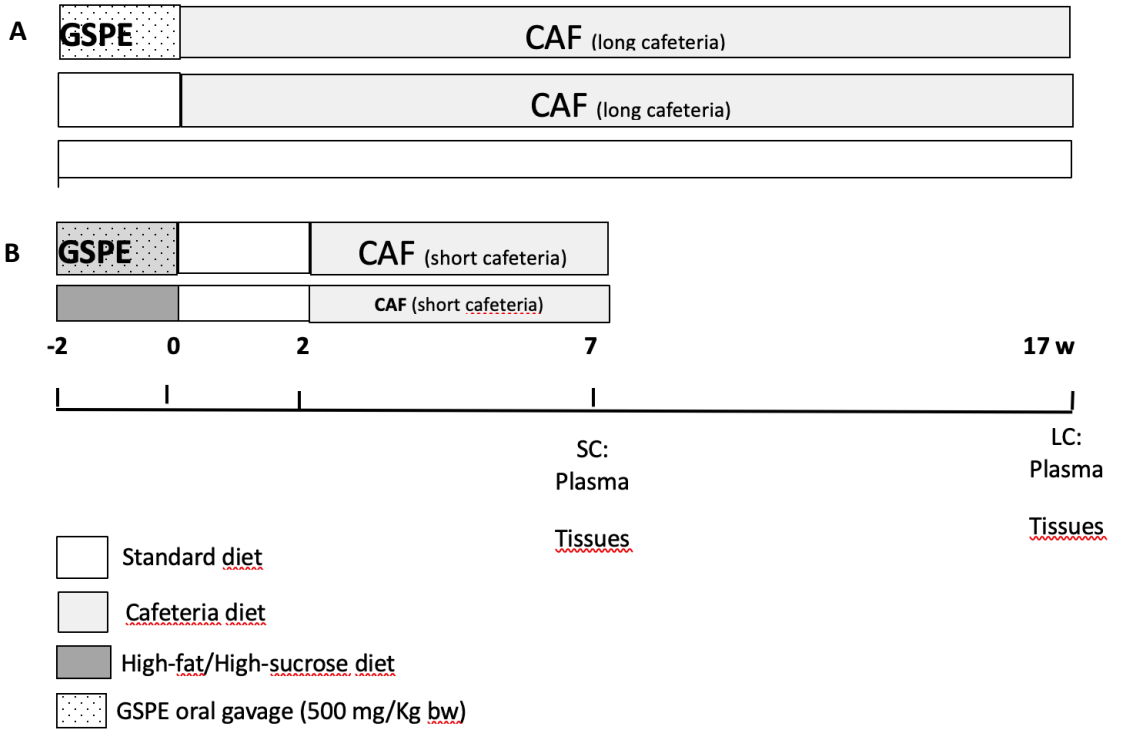

Figure 1. Schematic diagram of the experimental design. **(a)** CAF-LC: rats receiving a GSPE preventive treatment 10 days before the 17-week cafeteria intervention; **(b)** CAF-SC: rats receiving a GSPE preventive treatment for 10 days together with a high fat/high sucrose diet followed by an 18-day chow diet (standard diet) and then the 35-day cafeteria diet; GSPE: grape seed proanthocyanidin extract

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Objective

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Novel ex Vivo Experimental Setup to Assay the Vectorial Transepithelial Enteroendocrine Secretions of Different Intestinal Segments

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BIOACTIVITY OF FLAVANOLS ON THE MUCOSA OF THE INTESTINAL WALL: ENTEROENDOCRINE EFFECTS FOR PREVENTING DIET-INDUCED OBESITY AND ASSOCIATED PATHOLOGIES
Iris Ginés Mir

A novel *ex vivo* experimental setup to investigate how food components stimulate the enteroendocrine secretions of different intestinal segments

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Abstract

The enteroendocrine system coordinates gastrointestinal (GI) tract functionality and the whole organism. However, the scarcity of enteroendocrine cells and their scattered distribution make them difficult to study. Here, we glued segments of the GI wall of pigs to a silicon tube, ensuring that the apical and the basolateral sides were kept separate. The fact that there was less than 1% of 70-kDa fluorescein isothiocyanate (FITC)-dextran on the basolateral side proved that the gluing was efficient and the lactate dehydrogenase leakage lower than 0.1% proved that the tissue was viable. The intestinal barrier function was maintained as it is in segments mounted in Ussing chambers. Finally, apical treatments with a polyphenol-rich extract or an animal derived extract produced differential basolateral enterohormone secretions. In conclusion, we report an *ex vivo* system called "Ap-to-Bas" for assaying vectorial transepithelial processes that makes it possible to work with several samples at the same time.

1. Introduction

As sources of energy and the building blocks of essential constituents, food components play a key role in building and renewing the body. Also, through chemical and mechanical signalling in the gastrointestinal (GI) tract, they provide essential information for the homeostasis of the whole body. The enteroendocrine system is one of the largest endocrine organs in the body. It collects information at the entrance of the organism about what is being taken in and secretes signalling molecules in response. This information is sent to central control systems and used to coordinate homeostatic systems (for example, body energetics). The role, function and mechanisms of the enteroendocrine system are only partially understood [1,2]. One of the reasons for this lack of understanding is its distribution: it is the sum of lots of cells scattered throughout the intestine, with a low abundance of each type of enteroendocrine cell [3].

The most physiological and integrative approach to studying the enteroendocrine system works with the whole animal. Some authors administer the substances to be tested to specific areas of the GI and then obtain samples of blood from specific draining blood vessels and/or cut nerve communication with the central nervous system [4,5]. This approach requires a huge number of animals, which is problematical from an ethical point of view, and researchers who are highly skilled in surgical procedures. A different approach is to work in vitro with enteroendocrine cell lines. These are useful for highly controlled mechanistic studies, but the physiological response is sometimes quite different from the in vivo response. The most common enteroendocrine cell lines are STC-1 [6] and Glutag [7], which mimic L-cells. Additionally, ghrelin can be studied with attached MNG-3 (derived from mice gastric ghrelinoma [8]) and the unattached (SG-1 or PG-1 [9]) cell lines. One of the reasons for the different responses from animals and cell lines may be the lack of vector flux in the treatments. Most studies were carried out in cells attached to the

surface in a 2D situation, quite different from the polarised epithelial position in vivo. To overcome these culture limitations, 3D strategies such as gut-on-a-chip [10] have evolved to mimic intestinal fragments, although there are no reports on how effective they are in enteroendocrine studies.

Ex-vivo approaches, such as everted sacs, perfused intestinal loops, Ussing chambers, intestinal punches, precision-cut intestinal slices (PCIS) and organoids [11], are in between the previous options, as they use natural intact tissue structures in different controlled approaches [12]. Ussing chambers are widely used to address the need for vectorial processes [13,14]. They locate the mucosal epithelium in an apical to basolateral position in a hermetic situation with concomitant control of barrier properties. The main drawback is that it is difficult to have enough samples to minimise variability, largely because each device only has a few chambers and is also usually very expensive [15–17]. To overcome these limitations some authors work with *ex vivo* tissue fragments from animal intestines [18,19]. These crude explants from animal intestines make it possible to produce numerous replicates, depending on the animal's size. Although the treatment reaches all the exposed areas of the tissue, it does not mimic the effect of the apical stimulation that takes place in the in vivo gastrointestinal tract.

We have developed a setup called Ap-to-Bas (AtB). It is an *ex vivo* system that combines the tissue that is readily available in the intestine of pigs, an animal with a metabolism that is similar to that of a human being [20], together with the vectoriality provided by a system that mimics a Ussing chambers approach. Our setup could be a useful tool to screen agents that modulate enteroendocrine secretions throughout the apical and/or basolateral epithelial intestinal areas.

2. Materials & Methods

2.1 Chemicals

Most of the chemicals used – formaldehyde, ethanol, xylol, dimethyl benzene, paraffin, D-mannitol, D-glucose, HEPES, $CaCl_2$, $MgCl_2$, KCl, NaCl, $NaHCO_3$, NaH_2PO_4, 70-kDa fluorescein isothiocyanate (FITC)-dextran, IBMX (I7018) – were purchased from Sigma-Aldrich (Madrid, Spain). The tissue adhesive used was 3M Vetbond (Cat 1469SB, St. Paul, USA). Lucifer Yellow (LY-452 Da) was from BTIU 80016, Merck, (Darmstadt, Germany). The lactate dehydrogenase (LDH) kit was obtained from QCA (Amposta, Spain).

The ELISA kits for total GLP-1 (GLP-1-T) (Cat. # EZGLPT1-36k), active GLP-1 (GLP-1-A) (Cat. # EGLP-35K) and acyl-ghrelin (cat. # EZRGRA-90K) were purchased from Millipore (Billerica, MA, USA). We obtained Elisa kits for CCK (Cat. No: EKE-069-04) and PYY (Cat. No: FEK-059-03) from Phoenix Pharmaceuticals (Burlingame, CA, USA).

2.2 Collection of the tissue

Intestinal tissues were obtained from female pigs (*Sus scrofa domesticus, LANDRACE X LARGEWHITE*) that were killed for meat production at a local slaughterhouse. Forty-eight pigs were used in the study, all from the same farm. For each assay the number of replicates has been indicated as “n”. Pigs were commercial breeds (18% protein; 5.7% lipid; 4.9% fibre; 6.7% ashes; 1.03% Lys; 0.3% Met; 0.78% Calcium; 0.73 Phosphorus; 0.20% sodium, Coperal, Santa Coloma de Queralt, Spain) that weighed approximately 120 kg at slaughter and had been fasted for approximately 24 h prior to slaughter. Just 5 min after slaughter, the intestines were excised, and segments of various anatomical regions were stored in ice-cold oxygenated (95% O2,5%CO2) KRB buffer (Hepes 11.5 mM, $CaCl_2$ 2.6 mM, $MgCl_2$ 1.2 mM, KCl 5.5 mM, NaCl 138 mM, $NaHCO_3$ 4.2 mM, NaH_2PO_4 1.2 mM) with D-Manitol 10 mM. Duodenum (10 cm of intestine taken from the pylorus), distal Ileum (10 cm of intestine taken from the

ileocaecal junction) and proximal colon (10 cm of intestine taken downstream of the ileocaecal junction) were collected for the experiments.

Tissues were transported in KRB buffer to the laboratory at 4 ºC and immediately used for *ex vivo* experiments. The time between excision and the beginning of the experiments was approximately 30 min.

In the laboratory, the intestine was rinsed with cold KRB buffer (with D-Manitol 10 mM) and mounted in a plastic tube to facilitate the removal of the outer muscle layers. Then, the intestinal tube was cut open longitudinally, and the mucosal tissue was placed apical side up. Circles of tissue with a diameter of 14 mm (approximately 1.54 cm^2) were punched out using a biopsy punch (Figure 1a). Twelve circles were taken from each segment from each animal. The intestinal segments were randomized, per region, in a beaker glass. The entire process took around 20 minutes, and the whole time the sample was kept at a low temperature with cold buffer and an ice-cold bath.

2.3 Building the Ap-to-Bas (AtB) system

We cut a silicon tube with an internal diameter of 8 mm and an external diameter of 12 mm into pieces 1.5 cm long with a perfectly flat surface. Tissue adhesive for animal use was lightly applied to the flat side of the tube, which was then gently pressed onto the apical side of the intestinal segment [21]. After 10 seconds, the intestine was placed inside a cell culture insert with no bottom membrane (Cat MCRP12H48, 12-well hanging inserts) (Figure 1b). The entire insert containing the tissue segment and the piece of tube was placed in one of the wells of a 12-well plate prefilled with 1 ml of KRB buffer (with D-Glucose 10 mM). Apically, the tube was filled with 400 µl of KRB buffer (with D-Mannitol 10 mM). The tissues were then pre-incubated at 37 °C for 15 min in a humidified incubator (5% (v/v) CO_2) (Figure 1c).

A 70-kDa fluorescein isothiocyanate (FITC)-dextran was used to assess the efficiency of the gluing process. FITC-70 kDa was added apically (0.10 mg/mL), and after 60 minutes of incubation, the apical and the basolateral media were collected, centrifuged to precipitate the debris and stored at -20 ºC for further analysis. The amount of fluorescent dye that crossed to the basolateral side was measured using a Perkin-Elmer LS- 30 fluorimeter (Beaconsfield, UK) at λexc 430 nm; λem 540 nm.

Figure 1a

Figure 1c

Figure 1b

Figure 1. Representative pictures describing the building of the AtB a) After the outer muscle layers had been removed, the intestinal tube was cut open longitudinally. Circles of tissue with a diameter of 14 mm (approximately 1.54 cm^2) were punched out using a biopsy punch; b), the intestine was placed in a cell culture insert with no bottom membrane; c) Finally, the whole insert was placed in a well of a 12-well plate prefilled with 1 ml of KRB buffer (with D-Glucose 10 mM). Apically, the tube was filled with 400 µl of KRB buffer (with D-Mannitol 10 mM).

2.4 Ussing chamber methodology

Intestinal segments of 0.5 cm^2 were mounted in Ussing chambers apparatus (DIPL.-ING. K. MUSSLER-SCIENTIFIC INSTRUMENTS, Aachen, Germany). Up to 6 segments from each animal were used. Mucosal compartments were filled with 1.5 ml KRB buffer (with D-Mannitol 10 mM) and the serosal compartments were filled with KRB

buffer (with D-Glucose 10 mM) [22]. The chambers were kept at 37 °C and continuously oxygenated, 95% O_2 /5% CO_2, with a circular gas flow. Before the experiments were started, the tissues were equilibrated for 15 min in the chambers to achieve steady-state conditions in transepithelial potential differences.

The transmucosal potential difference was continuously monitored under open circuit conditions and recorded using 0.8 mm Ag/AgCl Glas-Electrodes. Ohm's law was used to calculate the basal transepithelial electrical resistance (TEER) from the voltage deflections induced by bipolar constant current pulses of 50 mA (every 60 s) with a duration of 200 ms and applied through platinum wires (Mussler Scientific Instruments, Aachen, Germany).

After the 20-minute equilibration period, the mucosal side of the biopsies was subject to treatment.

2.5 Paracellular transport (Lucifer Yellow assay)

To evaluate the integrity of the intestinal barrier in AtB and Ussing chambers, a solution of Lucifer Yellow (LY-452 Da,) was used [23]. In this study, 0.4 ml of LY 100 µM was added to the apical side and after 30, 60 and/or 90 minutes of treatment, the apical and the basolateral media were collected, centrifuged to precipitate the debris and stored at -20 ºC for further analysis. The amount of fluorescent dye that crossed to the basolateral side was measured using a Perkin-Elmer LS-30 fluorimeter (Beaconsfield, UK) at λexc 430 nm; λem 540 nm.

2.6 Viability test

Tissue viability was checked by measuring Lactate Dehydrogenase (LDH) with an LDH kit. Tissues were homogenized in ice-cold KRB buffer with a Tissue Lyser (Qiagen, Hilden, Germany) for 2 min at 50 oscillations/0.5 seg. After centrifugation, supernatant LDH was measured. Cell culture was centrifuged to eliminate debris, and

the supernatant was used for the LDH Assay. The amount of LDH activity found in the culture media was considered to indicate the health of the tissue sample throughout the incubation period. The percentage of LDH leakage vs total LDH was used as a viability test [23,16].

2.7 Study of the enteroendocrine function

To test the enteroendocrine function, we measured the basolateral presence of enterohormones in basal (unstimulated) or apically stimulated conditions. IBMX 20 μmol/L was used as positive control [25], and natural extracts of animal and plant origin were used to test the differential ability to stimulate enterohormone secretion in the AtB system. Animal protein homogenate was obtained from pork meat and diluted to 10 mg protein/mL in KRB with D-glucose and protease inhibitors. Vegetal Grape Seed Proanthocyanidin extract (GSPE) was diluted to 100 mg/mL in the same buffer. Treatments were initiated by replacing the apical KRB buffer solution with 400 µL of pre-warmed KRB buffer (37 °C) [25] containing the test compounds. KRB buffer with D-glucose was used as a control. After 30 minutes of the treatment, an aliquot of 200 µL was picked from the basolateral side of the AP-to-Bas system. Finally, 60 minutes (for ileum and colon) or 90 minutes (for duodenum) after the beginning of the experiment, the whole of the apical and basolateral sides was frozen and stored at −80 °C for further analysis of total and active GLP-1, PYY, CCK and acyl-ghrelin.

The enterohormones were assayed using commercial ELISA kits for total GLP-1 (GLP-1-T) (Cat. # EZGLPT1-36k), active GLP-1 (GLP-1-A) (Cat. # EGLP-35K), acyl-ghrelin (cat. # EZRGRA-90K), CCK (Cat. No: EKE-069-04) and PYY (Cat. No: FEK-059-03) (following the manufacturer's instructions.

2.8 Histology.

Intestinal segments of the duodenum, ileum and colon samples were fixed in 4% diluted formaldehyde. After 24 hours of fixation, successive dehydration (Alcohol/Ethanol 70%, 96% and 100%; plus xylol/Dimethyl benzene) and paraffin infiltration-immersion took place at 52ºC (Thermo Scientific). Then, sections 2 µm thick (Microm HM 355S. Thermo Scientific) were obtained, placed on slides (JP Selecta Paraffin Bath) and subjected to automated haematoxylin-eosin staining (Varistain Gemini. Shandom. Thermo) [26].

Figure 2 Haematoxilin-eosin staining of transversal thin sections from a pig's A) duodenum, B) ileum and C) colon mucosa (original magnification, ×6). The images show mucosa and submucosa of each section of intestine. The scale bar indicates 0.2 mm.

2.9 Statistical analysis.

Results were expressed as the mean ± standard error of the mean (SEM). Student's T-test was used to compare the treatments with the control. The one-way ANOVA test was used for multiple comparisons followed by a T3-Dunnett post-hoc. P-values < 0.05 were considered to be statistically significant. The calculations were performed using XL-Stat 2017 software.

3. Results

3.1 Structure, viability and barrier properties of the intestinal fragments in the AtB.

Our setup, called Ap-to-Bas (AtB), enables vectorial transepithelial processes prepared from different areas of the intestinal tract *ex vivo* to be assayed. As **Figure 2** shows, the intestinal mucosa and submucosa were easily separated from the muscularis.

The viability of the mucosa and submucosa was checked by measuring the amount of lactate dehydrogenase released to the basolateral side of the AtB setup. **Table 1** shows the amount of LDH activity of two representative segments (ileum and colon). At the beginning of incubation, it was similar for both tissues. After 30 minutes there was a significant increase in the amount of LDH, after which time it increased steadily. In both tissues, the ileum showed higher values of LDH basolaterally. However, the percentage of LDH in the basolateral side versus the total LDH (tissue plus basolateral) was lower than 0.1%. We also compared the LDH leakage of ileum samples mounted in the AtB to that of *ex vivo* free cultured ileum samples (of similar size). We found no differences (nKatals: 1.40 ± 0.17 (basolateral AtB); 1.38 ± 0.29 (free)).

Table 1. LDH leakage on the basolateral side of the AtB throughout the incubation

	0 MINUTES		**30 MINUTES**		**60 MINUTES**	
	nKatal	SEM	**nKatal**	SEM	**nKatal**	SEM
ILEUM	**0.46** ^{A}	0.04	**1.40** ^{B}	0.17	**1.92** ^{B}	0.18
COLON	**0.31** ^{A}	0.05	**0.74** ^{B}	0.07	**0.99** ^{B}	0.13

LDH in the basolateral media was measured at different time points. Values are the mean ± SEM. Statistical differences were calculated using one-way ANOVA followed by a T3-Dunnett post-hoc. Different superscripts mean statistically significant differences between time points. A p value < 0.05 was considered to be statistically significant.

For our study it is essential for us to be able to work on the apical-to-basolateral effect so, for this reason, the barrier function must be preserved. We initially used FITC-70 kDa to discount inadequate adhesion between biological tissue and the tube surface in the AtB. **Figure 3a** shows that the amount of FITC-70 was approximately 0.1% in the ileum samples and 0.5% in the colon samples, which suggests optimal adhesion. Afterwards, we checked the barrier properties and compared them to those found when a chambers system was used. Transepithelial electrical resistance (TEER) measurements of various intestinal segments, with similar characteristics and

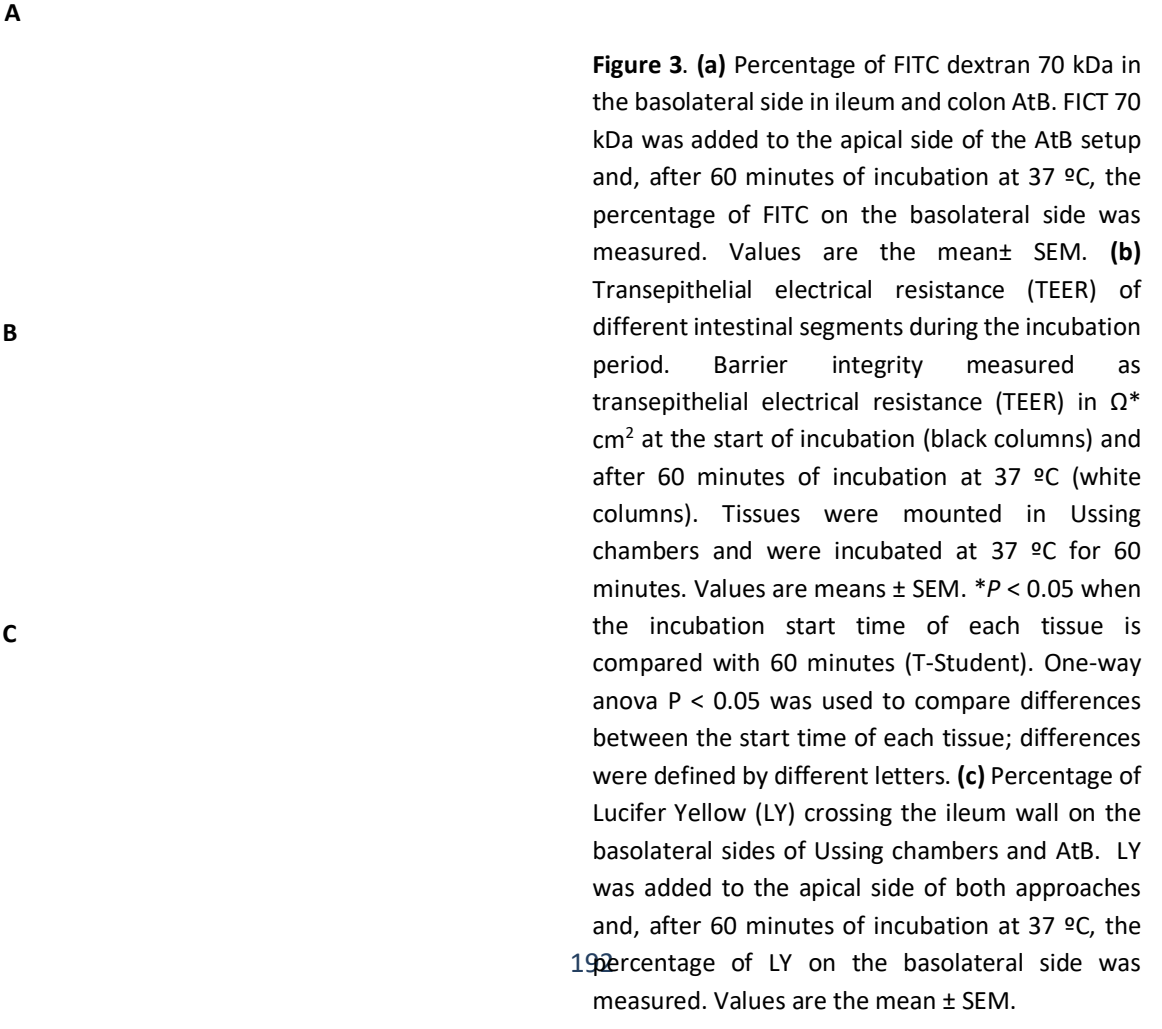

Figure 3. **(a)** Percentage of FITC dextran 70 kDa in the basolateral side in ileum and colon AtB. FICT 70 kDa was added to the apical side of the AtB setup and, after 60 minutes of incubation at 37 ºC, the percentage of FITC on the basolateral side was measured. Values are the mean± SEM. **(b)** Transepithelial electrical resistance (TEER) of different intestinal segments during the incubation period. Barrier integrity measured as transepithelial electrical resistance (TEER) in Ω* cm^2 at the start of incubation (black columns) and after 60 minutes of incubation at 37 ºC (white columns). Tissues were mounted in Ussing chambers and were incubated at 37 ºC for 60 minutes. Values are means ± SEM. *P < 0.05 when the incubation start time of each tissue is compared with 60 minutes (T-Student). One-way anova P < 0.05 was used to compare differences between the start time of each tissue; differences were defined by different letters. **(c)** Percentage of Lucifer Yellow (LY) crossing the ileum wall on the basolateral sides of Ussing chambers and AtB. LY was added to the apical side of both approaches and, after 60 minutes of incubation at 37 ºC, the percentage of LY on the basolateral side was measured. Values are the mean ± SEM.

assay conditions, but mounted in an Ussing chamber apparatus provides information on the integrity of the epithelia and their tightness.

Figure 3b shows that the TEER varies between the intestinal segments and that it decreased slightly only in duodenal segments after a 60-minute incubation. Since TEER cannot be measured in the AtB device, we measured the paracellular transport of Lucifer Yellow from apical to basolateral compartments. **Figure 3c** shows that the amount of Lucifer yellow crossing the ileum mucosa and submucosa is approximately 0.5% in both devices which had equal surface areas (Ussing chamber: 0.5 cm^2 and AtB: 0.5024 cm^2). The quantity of Lucifer Yellow in the duodenum mounted in AtB was 0.21% ± 0.06, and in the ascendant colon it was 1.62% ± 0.98.

3.2 Enteroendocrine function.

The relative abundance of enteroendocrine cells in the various intestinal segments depends on the species [27]. Here we show the differential basolateral secretion pattern obtained in response to different apical stimulatory signals. **Figure 4a** shows that non-stimulated secretion of PYY is higher in the duodenum than in the distal ileum. Moreover, **figure 4b** shows that the distal ileum produces more GLP-1 than the proximal colon. Since several enteroendocrine cells are located in the epithelium of the intestinal barrier, with the apical side in contact with the gastrointestinal duct, and the basolateral side draining the internal body fluids, apical stimulation by some agents should produce basolateral secretion of enterohormones (see **figure 4c).** Apically applied IBMX produces a statistically significant stimulation of basolateral secretion of active GLP-1 at the ileum and only a slight stimulation in colonic segments.

Figure 4. (a) Basolaterally secreted PYY under unstimulated conditions at different anatomical locations. Different Ap-to-Bas setups were mounted for each intestinal porcine duodenum (black column) and ileum (grey column) (n=8 for each section). After 60 minutes in the C- buffer, basolateral media were collected and hormone levels of peptide YY (PYY) were measured. Values are percentage ± SEM. * $p < 0.05$ vs duodenum. **(b)** Ileum and colon relative basal secretion into basolateral media of GLP-1. Different Ap-to-Bas setups were mounted for each intestinal porcine ileum (black columns) and colon (squared columns) (n=5 for each section). After 60 minutes in the C-buffer, basolateral media were collected and hormone levels of total and active GLP-1, were measured. Values are percentage ± SEM. * $p < 0.05$ vs ileum. **(c)** Sensitivity of ileum and colon segments to apical IBMX stimulation of active-GLP-1 secretion. Different Ap-to-Bas setups were mounted for each intestinal porcine Ileum and colon (n=5 for each section). IBMX (20 μM) was apically applied (white columns). Black columns refer to unstimulated controls. At the end of the treatment (60 minutes), basolateral media were collected and active GLP-1 was measured. Values are percentage ± SEM. * $p < 0.05$ compared to negative (vehicle treated) control (C-)

To determine whether our setup could be used to screen enteroendocrine secretagogues, we subjected our AtB setup to two treatments with potential

bioactivity for stimulating enterohormone secretion. **Figure 5a** shows that an animal extract increased CCK and active ghrelin secretion at the duodenum and inhibited PYY secretion. The same extract did not lead to any change in the secretion of active GLP-1 in the ileum or in the colon. A plant extract, which has been proved to be a satiating agent in rats [28], produced a different profile of secretions. It increased

A

B

colonic active GLP-1 secretion statistically. It showed a tendency to increase CCK and PYY in the duodenum and had no effect on active GLP-1 secretion in the ileum segment (**Figure 5b)**.

Figure 5. (a) Basolateral enteroendocrine secretions after apical stimulation with homogenates of animal origin in AtB setups. Different Ap-to-Bas setups were mounted for each intestinal porcine duodenum (black columns), ileum (grey column) and colon (striped column) (n=8 for each section). Animal extracts (10 mg protein/ mL) were apically applied. At the end of the treatment (duodenum: 90 min; others: 60 min) basolateral media were collected and hormone levels of cholecystokinin (CCK), peptide YY (PYY), active ghrelin and active glucagon(-like) peptide 1 (GLP-1) were measured. Values are percentage ± SEM. * $p < 0.05$ compared to negative (vehicle treated) control (C-) **(b)** Basolateral enteroendocrine secretions after apical stimulation with plant extract in AtB setups. Different Ap-to-Bas setups were mounted on each intestinal porcine duodenum (black columns), ileum (grey column) and colon (striped column) (n=8 replicates). Plant extracts (100 mg extract/ mL)

were applied apically. At the end of the treatment, basolateral media were removed and hormone levels of cholecystokinin (CCK), peptide YY (PYY) and active glucagon such as peptide 1 (GLP-1) were measured. Values are percentage ± SEM. * $p < 0.05$, # $p<0.1$ compared to the negative control (C-).

4. Discussion

The study of enteroendocrine processes requires a sufficient amount of tissue to host enough endocrine cells to enable hormone secretions to be measured. These processes also need to be studied in different intestinal segments, since the enteroendocrine cell populations are distributed differently throughout the gastrointestinal tract [29]. Another key point is to preserve apical to basolateral separation, which is usually found in vivo. What is more, many signals cannot cross the intestinal barrier [25]. Ussing chambers are the gold standard procedure for this purpose, but they are expensive and the number of chambers is limited [14].

Our Ap-to-Bas setup is an ex vivo system derived from the pig's intestinal wall, which enables vectorial transepithelial processes to be assayed. This system has three main advantages over the gold standard Ussing chambers method. It makes it possible to work with more samples at the same time while maintaining transepithelial activity affordably; it makes it possible to work with an animal model that is more similar to human beings [20], and the size of the mucosal sample guarantees that the enterohormones will be detected.

Ex vivo systems have limitations, such as the short-time viability of the tissue. We have shown that when healthy intestines are mounted in the AtB system there is enough time for the changes in enterohormone secretions to be measured. Westerhout and col showed that LDH leakage of the intracellular enzyme into the basolateral media from pig jejunal tissue segments mounted in their setup was 3.5 ± 0.8% [30]. The percentage of leakage we found was lower than this, and the amount

of LDH in the basolateral media increased as it did in free cultured equivalent segments, which suggests that the tissue was not damaged any further by being mounted in the AtB setup.

Our system maintained the vectoriality required for processes that occur across a wall. As we were working with glued surfaces, we discounted any problems in the adhesion of the tissue by apically applying fluorescein (FITC)-labelled dextran (70 kDa), an agent that is unable to cross the intestinal barrier [31] and is typically used to measure gastrointestinal transit [32]. The absence of fluorescence from the basolateral side of the AtB setup showed that the apical side had been optically insulated from the basolateral side. Pierre et al. [21] also used this same approach to develop an ex vivo intestinal segment culture (EVISC) model for studying the ex vivo effects of parenteral nutrition on the susceptibility of the ileum to invasion by extra-intestinal pathogenic Escherichia coli (ExPEC).

Evidence of the quality of the intestinal barrier was provided by various complementary approaches. Lucifer Yellow unidirectional permeable paracellular marker [13] showed that ileum segments were similarly permeable regardless of whether they were mounted in AtB or the Ussing chamber. And the permeability of the ileum and colon was similar. This similarity was also shown by Rozenhal working with Ussing chambers and an area of exposure that was quite similar to our own (0.46 cm^2) [13]. The percentage of LY leakage was also in the range that corresponded to an intact intestinal barrier (0.5%) according to Westerhout, who was working with porcine jejunal tissue and paracellular marker fluorescein isothiocyanate–dextran (FD4: MW 4 kDa) [30]. In fact, our values were slightly higher than those obtained by Westerhout et al, but our compound was smaller (LY: MW 0.54 kDa) than theirs. They also worked with different intestinal segments. Lennernäs [33] showed that MW correlated closely with the permeability coefficients of hydrophilic drugs and that high permeability drugs (BCS class I–II)

showed a slightly higher permeability in the colon than the jejunum and ileum when passive diffusion is the dominant transport mechanism.

To reinforce the integrity of the intestinal barrier and its standard state we compared the TEER measures of various intestinal fragments. As we were unable to measure TEER in our AtB setup, we worked with the same samples in the Ussing chamber apparatus, which we have also used to measure human colonic samples for other unpublished studies (Ω* cm^2: 39.5 ± 2.2). Although the TEER measurements were highly dependent on the assay condition and the best approach was to compare them in the same study, the range of units obtained did not significantly differ from those obtained by Westerhout et al. [30], who found a TEER of 58 ± 7 Ω cm^2, which remained stable for 120 min when they used their device to work with porcine jejunal tissue. Also working with Ussing chambers, Gleeson et al. [34] obtained a TEER of 37 ± 9 Ω cm^2 (n=40) in jejunal mucosae, which was within the acceptable range [30]. Jejunal TEER gradually decreased over 120 min to 70–80% of the initial value. The lowest TEER values we found were in colon segments, the result of different barrier properties between intestinal segments. Permeability to small molecules and electrolytes was lower in the duodenum, higher in the ileum and highly increased in the ascendant colon. Hamilton et al. [35] and Moyano et al. [36] have shown that permeability to FITC KD4 (and also various hydrophilic drugs [33]) follows a similar pattern in rat samples..

Our main reason for designing this setup was to be able to test the effects of compounds on enterohormone secretion in a situation that more closely resembles the physiological situation (i. e. several molecules in the gastrointestinal tube can only stimulate enterohormone secretion by interaction with the apical side of these cells). Very few studies have used ex-vivo approaches to determine vectorial enterohormone secretion [14,24]. Most studies use *ex-vivo* incubation of the intestine segment with treatment in a multiwell plate [36, 37]. Pig intestine makes it

possible to obtain samples from various intestinal sections in sizes that are big enough to produce a concentration of hormones secreted on the basolateral side that can be measured by standard ELISA kits. Holst et al. described the enteroendocrine hormone abundance of the various intestinal segments in different animal species [27]. The basolateral secretion of PYY in duodenum and GLP-1 in ileum was higher in our study than in their description. We should point out that we are working with basolateral secretions, although most of the available data has been published on the amount of hormone in each intestinal segment, not secreted on the basolateral side [27,39,40]. Ripken and Col studied the GLP-1 and PYY released by pig intestinal sections, but they did not compare them [16]. Similarly, Agersnap and col [41] assayed the relative presence of CCK throughout the small intestine, and showed that it was more abundant in the first 20 centimetres after the pyloric sphincter, and Vitari and col [42] proved the presence of ghrelin-producing cells in the duodenum of pigs. When we assayed an extract rich in protein, we found a stimulation in CCK. Similarly, Sufian et al. compared this effect between protein-derived extracts from different animals [43]. And, in fact, protein is a very well defined secretagogue for CCK [44], [45]. We found that this protein-rich extract had the specific effect of reducing PYY and stimulating acyl-ghrelin production, although analysing this effect is beyond the scope of this manuscript.

To determine the possible physiological effects of this screening tool , we checked the profile produced by an extract (GSPE [28]), which has been shown to have satiating properties. The main components of this extract are flavanols and phenolic acids. GSPE significantly increased GLP-1 secretion, as previously shown *in vivo* [28] and *ex-vivo*, by intestine tissue culture [46]. PYY, which significantly increased in our previous *ex-vivo* approach [46], tended to increase too. In contrast, our different systems gave different results for CCK. There may be several reasons for these

differences: for example, different responses between rat and pig, or the method for stimulating cells (apically in the AtB vs around all the cells in an *ex-vivo* system).

In conclusion, our AtB setup is a tool for screening new agents that can act apically on enteroendocrine cells in a physiological approach. This tool could be useful for identifying new agents that can have an effect on the gut-brain axis.

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Author Contributions Statement

I.G., K. G-C. and P. R. have run all the laboratory tasks. M.P., A.A., X.T. and M.B. designed the experiments and collected tissues. Ll. A. provided the tested samples. All the authors discussed the results. I.G., M.P. and A.A. wrote the manuscript, which was checked and discussed by all authors.

Competing financial interests

The investigators have no conflict of interest relating to this study.

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UNIVERSITAT ROVIRA I VIRGILI
BIOACTIVITY OF FLAVANOLS ON THE MUCOSA OF THE INTESTINAL WALL: ENTEROENDOCRINE EFFECTS FOR PREVENTING DIET-INDUCED OBESITY AND ASSOCIATED PATHOLOGIES
Iris Ginés Mir

GENERAL DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
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General Discussion

In an initial analysis of the best grape seed proanthocyanidin (GSPE) treatment for combating obesity and its associated pathologies, a GSPE dose of 500 mg/kg body weight (BW) administered to prevent or correct cafeteria diet-induced damage led to a reduction in respiratory quotients (RQs). Moreover, administering GSPE from the beginning of the experiment, as in preventive (PRE-CAF) and simultaneous-intermittent treatments (SIT-CAF), led to a reduction in adipose accrual and BW in the first weeks of the cafeteria (CAF) diet and/or at the end of it [1]. Modulating the enteroendocrine system has also been shown to be useful for maintaining energy balance [2]. We have previously reported that GSPE compounds can interact with the luminal surface of the gastrointestinal (GI) tract, thereby modifying enteroendocrine cells functions [3,4]. However, the molecular mechanisms that explain it, and the role of GSPE in the enteroendocrine system under a CAF diet, remain unclear. We therefore studied whether the beneficial effects of GSPE on cafeteria diet-induced obesity were partly mediated by modulating the enteroendocrine system and found that the CAF diet altered the function of the enteroendocrine cells (EECs). We also found that, depending on the period of administration, GSPE-modulated enterohormone profiles helped to reduce BW and, in some cases, maintained their effects on food intake (FI).

The effects of GSPE on the enteroendocrine system and its involvement in the modulation of BW and FI can be observed from multivariate linear regressions (MLR) derived from the plasma levels of the enterohormones and main pancreatic hormones (insulin, glucagon and amylin) analysed (see figure 1). In these analyses, every treatment showed a different set of enterohormones that were related to changes in food intake and BW. Insulin and glucagon are two pancreatic hormones that participate actively in the modulation of BW gain [5,6]. Our study shows that

both hormones explain changes in BW in the standard (STD) and CAF groups but that these changes disappear when GSPE has recently been administered. Moreover, CAF differs from ST in the appearance of ghrelin, which also significantly helps to explain the increase in BW in these animals though BW does not increase in animals recently treated with GSPE. Although ghrelin is well known as an orexigenic enterohormone, its physiological role and action mechanisms under an obesogenic state are unclear and still under investigation [7,8]. A certain ghrelin resistance has been observed in obese subjects [8]. One of the characteristics of this resistance is the inability to reduce the circulating levels of ghrelin in response to a meal, which may lead to greater food consumption and increased body weight in obese people [9]. When observing animals recently treated with GSPE, we see that ghrelin loses its involvement in BW explanation. Since it was impossible to detect ghrelin secretions through the Ussing Chamber method, we quantified the content of acylated ghrelin inside the stomach and found that GSPE induced a stomach retention of ghrelin. This GSPE-induced stomach retention may be a mechanism for confronting ghrelin resistance and limiting its prolonged plasma levels. In fact, our research group has recently reported that sub-chronic treatment with GSPE reduces both ghrelin production in the stomach and ghrelin secretion in the intestine of healthy rats [4].

Interestingly, in the MLR all GSPE treatments show that various enterohormones participate in BW regulation (figure 1). The exception is the corrective treatment (CORR500-CAF), where none of the hormones assayed explained the BW of these animals. This is probably related to the lack of differences with respect to the CAF group in the final BW. Nevertheless, a reduction in FI is reflected in the MLR, which indicate that GLP-1, insulin and amylin participate in FI regulation. The roles of GLP-1 in increasing insulin release in response to meal initiation [10,11] and of insulin in reducing food intake [12] suggest that this could be one of the mechanisms by which these hormones act on food intake. Other authors (e.g. Ibars et al. [13]) have

observed the same effects on FI administering GSPE as a corrective treatment after a long-term CAF diet. The role of amylin in obesity is not fully understood. However, the MLR shows that the inverse relationship between amylin and FI induced by CAF is reversed by GSPE in the CORR500-CAF group and that, although the metabolic implications of amylin are still under study, it counteracts the effect of the cafeteria diet and returns it to a similar profile to that of the STD group.

As we mentioned earlier, the CORR500-CAF is the only GSPE group in which the role of enterohormones in BW is not observed. However, it is also the only group in which they have a role in FI. In fact, Serrano et al. showed that acutely-administered GSPE is able to reduce FI in animals with an adjusted FI control but that it has a greater effect when administered to animals whose FI control is disrupted, as occurs with a high-palatable diet [14] or, in the case of CORR500-CAF animals, with a cafeteria diet. However, it has been observed that when the treatment is extended over a long period flavanols, they tend to exert their anti-obesogenic effect by decreasing BW rather than by affecting FI (reviewed in [15]). The long-term overconsumption of palatable foods is reported to activate the reward system and be addictive, which in the end, leads to an overriding sensation of satiety [16]. After long-time exposure, it appears that the organism adapts to flavanols and that the effects associated with a high-fat palatable diet prevail over the beneficial effects the flavanols may induce. In the SIT-CAF group, we decided to administer treatment intermittently to avoid this adaptation to GSPE. However, we were unable to prevent it since the results of the recurring measurements of FI showed a periodic loss in the effectiveness of GSPE to inhibit FI. Nevertheless, in agreement with the studies reviewed by Pinent et al. [15], our long-term treatment with GSPE did reduce BW in comparison with the CAF group. Indeed, the MLR suggests a role for CCK and GLP-1 in this modulation (figure 1). With regard to CCK secretions in the duodenum, it is unclear whether the effects of GSPE on CCK help to meliorate energy homeostasis. The MLR reveals the

involvement of plasmatic CCK in reducing body weight in the SIT-CAF group. GSPE affected neither the expression nor the plasma levels of the hormone, though *ex vivo* studies showed that it reduced the basolateral secretion in comparison with the CAF group and actually returned it to the level of standard-fed control. Our research group has previously demonstrated a role for GSPE in inhibiting CCK secretion both in *ex vivo* explants [3] and *in vivo* [17]. It is unlikely that the reduction in secretion in the present study is related to acute effects since the final GSPE dose was administered 36 hours before sacrifice and the tissues were thoroughly washed. Moreover, during the *ex vivo* assay, the tissues were not subjected to any stimulus for inducing CCK secretion. Casanova et al. tested the main monomeric and dimeric structures of GSPE in STC-1 cells and their inhibition on CCK secretion and suggested that these molecules, which are well absorbed in the upper intestine, may be involved in this inhibition [3].

GL-1 is the other hormone seen to play a role in BW modulation. However, as with ghrelin, it was impossible to quantify *ex vivo* secretions from different intestine segments due to lack of detection. Although the Ussing chamber method is one of the most widely used methods for these studies, we were unable to measure ghrelin and GLP-1 secretion despite our efforts to optimize the system. Ultimately, this left us with only the gene expression and plasma levels. Since our research group focuses on understanding the role of the enteroendocrine system, we have been working with organoids (unpublished results) and crude explants [3,4], though these do not enable us to study vectorial enteroendocrine secretions. At first, we tried to reproduce the intestinal layer *in vitro* by developing a tri-culture but was unsuccessful because we were unable to obtain a proper percentage of the three cell lines and adequate basolateral enteroendocrine secretions. To overcome these limitations, we developed a setup named Ap-to-Bas (AtB) to study enterohormone secretions in a situation that more closely resembles the physiological situation,

differentiating between the apical and basolateral sides of the intestinal segments to enable the screening of new agents that can act apically on enteroendocrine cells in a physiological approach. With this setup we were able to quantify acyl-ghrelin, CCK, PYY, active GLP-1 and total GLP-1 from pig duodenum, ileum and colon. We were also able to maintain the intestinal barrier and the viability of the tissue in the same conditions as the Ussing Chambers throughout the time needed to obtain a suitable enteroendocrine secretion.

Returning to the physiological effects of GSPE, the other hormone that explains changes in the body weight of SIT-CAF animals is GLP-1. It has previously been shown that GSPE acutely modulates GLP-1 gene expression depending on the feeding state [3] and that chronic GSPE treatment with a lower (25 mg/kg bw) dose in rats fed a cafeteria diet increases colonic GLP-1 gene expression [18]. In our study, depending on the treatment, the 500 mg/kg BW dosage of GSPE was seen to regulate the GLP-1 gene expression in the ileum (as pre-treatment PRE-CAF did), in the colon (as CORR500-CAF treatment did), or in both tissues (as SIT-CAF treatment did). With regard to the effects observed in the SIT-CAF group, although GLP-1 mRNA levels were increased in both ileum and colon, they were considerably higher in the colon. Moreover, the colonic gene expression profile of the SIT-CAF group was similar to that of the CORR500-CAF group, where both GLP-1 and PYY gene expressions were increased. The GSPE molecules that reach the colon are known to be metabolised by the microbiota, thus creating new metabolites [19,20]. This presents the possibility that the GSPE molecules that reach the colon and increase their gene expression are the same for both hormones. Indeed, these metabolites are reported to act as bioactive compounds in colonic cells, thus stimulating GLP-1 and PYY secretions ex vivo [3]. In the ileum, however, since GLP-1 was unchanged in the CORR500-CAF group, we assumed that it may be regulated by other pathways and/or molecules. These effects did not seem to be attributed to an increase in enteroendocrine cell

differentiation, either for GLP-1 or PYY, due to discrepancies between the PYY, GLP-1 and ChGA gene expressions. Moreover, the lack of effects on PYY secretion on ileal explants neither supported an increase in enteroendocrine cells. Administering yacon root flour to STZ-induced diabetic rats for 90 days led to a significant increase in caecal GLP-1 content that was accompanied by important tissue enlargement [21]. The differences in intestinal length observed in the SIT-CAF group (from the ratio between the small intestine and the colon), together with caecum enlargement, confer a greater colonic surface. This may help to explain the higher enterohormone expression in the colon than in the ileum. Moreover, as we mentioned earlier, the metabolites that stimulate GLP-1 gene expression in the colon appear to be digested metabolites that have been metabolized by caecal microbiota [3]. Colonic enlargement would therefore favour a greater metabolization of these components.

Although it is described as a BW-reducing hormone [22], GLP-1 shows a positive relationship towards BW in the MLR of the SIT-CAF animals (figure 1). Our research group has previously found that a dose of 1000 mg/kg BW of GSPE administered as a sub-chronic treatment in healthy rats induced hypothalamic desensitization to GLP-1, though this did not occur when the dose was the same as that used in this study (500mg/kg bw) [23]. Although our dosage of GSPE was lower, it was administered for a longer period. This may eventually have induced the same GSPE-hypothalamic desensitization to GLP-1, causing it to lose some of its functionality. This hypothesis may help to explain the positive association found between GLP-1 and BW in the SIT-CAF group.

When we analysed the enduring effects of GSPE, we found that 17 weeks after the last dose of GSPE, the PRE-CAF morphometric parameters resembled those of CAF and that BW was one of the parameters that statistically did no differ between the PRE-CAF and the CAF group. Moreover, the MLR of the PRE-CAF treatment shows the involvement of the same hormones that appear in the CAF treatment, where

ghrelin and glucagon play the same role in explaining BW (figure 1). This shows that the PRE-CAF group follows a similar profile with regard to BW modulation. However, insulin changes its association, thereby differentiating one group from the other. In this case, higher levels of insulin are associated with a decrease in BW. This suggests that GSPE may affect certain metabolic pathways that still attempt to control the gain in BW associated with the CAF diet. Indeed, the correlation analysis between the methylation of the GLP-1 promoter and the plasma insulin, BW and RQ (all measured after 17 weeks of CAF diet) suggests that GLP-1 participates in the regulation of these physiologic parameters, where the increase in energy expenditure may also be an indicator of BW loss [24]. Moreover, as we mentioned earlier, one of the main incretin effects of GLP-1 is to increase glucose-dependent insulin release. In this case, the MLR suggest that insulin contributes to a decrease in BW, possibly due to its ability to reach the brain and trigger catabolic pathways that increase energy expenditure and reduce body weight.

As we stated earlier, GSPE induced different effects on GLP-1 gene expression depending on the treatment administered. In the PRE-CAF group, we found that 17 weeks after the final dose of GSPE, the GLP-1 mRNA levels were increased in the ileum but not in the colon. To determine the mechanisms behind the enduring effects of GSPE, we checked whether an epigenetic involvement could help to explain these changes, and observed that the pre-treatment with GSPE was able to downregulate the DNA methylation at the gene promoter of GLP-1 in the ileum. This persisted several weeks after GSPE treatment, which is consistent with the increased gene expression observed in the PRE-CAF and SIT-CAF groups. GSPE therefore increased GLP-1 gene expression via mechanisms that presented long-lasting effects. Since flavonoids have been shown to modulate DNA methylation by attenuating the effect of (DNA methyltransferases) DNMTs, thus leading to a reduction in global DNA methylation [25–28], GSPE may exert its effects by interfering at some point with

DNMTs activity via, for example, direct enzymatic inhibition, indirect enzymatic inhibition, a reduction in DNMTs expression and translation, an interaction with methyl-CpG binding domain proteins, etc. [25]. We also checked the involvement of other epigenetic mechanisms, such as histone modifications, to determine whether they were involved in the differences observed between the ileum and the colon in the SIT-CAF and CORR500-CAF groups. However, since the few changes observed were not linked to the results of gene expression, we were unable to report their involvement. Nevertheless, we do not discard the participation of other epigenetic mechanisms in the modulation of GLP-1 gene expression in the ileum or colon. Moreover, since the rapid replacement of the intestinal epithelium [29,30] complicates the presence of long-term changes, and since epigenetic status is reported to remain almost unchanged upon intestinal stem cell differentiation [31,32], we postulate that these epigenetic changes occur in the stems cells located in the crypts. These may later be differentiated into the enteroendocrine L-cells, reach the brush border of the villus, and exert their activity, though further studies should be carried out to confirm this hypothesis.

As well as the long-lasting effects on GLP-1 gene expression, we also observed a clear long-term anti-obesity effect [1]. Indeed, we detected a limitation in adipose accrual that was partly attributed to inhibition of the gene expression of the white adipose tissue LPL. GSPE was seen to make up for the limitation in adipose storage by increasing FA oxidation in the liver and muscle. After 17 weeks of cafeteria diet, this compensatory mechanism was no longer working and the liver acquired a lipid-storing role because the increase in DGAT-2 favoured the production of TAG and the decrease in CPT-1 limited the entrance of lipids to the mitochondria to be oxidised. These data show that the lipolytic effect of GSPE has a limited duration and that what remain after 17 weeks are the cafeteria effects. Since, long-term GSPE intake has been shown not to trigger the accumulation of flavanol tissue, indicating a clearance

of products at each daily dosage [33], the long lasting-effects due to GSPE remainders are discarded. Another explanation for the 7-week duration of the GSPE effect may therefore be an epigenetic involvement. It has been reported that proanthocyanidins can modify hepatic miRNAs [34,35] and HDACs *in vivo* [36,37]. These effects have been associated with a significant protection against hepatic triglyceride and cholesterol accumulation in healthy rats [37]. Moreover, isolated pancreatic islets are also reported to be a target for epigenetic changes induced by GSPE. Castell-Auví et al. observed that a daily dose of 25 mg GSPE/kg BW for 45 days significantly altered the expression of some miRNAs, a result that is related to ion transport and response to glucose [38].

Considering all the information from the various studies conducted during this thesis, the treatment that most favourably modulates the enteroendocrine system appears to be SIT-CAF since it retains the long-lasting epigenetic effects on GLP-1 gene expression in the ileum (which are also induced in the PRE-CAF group) while also modulating its gene expression in the colon (like the CORR500-CAF group). Moreover, it downregulates the enzyme responsible for ghrelin activation and induces and acts against possible ghrelin resistance through retention in the stomach. The simultaneous-intermittent treatment is also shown to be the most effective in limiting BW gain, associating some effects on the enteroendocrine system with the BW modulation. However, the possible resistance of FI and GLP-1 to the treatment should be taken into account. Given that GSPE exerts its beneficial effects on BW several weeks after the final dose, a simultaneous-intermittent treatment with a prolonged resting period between administrations should be tested to prevent accommodation to treatment and loss of effectiveness. For example, the effect of administering GSPE one week every 2-3 weeks rather than every other week could be tested. Also, in relation to future extrapolation to humans, although the use of natural compounds as a dietary strategy against obesity has been widely studied

(reviewed in [2]), most chronic studies on overweight and obese subjects have demonstrated the effectiveness of these compounds against BW gain but not against FI. Some studies, however, have reported modulations of enteroendocrine secretions, a situation that resembles our SIT-CAF treatment. Further studies are needed to assess the effectiveness on FI of long-term treatments with food components. In addition, the toxicity of GSPE has been assessed in rats with both a higher concentration and the same concentration ours. Neither concentration proved toxic to rats when administered for a long period. However, further studies of the effects of long-term treatment on metabolism should be conducted in order to discard negative metabolic effects on the organism.

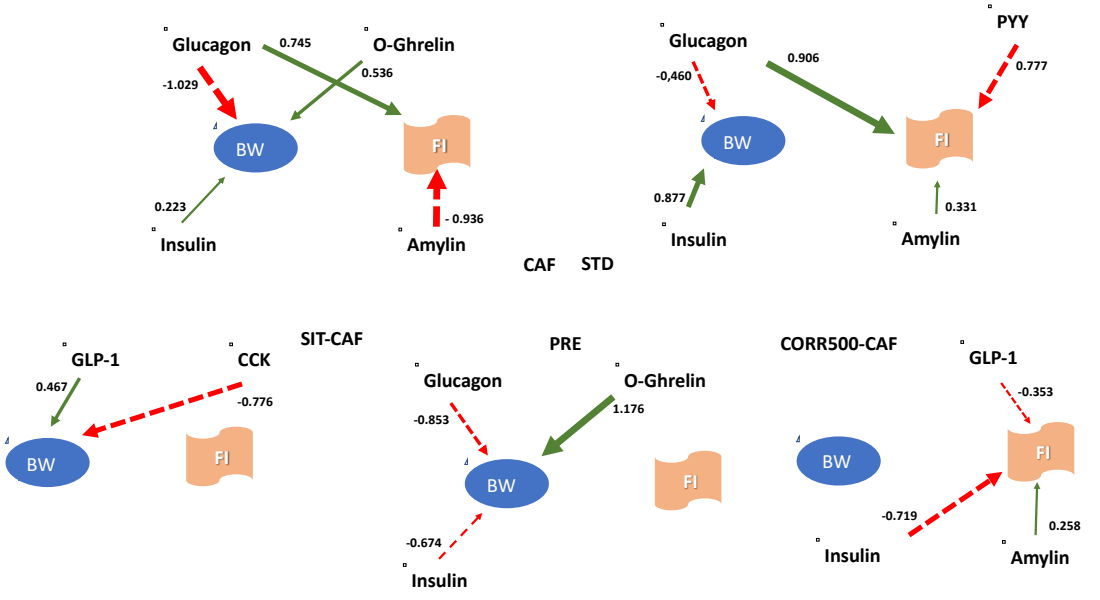

Figure 1. Diagram of modelled relationships between hormones and body weight or food intake, derived from multivariate linear regressions of the cafeteria and standard treatments, and the grape seed proanthocyanidin extract (GSPE) treatments: simultaneous-intermittent treatment, preventive treatment and a corrective treatment of 500 mg/bw GSPE. Solid lines represent a positive contribution to the dependent variable, while dashed lines represent a negative contribution. Numbers correspond to beta coefficient.

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CONCLUSIONS

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Conclusions

1. A 500 mg GSPE/kg BW dose modulates the enteroendocrine system of animals subjected to a 17-week cafeteria diet differently depending on the period of administration:
    a. A 10-day preventive treatment upregulates GLP-1 gene expression in the ileum.
    b. A 17-week simultaneous-intermittent treatment downregulates ghrelin acylation gene expression and increases ghrelin accumulation in the stomach, decreases CCK secretion in the duodenum, and increases GLP-1 and PYY gene expression in the ileum and the colon.
    c. A 15-day corrective treatment increases ghrelin accumulation in the stomach, decreases PYY gene expression in the ileum, and increases GLP-1 and PYY gene expression in the colon.

2. A 10-day preventive treatment with 500 mg GSPE/kg BW induces hypomethylation of the GLP-1 promoter that helps to explain the up-regulation of GLP-1 gene expression in the ileum after 17 weeks of cafeteria diet.

3. Seven weeks after the final dose, 500 mg/kg BW of GSPE maintains a fat redistribution in the peripheric tissues that prevents its accumulation in the adipose tissue and is compensated by increased lipid oxidation in the liver and the skeletal muscle.

4. A novel device named Ap-to-Bas, which enables separation of the apical side from the basolateral side of an intestinal segment, allows more accurate *ex vivo* quantification of enterohormone secretions in the duodenum, the ileum and the colon.

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ANNEXES

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Annex 2

LIST OF PUBLICATIONS

Ginés I, Gil-Cardoso K, Serrano J, Casanova-Martí À, Blay M, Pinent M, Ardévol A, Terra X. (2018) Effects of an intermittent Grape-Seed Proanthocyanins (GSPE) treatment on a cafeteria diet obesogenic challenge in rats. Nutrients. *10*(3), 315.

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SUBMITTED PAPERS

Ginés I, Gil-Cardoso K, Serrano J, Casanova-Martí À, Lobato M, Terra X, Blay M, Ardévol A, Pinent M. Long-lasting effects of proanthocyanidins in rat prevent cafeteria diet effects by limiting adipose tissue accrual. [Submitted to Food and Function]

Ginés I, Gil-Cardoso K, Terra X, Blay M, Pérez-Vendrell AM, Pinent M, Ardévol A. Modulation of the enteroendocrine system by grape seed proanthocyanidin extract in cafeteria diet-fed rats. [Submitted to Molecular Nutrition and Food Research]

Ginés I, Gil-Cardoso K, D'Addario C, Falconi A, Bellia F, Blay M, Terra X, Pinent M, Ardévol A. Long-lasting effects of GSPE on ileal GLP-1 gene expression are associated to a hypomethylation of the glp-1 promoter. [Submitted to The Journal of Nutritional Biochemistry]

COMMUNICATION POSTERS

Grau C, **Ginés I**, Gonzalez-Quilen C, Blay M, Terra X, Beltran R, Pinent M, Ardévol A. El extracto de proantocianidinas derivado de semillas de uva estimula la secreción de enterohormonas en distintas secciones del tracto gastrointestinal de cerdo. Congreso Nacional de Biotecnología del Vino. 2018

Ginés I, Gil-Cardoso K; Robles P, Terra X, Blay M, Ardévol A, Pinent M. Ap-to-Bas, an intestinal dispositive to measure pig mucosa enteroendocrine secretory activity in response to apical stimulation. The 14th NuGO week Congress. 2017

Ginés I, Gil-Cardoso K; Robles P, Terra X, Blay M, Ardévol A, Pinent M. Diseño de un dispositivo con el que testar los efectos bioactivos de extractos naturales sobre secreciones enteroendocrinas y su posible efecto saciante. IX Seminario sobre Alimentación y Estilos de Vida Saludables. 2017

Robles-Bolivar P, Gonzalez-Quilen C, **Ginés I**, Blay M, Terra X, Pinent M, Ardévol A. Identification of pure bioactive molecules on enteroendocrine secretions in different pig intestine segments. XL SEBBM Congress. 2017

Gil-Cardoso K, **Ginés I**, Pinent, M, Ardévol A, Terra X, Blay M. A rat model of moderate intestinal inflammation to study molecular bioactivity of food. The 1st International Conference on Food Bioactive and Health. 2016

Gil-Cardoso K, Casanova-Martí À, Serrano J, **Ginés I**, Pinent M, Ardévol A, Blay M, Terra X. Chronic supplementation with proanthocyanidins protects from diet-induced intestinal alterations in obese rats. XXV Congrés de la Societat Catalana de Digestologia. 2016

Gil-Cardoso K, Casanova-Martí À, Serrano J, **Ginés I**, Gual A, Pinent M, Ardévol A, Blay M, Terra X. Chronic supplementation with proanthocyanidins protects from diet-induced intestinal alterations in obese rats. 12th NuGO week Congress. 2015

UNIVERSITAT ROVIRA I VIRGILI
BIOACTIVITY OF FLAVANOLS ON THE MUCOSA OF THE INTESTINAL WALL: ENTEROENDOCRINE EFFECTS FOR PREVENTING DIET-INDUCED OBESITY AND ASSOCIATED PATHOLOGIES
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Obesity is one of the most prevalent diseases affecting the global population. It entails metabolic disruptions that affect the whole organism, including the correct functionality of the gastrointestinal (GI) tract. Proanthocyanidins have already proved to be effective at stimulating the enteroendocrine system in healthy conditions, but their effects under an obesogenic challenge have still to be determined. For this reason, this thesis was designed to study the effects of a grape seed proanthocyanidin extract (GSPE) on the enteroendocrine system in rats fed with a long-term cafeteria diet.

Our results showed that a preventive treatment, a synchronic intermittent treatment and a corrective treatment were all capable of modulating the enteroendocrine system differently. Furthermore, each GSPE treatment showed different enteroendocrine profiles associated with changes in body weight and/or food intake. However, we had certain difficulties regarding the quantification of enterohormone secretions, which led us to develop a new *ex vivo* methodology that stimulated different segments of the GI tract and quantified their enterohormone secretion response, thus keeping their vectoriality.

We also found that a 10-day pre-treatment with GSPE induced a long-term upregulation of GLP-1 gene expression in the ileum that was partly mediated by the hypomethylation of its GLP-1 promoter. Moreover, these effects were maintained when GSPE was administered every other week during the seventeen weeks of cafeteria diet. In addition, since this preventive GSPE treatment presented a decreased respiratory quotient and tended to reduce the body weight gain, we evaluated if there were also long-lasting GSPE effects on lipid management in the peripheric tissues. The results showed a limitation on adipose storage and an increase in lipid oxidation in the liver and skeletal muscle that lasted seven weeks after the last GSPE dose.

To sum up, this thesis revealed that grape seed proanthocyanidins are capable of modulating the enteroendocrine system and improving the energetic state altered by a cafeteria diet, thus demonstrating that they are good agents for treating metabolic alterations induced by obesity.