REGULATION OF ENTEROENDOCRINE FUNCTION BY BIOACTIVE COMPONENTS THROUGH THEIR INTERACTION WITH BITTER TASTE RECEPTORS

Carme Grau Bové

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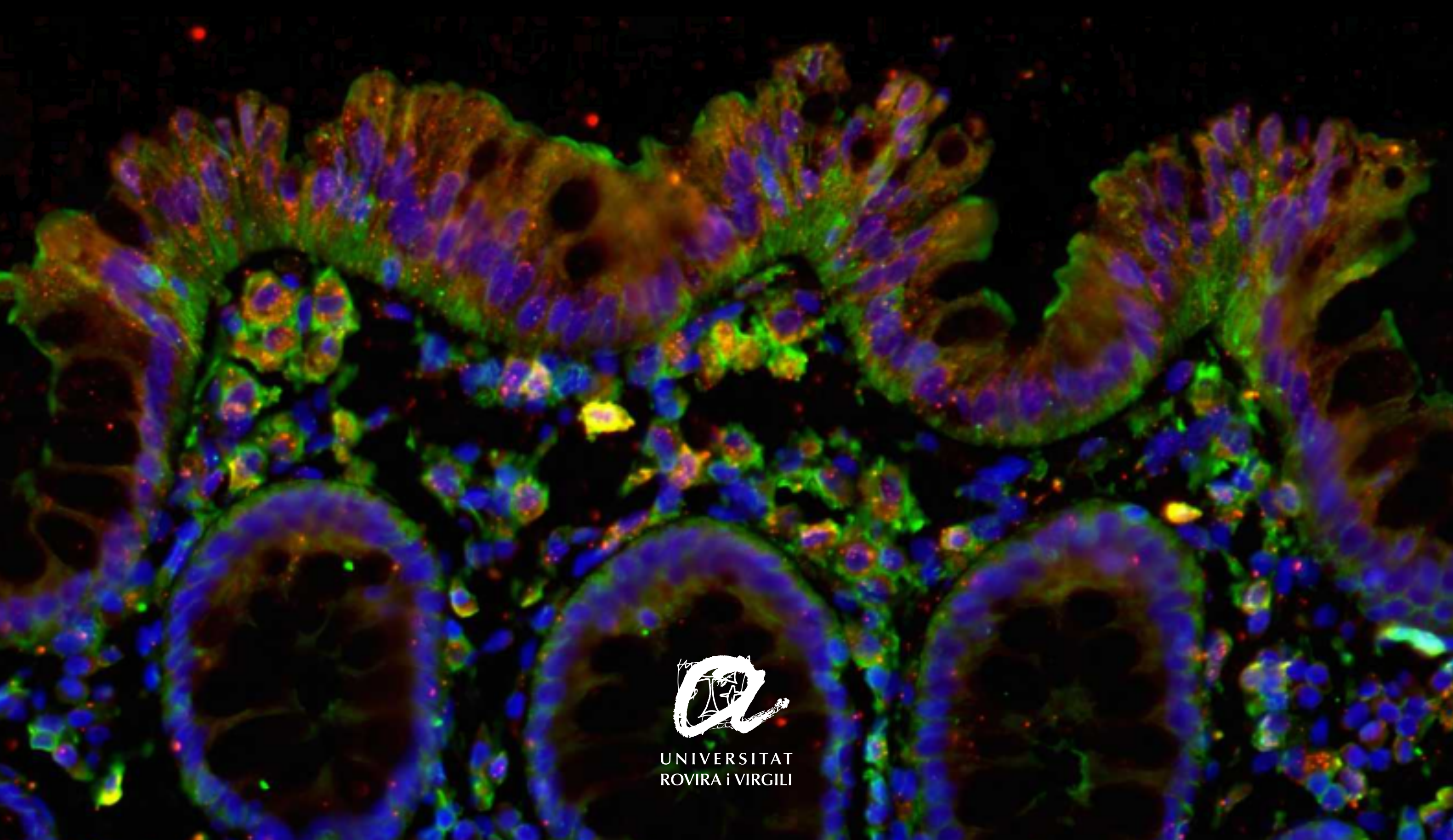
Doctoral thesis

Regulation of enteroendocrine function by bioactive components through their interaction with bitter taste receptors

Carme Grau Bové

2021



Carme Grau-Bové

Regulation of enteroendocrine function by bioactive components through their interaction with bitter taste receptors

Doctoral Thesis

Thesis supervised by Dr. Anna Ardévol Grau and Dr. Montserrat Pinent Armengol

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UNIVERSITAT
ROVIRA i VIRGILI

Tarragona 2021

UNIVERSITAT ROVIRA I VIRGILI
REGULATION OF ENTEROENDOCRINE FUNCTION BY BIOACTIVE COMPONENTS THROUGH THEIR INTERACTION WITH BITTER TASTE RECEPTORS
Carme Grau Bové

UNIVERSITAT
ROVIRA i VIRGILI

FAIG CONSTAR que aquest treball, titulat "Regulation of enteroendocrine function by bioactive components through their interaction with bitter taste receptors", que presenta Carme Grau-Bové per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado "Regulation of enteroendocrine function by bioactive components through their interaction with bitter taste receptors", que presenta Carme Grau-Bové para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Bioquímica i Biotecnología de esta universidad.

I STATE that the present study, entitled "Regulation of enteroendocrine function by bioactive components through their interaction with bitter taste receptors", presented by Carme Grau-Bové 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.

Tarragona, 02/09/2021

El/s director/s de la tesi doctoral

El/los director/es de la tesis doctoral

Doctoral Thesis Supervisor/s

Dr. Anna Ardévol Grau

Dr. Montserrat Pinent Armengol

Als de casa.

A l’alegria dels tiets, el somriure de la iaia i el cant del padrí.

"Amb totes dues mans alçades a la lluna, obrim una finestra en aquest cel tancat."

Maria Mercè Marçal

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SUMMARIES

SUMMARY

The enteroendocrine system is located in the gastrointestinal tract and controls appetite and endocrine pancreatic activity, among other functions. Thus, bioactive compounds that stimulate the enteroendocrine system are therapeutic candidates for treating pathologies related to these functions. Previous research has identified a grape-seed proanthocyanidin extract (GSPE) as antidiabetic for its β-cell function enhancement abilities and its appetite-suppressing activity at least partly through activating the enteroendocrine system. Moreover, our group has linked the polyphenol-induced enteroendocrine secretions to the stimulation of some bitter taste receptors (TAS2R) *in vitro*, but whether it results in an altered food intake has not been studied yet. Since little is known of the mechanisms used by polyphenols to stimulate secretory mechanisms of the enteroendocrine system, there is a need to fully comprehend this system to specifically target it with a therapeutic strategy.

For this reason, this thesis addressed whether GSPE-induced enterohormone secretions modulate pancreatic glucagon production, and whether these secretions are regulated through the specific stimulation of TAS2R leading to a differential control of food intake. This hypothesis was assessed with *in vivo* studies in rats and *ex vivo* studies in intestinal samples.

We found that glucagon was higher sensitive than insulin to GSPE which correlates with enhanced ileal GLP1 secretion. Moreover, we identified that while some polyphenols stimulate basolateral secretion of enterohormones differentially depending on the location in the gastrointestinal tract; but also that GLP1 and PYY are secreted and act into the lumen of human colon. Finally, we showed that flavanols act through TAS2R, the selective stimulation of which can be used to increased or reduced food intake.

To sum up, this thesis shows that polyphenols influence pancreatic glucagon production through a stimulation of the enteroendocrine system, which occurs through the activation of TAS2R. Polyphenols' activation of TAS2R in turn also modulates the food intake.

RESUM

El sistema enteroendocrí es troba al tracte gastrointestinal i controla la gana i l'activitat pancreàtica endocrina, entre altres funcions. Els compostos bioactius que estimulen aquest sistema són candidats terapèutics per tractar patologies relacionades amb aquestes funcions. Prèviament s'ha identificat que un extracte de proantocianidines de llavors de raïm (GSPE) és antidiabètic per les seves capacitats de millora de la funció de les cèl·lules β i la seva capacitat saciant, com a conseqüència en part de l'activació del sistema enteroendocrí. El nostre grup ha relacionat les secrecions enteroendocrines induïdes per polifenols amb l'estimulació de receptors del gust amarg (TAS2R) *in vitro*, però si això es reflecteix en una ingesta alterada encara és deconegut. Per això, és necessari comprendre millor aquest sistema per poder desenvolupar millors estratègies terapèutiques.

Aquesta tesi aborda si les secrecions d'intererohormones induïdes per GSPE modulen la producció de glucagó pancreàtic i si aquestes secrecions es regulen mitjançant l'estimulació específica de TAS2R que condueix a un control diferencial de la ingesta d'aliments. Aquesta hipòtesi s'ha avaluat amb estudis *in vivo* en rates i estudis *ex vivo* en mostres intestinals.

Hem identificat que el glucagó és més sensible que la insulina a GSPE, fet que es correlaciona amb una secreció il·leal de GLP1 millorada. Hem identificat que, si bé alguns polifenols estimulen la secreció basolateral d'enterohormones de manera diferent segons la ubicació al tracte gastrointestinal; GLP1 i PYY se secreten i actuen a la llum del còlon humà. Finalment, hem demostrat que els flavanols actuen mitjançant TAS2R, l'estimulació selectiva des quals es pot utilitzar per augmentar o reduir la ingesta.

En resum, aquesta tesi mostra que els polifenols influeixen en la producció de glucagó pancreàtic mitjançant una estimulació del sistema enteroendocrí, que es produeix mitjançant l'activació de TAS2R. L'activació dels polifenols de TAS2R al seu torn també modula la ingesta.

RESUMEN

El sistema enteroendocrino se encuentra en el tracto gastrointestinal y controla el apetito y la actividad pancreática endocrina, entre otras funciones. Los compuestos bioactivos que estimulan este sistema son candidatos terapéuticos para tratar patologías relacionadas con estas funciones. Previamente se identificó que un extracto de proantocianidinas de semillas de uva (GSPE) es antidiabético por sus capacidades de mejora de la función de las células β y su capacidad saciante, como consecuencia en parte activar del sistema enteroendocrino. Nuestro grupo relacionó las secreciones enteroendocrinas inducidas por polifenoles con la estimulación de receptores del gusto amargo (TAS2R) *in vitro*, pero si esto se refleja en una ingesta alterada aún se desconoce. Por esto, es necesario comprender mejor este sistema para poder desarrollar mejores estrategias terapéuticas.

Esta tesis aborda si las secreciones enteroendocrinas inducidas por GSPE modulan la producción de glucagón pancreático y si estas se regulan mediante la estimulación específica de TAS2R que conduce a un control diferencial de la ingesta. Esta hipótesis se ha evaluado con estudios *in vivo* en ratas y estudios *ex vivo* en muestras intestinales.

Hemos identificado que el glucagón es más sensible que la insulina a GSPE, lo que se correlaciona con una secreción ileal de GLP1 mejorada. Hemos identificado que, si bien algunos polifenoles estimulan la secreción basolateral de enterohormones de manera diferente según la ubicación en el tracto gastrointestinal; GLP1 y PYY se secretan y actúan a la luz del colon humano. Hemos demostrado que los flavanoles actúan mediante TAS2R y que su estimulación selectiva se puede utilizar para aumentar o reducir la ingesta.

Esta tesis muestra que los polifenoles influyen en la producción de glucagón pancreático mediante una estimulación del sistema enteroendocrino, que se produce mediante la activación de TAS2R. La activación de los polifenoles de TAS2R a su vez también modula la ingesta.

LIST OF ABBREVIATIONS

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5-HT	Serotonin
ACTH	Adrenocorticotropic hormone
AGRP	Agouti-related protein
AP	Area postrema
ARC	Arcuate nucleus
ASC	Adipose stem cells
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
CART	Cocaine and amphetamine-regulated transcript
CasR	Calcium receptor
CCK	Cholecystokinin
CCKAR (or CCK1R)	Cholecystokinin receptor A (or cholecystokinin receptor 1)
CCKBR (or CCK2R)	Cholecystokinin receptor B (or cholecystokinin receptor 2)
CNS	Central nervous system
DB	Denatonium benzoate
DPP4	Dipeptidyl peptidase 4
DSS	Dextran sodium sulphate
EC	Epicatechin
EC	Epicatechin
EC50	Half-maximal concentration
ECG	Epicatechin gallate
EECs	Enteroendocrine cells
EGCG	Epigallocatechin gallate
GCGR	Glucagon receptor
GH	Growth hormone
GHS-R	Ghrelin receptor
GI tract	Gastrointestinal tract
GIP	Gastric inhibitory polypeptide
GIPR	Gastric inhibitory polypeptide receptor
GLP1	Glucagon-like peptide 1
GLP1R	Glucagon-like peptide 1 receptor
GLP2	Glucagon-like peptide 2
GLP2R	Glucagon-like peptide 2 receptor

GLUT	Glucose transporter
GOAT	Ghrelin O-acyltransferase
GPCR	G-protein coupled receptors
GPx	Glutathione peroxidase
GSIS	Glucose-stimulated insulin secretion
GSPE	Grape-seed proanthocyanidin extract
INSL5	Insulin-like peptide 5
KO	Knock out
LPS	Lipopolysaccharide
miRNA	Micro RNA
NAFLD	Non-alcoholic fatty liver disease
NEFA	Non-esterified fatty acids
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
OXM	Oxyntomodulin
PACs	Proanthocyanidins
PC1/3	Prohormone convertases 1 and 3
PC2	Prohormone convertase 2
PGG	Pentagalloylglucose
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
PTU	Phenyltiourea
PYY	Peptide YY
SCFA	Short chain fatty acid
SGLT1	Sodium-glucose transporter 1
SLC	Sodium-coupled transporters
SOD	Superoxide dismutase
SSRT2	Somatostatin receptor 2
SSRT5	Somatostatin receptor 5
SST	Somatostatin
T2D	Type 2 Diabetes Mellitus
TAG	Triacylglycerol
TAS1R	Taste receptor type 1
TAS2R	Taste receptor type 2
TEER	Trans epithelial electrical resistance

TNBS	Trinitrobenzene sulphonic acid
TRL	Toll-like receptor
TRPM5	Transient receptor potential cation channel subfamily M member 5
VLDL	Very low-density lipoprotein

INTRODUCTION

INTRODUCTION

1. Enterohormones, the intestinal signalling system

In the recent decades, the conception of the gastrointestinal tract (GI tract) has evolved greatly, from being merely responsible for nutrient digestion and absorption to being the largest endocrine organ in the body, which integrates external and internal neural and hormonal signals, by producing more than 30 hormones. This intestinally produced hormones are called enterohormones, and their producing cells, enteroendocrine cells (EECs). Together, they constitute the enteroendocrine system [1]–[3] (**Figure 1**). The enteroendocrine system responds to food intake and controls metabolism by regulating digestion, nutrient absorption and homoeostasis and appetite in both fasting and fed states[1]. The enteroendocrine system therefore meets with a great variety of stimuli that compose the ingested food, which is sensed through different chemosensors expressed in the EECs and, for most cases, more than one secretory stimulus has been identified for each enterohormone. Enterohormones, among numerous other functions in the body, play a key role in the regulation of insulin secretion in response to food arrival into the GI tract and the regulation of appetite and food intake. Enterohormones are commonly divided in anorexigenic and orexigenic, being those that inhibit appetite and those that stimulate it, respectively. While only ghrelin has been confirmed as an orexigenic enterohormone, cholecystokinin (CCK), glucagon-like eptide-1 (GLP1) and peptide YY (PYY) among others are known to be anorexigenic, since their levels in blood rise short after food intake and induce satiety [4].

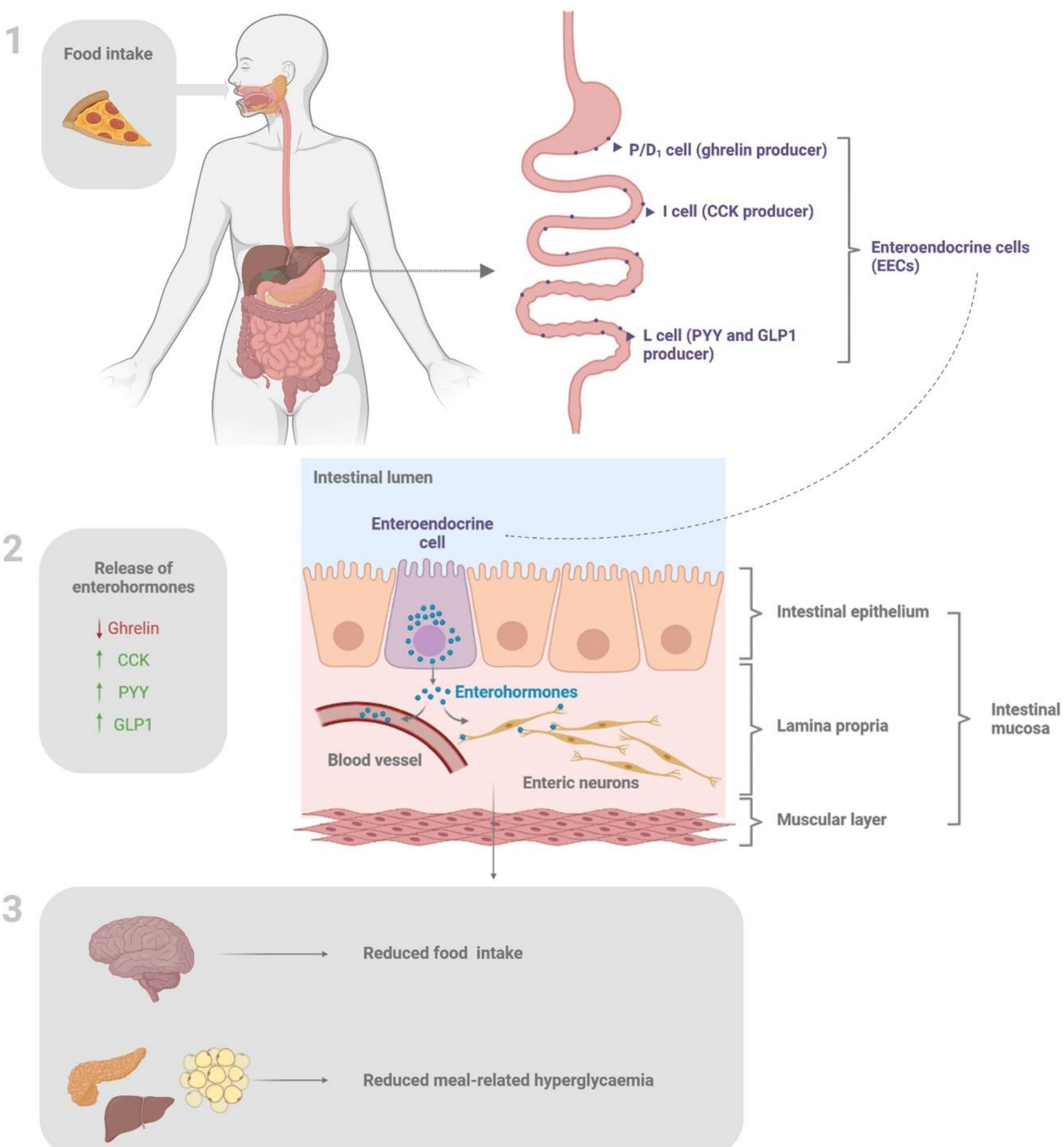

Figure 1. Overview on the mechanism by which the enteroendocrine system responds to food intake. Created with Biorender.com.

As opposed to other endocrine systems, the morphology of the enteroendocrine system is not glandular. Instead, EECs are present from the stomach to the rectum, and, in total, they constitute only 1% of the cells in the total GI tract epithelium [5]. Despite, it is still the largest endocrine system in the body. EECs, as other intestinal types do, are formed from stem cells in the crypts of Lieberkühn, found along the GI tract [6]. First, they differentiate into mature migratory cells and then they migrate to the villus, where they

live for approximately 5 days before they undergo anoikis, when they lose contact with the extracellular matrix of the neighbouring cells and shed into the lumen. The process of differentiation and migration from the crypt-villus axis is orchestrated by opposed gradients of the differentiating factors WNT and BMT expressed by stem cells and other cell types in the crypt, that push the EECs towards the villus (**Figure 2**).

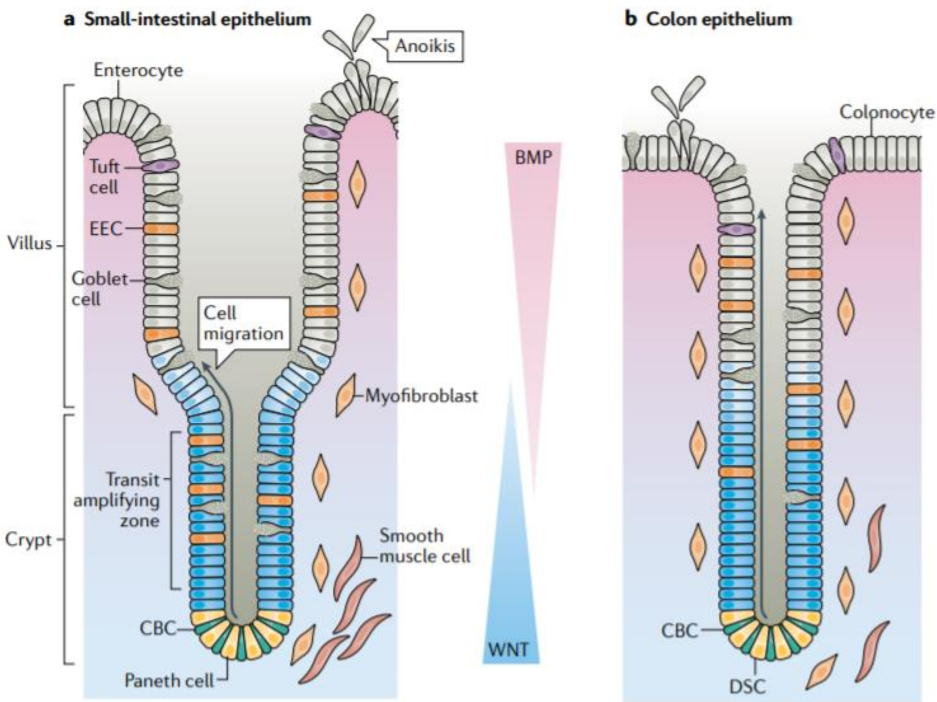

Figure 2. Regulatory signals from intestinal stem cells in the differentiation and migration of different intestinal cell types in the small intestine (a) and colon (b). EEC: enteroendocrine cell; CBC: crypt-base columnar cells; DSC: deep secretory cells. Adapted from [7].

EECs are classified in two types according to their morphology: the open type that has an apical and a basolateral membrane and senses luminal content through apical chemoreceptors, and the closed type that does not have an apical surface and senses luminal content through neuronal or humoral mechanisms. In the stomach, most EECs are closed-type while in the small intestine most are open-type EECs. Traditionally, EECs were assigned a nomenclature depending on which enterohormone produced. However, it is now clear that there is an overlapping co-production of different enterohormone by the same cell type, in addition to an overlap in the secretion of the same enterohormone by different cell types. In the proximal gut, in humans, some cells produce CCK, gastric inhibitory polypeptide (GIP) and GLP1, whilst in the distal gut GLP1 is produced together with PYY and insulin-like peptide 5 (INSL5) [1] (**Figure 3**). In this sense, labelling of K and

L cells of mouse and pig small intestine and colon revealed that there is a subset of these cells that are labelled as both [8]. Therefore, which enterohormone is predominantly secreted by a particular EECs seems to be determined by the position in the GI tract and the specie. Despite the overlap of enterohormone production among EECs, in humans, ghrelin is manly produced in the stomach, GIP in the duodenum, CCK in the duodenum and jejunum, GLP1 in the jejunum, ileum and colon, and PYY in scattered cells in the small intestine but predominantly in the colon. Although the crypt-to-axis gradient of differentiating factors by which EECs reach their position in the villus to exert their function is well studied, little is known about how the gradient of different EECs is established and maintained along the GI tract.

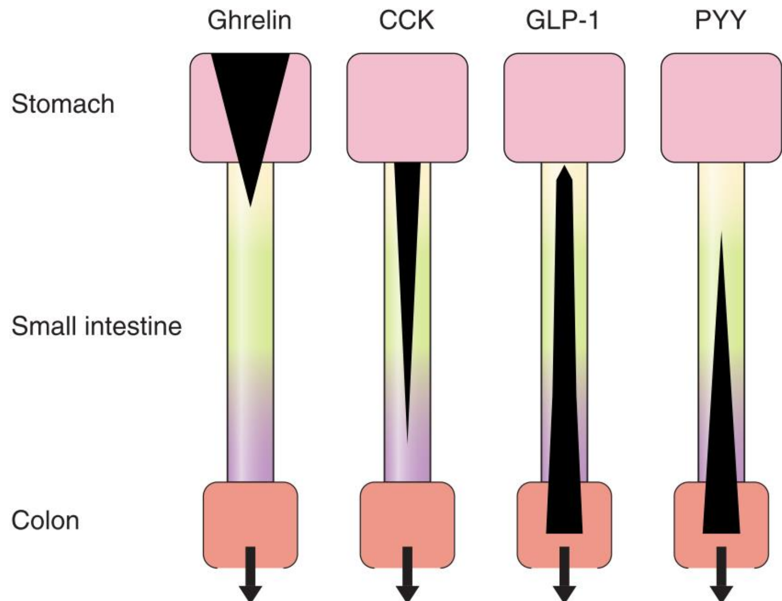

Figure 3. Distribution of EECs secreting ghrelin, CCK, GLP1 and PYY along the human GI tract. Adapted from [4].

Enterohormones respond differently to the various nutrients, showing different susceptibilities to proteins, lipids and carbohydrates. Moreover, the stimuli needed to induce the secretion of a particular enterohormone also vary depending on where the secretory cell is located; whilst nutrients induce secretions from EECs in the small intestine, microbiota metabolites among others non-nutrients are responsible for the stimulation of EECs in the colon.

Some EECs have synaptic features: L-cells, which produce GLP1, PYY and INSL5 in the distal gut, have synaptic features (interact with glia, nerve endings in the mucosa). In addition to luminal sensing, different paracrine mechanisms involving different EECs and other intestinal epithelium cell types have been defined [5]. Due to the importance of

enterohormones in the complex metabolic, neuronal and humoral interplay in the regulation of nutrient homeostasis, they have widely been considered appropriate candidates for drug design to treat metabolic diseases[9]. However, the evolution of the knowledge about the enteroendocrine system is hindered by the important challenge of the study of EECs. Their scattered and irregular localization along the GI tract makes difficult the design of appropriate experimental models [10].

1.1 Anorexigenic enterohormones

1.1.1 GLP1 and the other derived peptides from proglucagon

GLP1 in the intestine is produced by L-cells. Together with GIP, GLP1 is considered an incretin, because they stimulate postprandial secretion of insulin. GLP1 is secreted upon food intake in a response period of 10-15 minutes and stimulates glucose-stimulated insulin secretion (GSIS)[11] while inhibiting glucagon production, producing an inhibition of hepatic gluconeogenesis and glycogenolysis [12], [13]. Moreover, GLP1 slows gastric motility, increasing glucose absorption [14]. In obese and type 2 diabetes mellitus (T2D) patients, the postprandial increase of GLP1 and its insulinotropic effect is maintained [15], [16]. Thus, GLP1 is an interesting therapeutical target in the treatment of this pathology and currently, many GLP1 based drugs have been developed [17], [18]. Besides, GLP1 has an important role in the control of energy balance in a variety of metabolic pathways. GLP1 is implicated in the regulation of adiposity and in the control of food intake, by activating its receptors found on the vagal afferent nerves and on the hypothalamus promoting an acute anorectic effect. It has been suggested that the improved control of satiety in gastric bypass surgery patients is partly explained by an enhanced production of GLP1 [17], [19]. However, a recent study has shown that infusion of GLP1 agonist exendin 9-39 in gastric-bypass surgery patients did not have an effect in satiety and the effects were attributed to increased levels of PYY [17]. Nevertheless, GLP1-based drugs, being receptor agonists or inhibitors of DPP4, the protease that cleaves GLP1, are currently being used as a treatment for T2D and obesity, for their combined abilities to lower blood glucose levels and body weight [20]. In addition, current research is focused on the study of compounds that act as GLP1 secretagogues, stimulating the endogenous production of GLP1, and their anti-diabetic potential [21]. The most reported side effect of GLP1-based drugs is nausea, when they are administered at supraphysiological concentrations [22]. Current findings on the activity of GLP1 cleaved form, which was initially believed to be inactive, on the cardiovascular system have shifted the focus of GLP1 research in deciphering which functions of GLP1

are due to the full of the cleaved form. The cleaved form of GLP1, GLP1(9-36), is originated by the cleaving of the full form, GLP1(7-36), by the protease DPP4, which sets the half live of GLP1(7-36) to 1-2 minutes. Whilst GLP1(7-36) is active through GLP1 receptor (GLP1R), a recent study by Guida *et al.* [23] shows that GLP1(9-36) is able to activate glucagon receptor (GCGR) expressed in pancreatic α-cells and inhibit glucagon production.

GLP1 is a product of cleavage of proglucagon by intestinal prohormone convertases 1 and 3 (PC1/3), which can also generate GLP2, oxyntomodulin, glicentin and two other intervening peptides (**Figure 4**). Physiological roles have only been defined for GLP1, GLP2 and oxyntomodulin. Proglucagon is also expressed in certain areas of the brain and α cells in the pancreas. While the brain expresses PC1/3 that generate the same products as in the intestine, α-cells express PC2, which generates different peptides among which glucagon stands out [24].

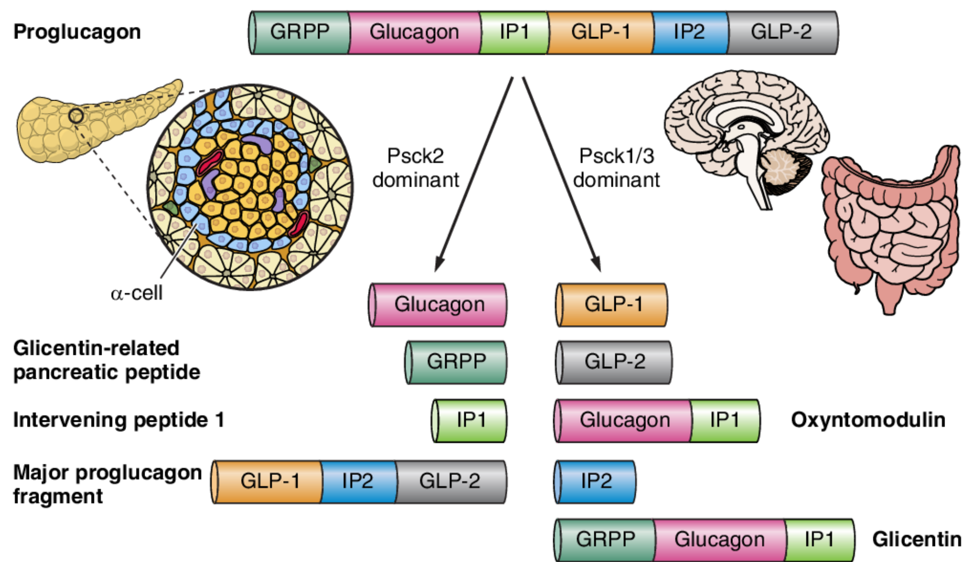

Figure 4. Post-translational processing of proglucagon. Adapted from [24]

Increasing evidence suggests that intestinal proximal L-cells are different from distal L-cells. Around 20% of proximal L-cells co-express GIP and 40% of them co-express CCK. However, distal L-cells hardly express neither GIP nor CCK, whilst 45% of ileal and 70% of colonic L-cells co-express PYY [25], [26]. Moreover, although the percentage of L-cells in the colon is higher than in the distal small intestine, the increased blood levels of PYY and GLP1 in the postprandial state are due to the sensing of nutrients in the distal small intestine, because by the time that ingested food reaches the colon, these

enterohormones have already risen. Thus, L-cells in the colon respond to different stimuli than those in the distal small intestine, being those of microbiota and neurohormonal stimuli. In general, L-cells respond to feeding in two stages: first, vagus nerves through muscarinic receptors activate enterohormone release and, next, nutrients stimulate enterohormone release in a more prolonged way. The secretion mechanisms of L-cells resemble that of pancreatic β-cells regarding glucose and fatty acid responses[27].

In contrast to GLP1, GLP2 is not an incretin and has more focalized effects on the GI tract. GLP2 is also susceptible for DPP4 cleaving, which reduces its half live to 7 minutes. GLP2R is predominantly expressed in the GI tract, although it is also expressed in the brain and vagus nerve. GLP2 main function is in the intestinal epithelium, where it enhances nutrient absorption by ensuring a good intestinal function. GLP2 increases the expression of digestive enzymes, the intestinal blood flow and the surface area of the small intestine and the colon. Moreover, it delays gastric emptying, although to a lesser extent than GLP1. GLP2 reduces intestinal inflammation and increases barrier function, thus protecting the intestinal epithelium. The mechanism of GLP2 has not been fully elucidated, because while GLP2R is expressed subepithelially, the paracrine role of GLP2 in the epithelium has yet to be elucidated. GLP1 and GLP2 do not have additive effects in suppressing food intake [28].

Oxyntomodulin (OXM) is produced in L-cells, and although no specific receptor has been identified for it until now, it can exert its known functions through a weak activation of GLP1R and glucagon receptor (GCGR) [29]). Differently than GLP1 and GLP2, OXM is less susceptible to DPP4 degradation. While GCGR activation leads to glucose release into the bloodstream, the simultaneous GLP1R activation counteracts this effect, by stimulating insulin secretion and producing an overall glycaemic control. However, the affinity of OXM for the receptor of GLP1 and glucagon are much lower than the affinities of these hormones. The affinity of OXM is 100-fold and 10-fold lower for GLP1 and GCGR, respectively, in comparison to GLP1 and glucagon. However, pharmacologically doses of OXM that activate both receptors have anti-obesity effects by reducing food intake and increasing energy expenditure [29]–[31]. Besides, OXM also is able to delay food intake. In obese patients, independent of whether they had T2D, infusion of OXM is able to stimulate GSIS and reduce glycaemia [32]. These recent findings have raised the development of GLP1R/GCGR co-agonists and GIPR/ GLP1R/GCGR tri-agonists to treat T2D that, so far, have had promising anti-obesity effects in preclinical studies and are being studied in clinical trials [33].

1.1.2 PYY

PYY is co-expressed with GLP1 from L-cells. In contrast to GLP1, PYY abundance in the upper part of the GI tract is very low and is predominantly secreted in the colon [34], [35](Figure Steinert 17). Hence, postprandial increase of PYY circulating levels is most probably accomplished though paracrine and neural mechanisms, instead of direct luminal stimulation of L-cells. PYY can be found in circulation in two forms: PYY(1-36) and PYY(3-36). PYY (1-36-NH_2) is secreted from L-cells after being excised from pro-PYY. Full PYY binds many receptors of the Neuropeptide Y (NPY) family, named Y1, Y2, Y3, Y4 and Y5 receptors. Both full PYY and NPY stimulate food intake. However, the cleaving product of PYY by DPP4, PYY (3-36-NH_2) is the main form and selectively binds Y2 receptor, which is the responsible for the anorectic effect. Increased satiety after a gastric-bypass surgery has been attributed to elevated levels by several folds of postprandial PYY[22]. There are probably different regulatory mechanisms for Y2R depending on the species, since Y2R seems to be activated in the vagus nerves in rats but it appears to be different in humans [36]. Nevertheless, both PYY(1-36) and PYY(3-36) are responsible for the so-called “ileal break”, which consists on the inhibition of gastric and pancreatic secretions and the motility in the proximal gut and contributes partially to the satiating effect of PYY [37], [38]. Administration of exogenous PYY potently decreases food intake in lean and obese individuals [37], [38]. However, at high doses, it produces nausea and, for this reason, PYY is not considered as a valid candidate for an anti-obesity drug [39]. PYY does not have any effect in glycemia control or insulin secretion. However, it has been suggested that it has protective effects on pancreatic β-cells [40].

1.1.3 CCK

Cholecystokinin (CCK) is produced by I-cells, located densely in duodenum and jejunum, more densely expressed in the proximal than in the distal jejunum, and less expressed in ileum [41], [42]. Besides, it is also expressed in the brain. The various forms of CCK are derived from a 95-amino acid peptide, pro-CCK, and are classified regarding their number of amino acids: CCK-5, CCK-8, CCK-22, CCK-33, CCK-39, CCK-58, CCK-83. Whilst in circulation and in the intestine the most abundant forms are CCK-22 and CCK-33, CCK-8 is the most abundant form in the brain. CCK binds two receptors: CCKAR (or CCK1R) and CCKBR (or CCK2R) [43]. CCK suppresses food intake and delays gastric emptying [44]. In contrast to other anorexigenic enterohormones, CCK induces satiety by reducing meal size without having an effect in the sensation of fullness and other meal-related

sensations [45]. Although infusion of exogenous CCK reduces glucose after an oral glucose load, it does not have an effect on insulin secretion. Moreover, the glucose-lowering ability is lost when the glucose load is administered in the duodenum. This finding indicates that CCK does not have a direct insulinotropic effect and suggests that it has an indirect effect on glucose levels through the delay of gastric emptying [46]. In T2D patients, CCK secretion is reduced [47] and exogenous CCK infusions improve their insulinemic and glycaemic profile [48]. Although studies on whether CCK levels are altered in obesity have controversial results [49] there is extensive evidence that deregulation of CCK is associated with obesity. Certain CCKAR polymorphisms are related with increased appetite and obesity [50]. Moreover, some allelic variations in the CCK are more prevalent in obese subjects [51]. Studies in rodents revealed that obese subjects have reduced responsiveness of exogenous CCK. Mice fed with high-fat diet exhibit smaller electrophysiological response in the vagal afferent nerves than lean mice [52] and so did leptin-resistant high-fat fed rats [53]. Since CCK infusion has not shown any adverse effects, CCK receptor agonists could be an adequate therapeutic target for T2D and obesity.

1.2 Orexigenic enterohormones

Up to date, only ghrelin has been well characterized as an orexigenic enterohormone. In humans, ghrelin is mainly produced in the stomach and proximal small intestine by P/D_1 cells, which are also referred as X/A cells in rodents. While P/D_1 cells are are mostly of the closed type in the stomach, in the proximal small intestine, they are open cells. Ghrelin is active through Growth hormone secretagogue receptor (GHS-R), also known as ghrelin receptor, when it is acylated with an eight-carbon octanoyl group by the enzyme ghrelin O-acyltransferase (GOAT) within P/D_1 cells [54], [55]. However, nonacylated ghrelin is also secreted by P/D_1 cells, although its physiological role is unclear. Ghrelin is produced before food ingestion and, although the particular mechanism by which ghrelin levels rise are not fully understood, it has been observed that its levels are risen particularly when food intake occurs at a regular time every day and it has been suggested that the sight or smell of food could act as stimulants. Since *in vitro* noradrenalin stimulates ghrelin secretion, and this and β1-adrenergic inhibitors supressed this stimulation, it is believed that the sympathetic nervous system can be involved in the control of ghrelin secretion. Levels of circulating plasma are regulated by levels of GOAT, and they are reduced upon food intake. In cultured gastric epithelium,

ghrelin secretion is stimulated by 1mM glucose (blood glucose levels below the normo-glucose concentration of 5mM). Instead, 10 mM glucose inhibits ghrelin secretion. In addition, fatty acids also suppress ghrelin secretion [56], [57]. Moreover, ghrelin secretion seems to be stimulated by paracrine mechanism involving somatostatin produced from neighbouring D-cells, in addition to other neuronal mechanisms. The mechanism of regulation of secretion of ghrelin resembles those of glucagon release, since its secretion from α-cells in the pancreas is inhibited with prandial glucose levels, insulin release and neuronally. Ghrelin levels are correlated with insulin resistance and development of diabetic mellitus type 2. However, it remains to be clarified whether antagonising ghrelin could be a therapeutic target to treat these metabolic diseases. GOAT inhibitors and GHS-R antagonists are being studied in mice, showing promising results in body weight reduction [58]. As discussed in the following section (2. Principal target organs of enterohormones), ghrelin is the enterohormone with the most known effects and that exerts regulatory functions in all body systems.

Besides ghrelin, recent findings about INSL5, which is produced in distal but not proximal L-cells, and motilin, produced in duodenal and jejunal M-cells, point at their possible orexigenic role, but these findings await verification [59].

1.3 Enterohormones without an effect on food intake

In addition to the above mentioned, there are other enterohormones which do not regulate food intake but that are also important for our physiology, including somatostatin (SST), glucose-dependent insulinotropic peptide (GIP) and serotonin (5-HT) [60]–[62]. SST is secreted in fasted conditions and acts as an inhibitory control of acid gastric secretion. GIP, alongside with GLP1, is vital for insulin secretion. 5-HT, which functions are widely studied in the regulation of neurological functions, has newly described functions in the gut, where it is predominantly produced. SST, GIP and 5-HT are produced by D-cells, K-cells and enterochromaffin cells, respectively. D-cells are located in the stomach, K-cells are located in the proximal small intestine and enterochromaffin cells constitute around 50% of the total EECs and are found equally scattered along the gut, from the stomach to the distal colon.

SST is released in the fasted state and inhibits acid gastric secretion, but it is rapidly inhibited upon food ingestion, as a response form vagal acetylcholine detected by muscarinic receptors on D-cells [60]. However, short after ingestion, during digestion,

SST levels rise again when stimulated by distal-proximal regulatory loops involving GIP, CCK and GLP1.

GIP, together with GLP1, acts as an incretin inducing 70% of the postprandial secretion of insulin [63]. GIP also affects insulin function through the stimulation of insulin biosynthesis, promotion of β-cell proliferation and inhibition of β cell apoptosis [64]. The effect of GIP on insulin are drastically attenuated in T2D individuals [65], probably because of the downregulation and desensitization of its receptor [66], [67], which may be a main cause of impaired insulin secretion in these patients. Contrastingly, GIP is often found elevated in obese individuals and has some obesogenic effects [68]. GIP stimulates osteopontin expression in adipocytes, which is related to systemic low-grade inflammation associated with obesity. Paradoxically, in hypo and euglycemic conditions, GIP also stimulates glucagon secretion in healthy individuals [69] but, in T2D patients, this glucagonotropic effect is much more accentuated [70]. Therefore, due to the conflicting effects of GIP in metabolism, it is not considered a viable therapeutic target for the treatment of T2D.

Besides around 90% of the total 5-HT is produced by enterochromaffin cells, 5-HT is also produced by platelets and the central nervous system (CNS). 5-HT has been widely studied as it is an important signalling molecule in both central and peripheral nervous systems, which deregulation is implicated in the basis of many neurological diseases like anxiety and depression. However, its role in the gut has been long only confined to the stimulation of gastric and intestinal motility [71]. More recently, it has been defined that 5-HT is implicated in the regulation of inflammatory response to pathogens in the gut [72] and plays an important role in metabolism. 5-HT, in fasting condition, acts on the liver similarly to glucagon in the regulation of euglycemia by increasing gluconeogenesis and glycogenolysis and inhibiting glucose uptake and glycogen synthesis. In white adipose tissue, 5-HT inhibits browning and stimulates lipolysis, which produces the substrates needed for hepatic gluconeogenesis: free fatty acids and glycerol. In brown adipose tissue, 5-HT attenuates thermogenesis, which results in energy conservation and increased weight gain [73]. In addition, enterochromaffin cells-derived 5-HT attenuates the secretion by peripheral organs of chemokines with glucose-lowering abilities like adiponectin from adipose tissue [74] and osteocalcin and lipocalin 2 from bones [75]. Although the clear involvement of 5-HT in metabolism, how it is related with obesity and T2D remains unclear. Since 5-HT receptors are heterogenous an widely expressed in many organs, they are not a viable therapeutic target [76].

2. Endocrine pancreas and food intake, two main physiological functions modulated by enterohormones.

Enterohormones exert their function through binding G-protein coupled receptors (GPCRs). For each enterohormones, there is at least one matching GPCR. GLP1 receptor (GLP1R) is responsible for the detection of GLP1(7-36) actions in many organs, while the receptor responsible for the detection of GLP1(9-36), which has GLP1R-independent roles, still under study [23]. PYY acts through the NPY receptor family, Y1,Y2, Y3, Y4 and Y5, which modulate different PYY functions [77]. CCK receptors CCKAR (or CCK1R) and CCKBR (or CCK2R), are also expressed differently in the body [78]. Lastly, ghrelin is detected exclusively by GHSR [54].

2.1 Food intake is regulated by enterohormones through the central nervous system and the vagus nerve

The CNS integrates the metabolic status signals in the hypothalamic arcuate nucleus (ARC). ARC expresses a large range of hormone and nutrient receptors and its strategic position close to the median eminence, a circumventricular organ with permeable capillaries and thus, not fully protected by the blood brain barrier, allows the arrival of the metabolic signals [79]. Subpopulations of ARC neuros haven been identified to express satiety regulating neuropeptides: the orexigenic peptides Agouti-related protein (AGRP) and NPY, and the anorexigenic peptides pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART). AGRP/NPY neurons and POMC/CART neurons mutually inhibit each other when activated [79]. The area postrema (AP) is another circumventricular organ analogous to the hypothalamic ARC, located in the caudal brainstem and integrates metabolic signals including enterohormones and other signals like leptin and amylin. The AP projects to the nucleus tractus solitarius (NTS), which responds activating other brain areas. The signalling result of enterohormone activation of the vagus nerve (explained below) is integrated in the NTS.

Some enterohormones can directly act on the neurons of ARC (**Figure 5**). In the case of GLP1, it has been reported that one GLP1 agonist, liraglutide, directly activates the anorectic POMC neurons in rodents and indirectly inhibits orexigenic AGRP/NPY neurons in the ARC to reduce appetite [80]. In an independent way from the regulation of food intake, GLP1 also inhibits the water intake via CNS-dependent mechanisms, although the exact mechanisms are not established yet [81]. In addition, the action of GLP1 in GLP1R expressed in the dorsomedial hypothalamic neurons increases the

energy expenditure by activating the brown adipose tissue (BAT) activity [82]. Besides, GLP1 modulates important neuronal activities such as learning and memory by the regulation of corticosterone, aldosterone, and adrenocorticotropic hormone (ACTH) secretion from the hypophysis-pituitary axis [83]. Although elevated plasma levels of PYY results in reduced food intake, injections of PYY on the hypothalamus revealed that depending on the particular receptor from the NPY family that is activated, PYY can exert an orexigenic or anorexigenic effect. While direct stimulation of Y1 and Y5 receptors and knockout of these receptors have an orexigenic effect, Y2 stimulation leads to an anorectic effect [84]. Similarly to GLP1, PYY infusion in humans has been related with increased energy expenditure and body temperature with could be an effect of increased thermogenesis, although the specific mechanism has not yet been defined [85]. CCK receptors are also expressed in the (CNS). While CCKAR is expressed in limited areas of the brain (NTS, AP, interpeduncular nucleus, posterior hypothalamus and nucleus accumbens), CCKBR are widely present and predominates in the CNS [86]. Despite the presence of CCK receptors in the brain, intestinally produced CCK cannot cross blood brain barrier, and it the exerts its anorectic effect through the receptors in the vagus nerve. However neocortical pyramidal neurons also express CCK that acts locally by regulating neurophysiological functions, such as neocortical development and regulating pain, anxiety and memory [87]. Accordingly, the orexigenic effect of ghrelin is regulated by activating AGRP/NPY neurons that express GHSR, while inhibiting POMC/CART neurons in the ARC [88]. Despite the hypothalamus is the main target of ghrelin in the CNS, it activates other areas of the brain where it exerts important roles of body development, including the stimulation of the GH and sex hormones [89]. Interestingly, it is through the appetite regulation neuronal pathway and not via the GH-mediated neuronal pathways that ghrelin increases adiposity [90].

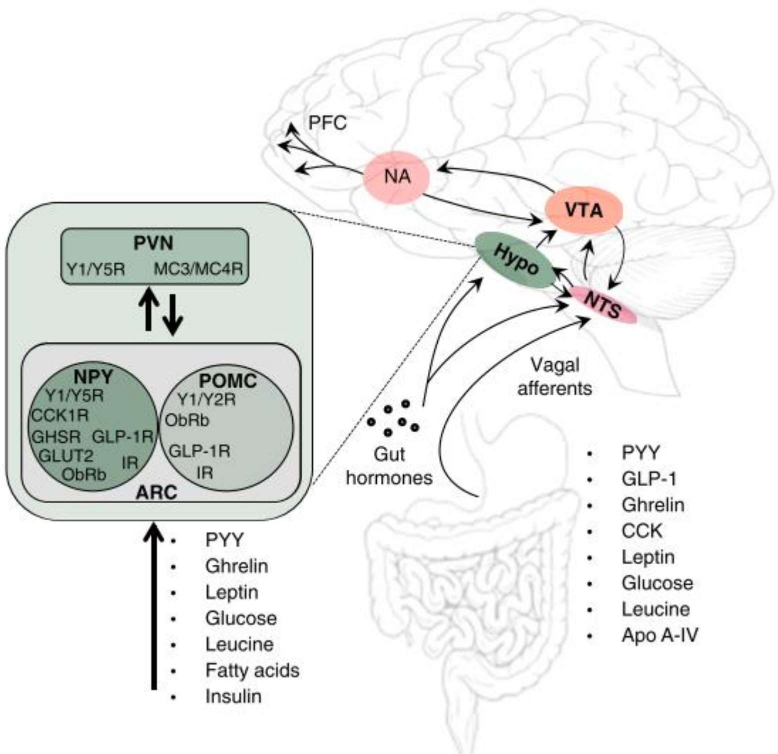

Figure 5. Integration of satiety signals by the central nervous system. ARC: arcuate nucleus; NA: nucleus accumbens; NTS: nucleus of the solitary tract; PFC: prefrontal cortex; VTA: ventral tegmental area; GLUT2: glucose transporter 2; IR: insulin receptor. Adapted from [91].

Brain responds not only to the direct effects of enterohormones, but EECS are the first level of interaction of nutrients with the neuronal circuits that will activate the signals that will be integrated in the brain [5]. Afferent terminals of neurons of the vagus nerve are infiltrated in the lamina propria of intestinal mucosa and receive enterohormones released from EECs in the intestinal epithelium.

GLP1 inhibits gastric emptying though GLP1R expressed in the vagus nerve, since GLP1R-KO [92], denervation of vagal afferent nerves [93], and GLP1R blocking with exendin (9-39) resulted in increased gastric emptying. Moreover, the GLP1 (7-36) agonist exendin-4 is not able to regulate gastric emptying in GLP1R-KO mice [93], which father confirms the GLP1R- dependency of GLP1 in the regulation of gastric emptying. Reduced gastric emptying as result is one of the various mechanisms by which GLP1 improves glycaemia [94].

The anorectic effect of PYY(3-36) requires the vagal sensing from the GI through Y2 receptors tract to the CNS. Despite several Y-receptors are expressed in the small

intestine and colon, only Y2 is expressed in the myenteric plexus neurons of the vagus nerve that can be found in the muscular layer of the intestine and is responsible for mediating the ileal break delaying gastric emptying [95]. As mentioned above, the anorectic effect of CCK is mediated only via the vagus nerve, through CCKAR, as vagotomy prevents this effect in rodents [96]. Vagal afferent nerves express CCKAR but not CCKBR in the lamina propria, mainly in small intestine. CCKARs are expressed in the vagus nerve in the intestinal mucosa, where they suppress motility of the proximal stomach, duodenum and colon, while they stimulate jejunum motility. Unlike other enterohormones, CCK does not only control intestinal motility through its receptors expressed in the afferent endings of the vagus nerve. CCK also exerts a break on gastric emptying through CCKAR expressed in the pyloric sphincter, the muscle ring located at the bottom of the stomach the controls the passage of stomach juices and partially digested food into the duodenum [44]. Ghrelin binds GSHR expressed in the gastric vagal afferent nerves which are partly involved in the regulation of ghrelin-induced food intake. In addition, ghrelin stimulates in the gastric acid secretion and gastric emptying via the vagal nerves. While when blocking the gastric afferent nerves, the effect of ghrelin on food intake is reduced, ghrelin analogues given after vagotomy revealed that the vagal afferent nerves are not essential for the orexigenic effect of ghrelin [97]. Moreover, atropine blocking of the vagal afferents and vagotomy abolished acid gastric secretion induced by ghrelin [98].

2.2 Regulation of glycaemia by enterohormones through effects on endocrine pancreas

Endocrine pancreas is the most studied target organ of GLP1, since GLP1 exerts its incretin actions through the modulation of pancreatic function. GLP1 induces glucose-dependent insulin synthesis and secretion, while inhibiting glucagon secretion. Moreover, it promotes β-cell proliferation and inhibits apoptosis and detrimental NO synthesis during diabetes. GLP1 exerts these functions on the pancreas by humoral and neuronal mechanisms. On the one hand, GLP1 directly exerts its insulinotropic effect via GLP1R expressed on β-cells. Moreover, GLP1 indirectly inhibits glucagon secretion from α-cells through GLP1R expressed in pancreatic δ-cells, which produce somatostatin that reaches α-cells through the somatostatin receptor SSRT2. On the other hand, upon GLP1 release from the intestine and activation of subjacent vagal afferent nerves, neurons of the dorsal medium vagus modulate endocrine secretion through neural input [99]. Contrasting findings in mice have suggested that GLP1 is not only produced in the

intestine, but also in α-cells through an alternative processing of the proglucagon gene, and that it could contribute to the incretin effect or have a paracrine role, but these findings await verification [100].

Although PYY does not have a direct role in the endocrine pancreas, PYY involvement in the regulation of pancreatic function is revealed by the elevated PYY together with GLP1 levels after bariatric surgery to treat T2D, which improves pancreatic function. However, several studies with GLP1 agonists in bariatric surgery models revealed that GLP1 is not responsible for the improvement on pancreatic islets [22], since it increases initially after the surgery but not significantly and in a sustained way. Thus, the focus has shifted to PYY, since chronic treatment of PYY in diabetic rats subjected to bariatric surgery has been shown to improve insulin and glucagon release. Reduced appetite and body weigh after bariatric surgery have been associated with increased PYY levels [17], that act through Y2 receptors in the hypothalamic ARC and in the vagus nerve. However, among the receptors expressed in pancreatic islets, Y2 is the least expressed, indicating that PYY acts on pancreatic function through a different pathway than appetite regulation. While chronic treatment with PYY (3-36) of obese and diabetic rodents has been seen to improve glucose homeostasis, acute treatment for 60 minutes with PYY has no effect on plasmatic glucose levels, revealing that the benefits of PYY in pancreatic function occur only in the long term [101]. Besides, PYY directly regulates exocrine secretion of enzymes from the pancreas by Y1 receptors [102].

Regarding ghrelin, it is involved in glucose homeostasis because pancreatic islets, liver and, to a lesser extent, muscle express GHSR. In addition, it has been described that ghrelin inhibits glucose-induced insulin secretion and decreases insulin sensitivity, while increasing glucose tolerance. In sum, although the mechanisms are not still fully characterized, ghrelin is involved in a negative loop in the regulation of blood glucose levels. GHSR is expressed in δ-cells and activate somatostatin that inhibits insulin secretion via SSRT5 receptors expressed in β-cells. Besides, ghrelin inhibits pancreatic exocrine activity [103].

Regarding the role of CCK in the pancreas, both CCKAR and CCKBR are found in the pancreas [104]. Although CCK does not stimulate any endocrine secretion in the pancreas, it exerts an exocrine activity, stimulating meal-induced pancreatic enzymes secretions [99].

2.3 Enterohormones target several other tissues in the body to exert physiologic functions

In addition to the above-mentioned main effects of enterohormones mediated through receptors in CNS and pancreas, enterohormones have many physiological roles due to the wide distribution of their receptors in other tissues. In this section, we will briefly summarize them.

In the liver, GLP1 has been shown to inhibit hepatic gluconeogenesis and lipogenesis, while stimulating the uptake of exogenous glucose and lipid oxidation, which contributes to the its glycaemia-reducing effect. Since GLP1R is barely expressed in hepatocytes, it is suggested that the beneficial hepatic metabolic effects of GLP1 are mainly achieved by the modulation of pancreatic hormones [105]. On the contrary, ghrelin contributes to increasing adiposity in the liver by an increase in lipogenic and glucogenic enzyme expression [106], [107].

Enterohormones contribute to the regulation of adiposity through direct modulation in the adipose tissue. Controversial effects have been described GLP1 on adipocytes depending on the species. While GLP1 promotes lipogenesis and inhibits lipolysis in rodents [108], the GLP1-agonist liraglutide has a lipogenic-reducing effect in humans through the inhibition of cell growth and differentiation in human adipose stem cells (ASC) [109]. Other enterohormones seem to have fat-depot specific effects. PYY inhibits lipolysis through Y1 receptors [110] which are higher expressed in the subcutaneous fat [77]. Contrastingly, CCK promotes triglyceride storage in human adipocytes through CCKBR [111], in subcutaneous and visceral fat depots [112]. Ghrelin promotes adiposity through the increased expression of storage enzymes in adipocytes in the abdominal fat depot [107].

Both GLP1 and PYY exert functions within the GI tract different from those related to the vagus nerve activation. GLP1 increases intestinal surface, in an alternative pathway to that well-established of GLP2 [113]. PYY inhibits duodenal electrolytes and fluid secretion through the epithelial Y-receptors [114]. Regarding digestion, CCK is responsible for meal-induced bile acid secretion through CCKAR expressed in the smooth muscle layer of the gallbladder [44].

Enterohormones act as natural anti-inflammatory agents, reducing pro-inflammatory gene expression and macrophage adhesion, as established for GLP1, PYY, CCK and ghrelin [42], [115]–[118]. Moreover, ghrelin receptors expressed in lymphocytes directly

regulate the synthesis of anti-inflammatory cytokines and cellular differentiation and proliferation. In several organs such as heart, skin and kidneys, ghrelin exerts an antifibrotic action [118]

The improvement of the cardiovascular output and reduced blood pressure with a prolonged treatment of T2D with GLP1R agonists revealed the relevance of GLP1 in the regulation of cardiovascular function. GLP1 inhibits cardiomyocyte apoptosis, improves endothelial dysfunction related to insulin resistance and ameliorates cardiac outcome after heart failure [119], [120]. Some GLP1 cardioprotective effects occur independently of GLP1R, as revealed by knockout studies [121], and are attributed to its cleaved form, GLP1(9-36). While GLP1(7-36) has been shown to controls cardiac contractility through GLP1R-dependent signalling, GLP1(9-36) has a GLP1-R independent role in vasodilation and improving recovery after cardiac injury [122]. The increased vasodilation induced by GLP1 has also a direct effect on other physiological functions such as renal function, since it increases glomerular filtration rate [123], and improves insulin sensitivity in skeletal muscle, which enables a better irrigation of insulin [124], [125]. Moreover, while PYY has been shown to have a vasoconstrictive effect [114], ghrelin stimulates of vasodilation and have other cardioprotective effects such as above-mentioned antifibrotic activity that reduces prevents atherosclerotic plaque formation, and inhibition of cardiomyocyte apoptosis [118].

Finally, enterohormones are also important regulators of bone density. While osteoclast activity is activated by PYY [126], GLP1 has been shown to reduce it [127]. Similarly, ghrelin promotes the increase of bone density by activating osteoblasts [128].

3. Enterohormone secretion: stimulators of enterohormones release and the mechanisms to sense them in enteroendocrine cells

EECs release the enterohormones in response to various stimuli present in the lumen including nutrients, non-nutrients chemicals, food-born toxins and microorganisms[2], [5]. In the small intestine, digestion is a prerequisite for nutrient to activate most EECs. Carbohydrates, lipids and proteins seem to be sensed in the form of glucose, fatty acids and amino acids, respectively [129]. EECs sense contents in the intestinal lumen through an extensive array of chemosensors. The sensing of nutrients occurs through GPCRs, coupled to various G proteins, although nutrient receptors are predominantly coupled to G_q [27]. In addition to nutrient sensing in the lumen, nutrient absorption has also been

identified as an obliged step for monosaccharides and lipids to stimulate EECs. The importance of nutrient absorption on enterohormone secretion is evidenced by the incretin effect of GIP and GLP1 since they stimulate insulin secretion, which, at its turn, regulates the absorbed levels of glucose in blood. Therefore, the incretins are relevant when glucose reaches the bloodstream, rather when it is present in the lumen of the GI tract.

According to Steinert *et al.*, while all EECs are stimulated by the luminal content of the gut, this kind of stimulation is believed to only occur in the EECs from distal gut when absorption rate in proximal gut is exceeded by the infusion rate of food intake [4]. Accordingly, it is generally reported that CCK and GIP secretion are stimulated with low infusion rates whilst PYY and GLP1 need larger infusion rates to be directly stimulated by luminal content [130], [131]. However, proximal-distal regulatory loops are being studied to understand the rapid rise of GLP1 in plasma after intake, when nutrients have not already reached the distal gut.

3.1 Macronutrient stimulation of EE

3.1.1 Carbohydrates stimulation of enterohormone release requires glucose absorption

As before mentioned, digestion is essential for EECs recognition of carbohydrates, as revealed by the inhibition of carbohydrates digestion with α-2-glucosidase inhibitors [93]. Glucose is a strong stimulator of GIP and GLP1 release, and a milder stimulator of CCK release. L-cells are activated by glucose uptaken by the Sodium-glucose transporter 1 (SGLT1) and K^+ ATP channels (leading to a cell depolarization induced by Na^+ and K^+), in addition to sweet taste receptors [5] (**Figure 6**). As before mentioned, glucose absorption, that in this case occurs through SGLT1 is more relevant than luminal sensing for the stimulation of GLP1 secretion. Glucose uptake together with two Na^+ ions by SGLT1 triggers membrane depolarization and Ca^{2+} entry in the cell, required for GLP1 secretion. In this direction, *sglt1*-knockout mice showed reduced GIP and GLP1 plasma levels [132]. In a normal situation, glucose does not reach the distal part of the GI tract, where most of L-cells are located. However, after a bypass surgery and in the case of *sglt1*-knockout mice, more glucose reaches the distal gut. In addition, in these cases, higher concentration of both PYY and GLP1 are defined. Thus, distal L-cells seem to have an alternative glucose sensing mechanism, independent of SGLT1, that could detect glucose itself or microbial metabolites derived from glucose, as short chain fatty acids (SCFAs). The low Km of SGLT1 for glucose makes of it an excellent sensor of luminal

glucose after food intake, that stimulates the secretion of GIP and GLP1 at the same time as glucose is being absorbed in the blood. However, expression of glucose transporter (GLUT) transporters like GLUT2 in the basolateral membrane L-cells also play an important part in the interaction of EECs with postprandial glucose. The concentration of glucose inside L-cells seems to be dependent by GLUT-mediate uptake of elevated plasma glucose levels. In this regard, GLUT2 knockout mice exhibit impaired GLP1 intestinal content and secretion [132].

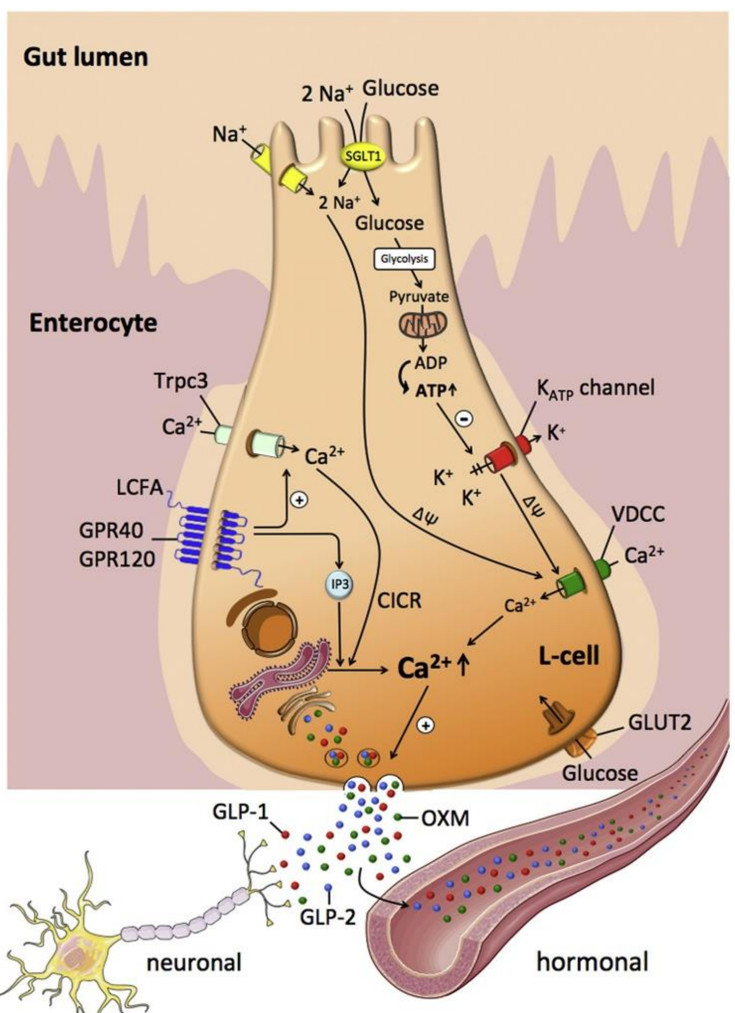

Figure 6. Nutrient-induced stimulation of GLP1 secretion in L-cells. CICR: calcium-induced calcium release; LCFA: long-chain fatty acids, GLUT2: glucose transporter 2; GLP-1: glucagon-like peptide-1; GLP-2: glucagon-like peptide-2; OXM: oxyntomodulin; Trpc3: transient receptor potential channel 3; VDCC: voltage-dependent calcium channel; SGLT1: sodium/glucose co-transporter 1. Adapted from [81].

In contrast to other EECs, digestion of carbohydrates may not be a requisite for I-cells to sense them since inhibition of their digestion with α-glucosidase had no effect in CCK response [133].

Luminal fructose induces GLP1 and CCK secretion, but not GIP, glucagon or insulin secretion [134]. Fructose is absorbed through GLUT2 and GLUT5, which translocate from the basolateral membrane to the apical side of enterocytes. Since the absorption does not generate a cell depolarization [135], it is suggested that its metabolization is required for the stimulation of enterohormone secretion.

Carbohydrates are also sensed by sweet taste receptors present in the gut. Sweet receptors belong to the taste receptor type 1 (TAS1R) family and are formed by combination of TAS1R2 and TAS1R3 [136]. As it happens in taste buds in the tongue, the heterodimeric taste receptor TAS1R2/TAS1R3 in the intestine is coupled to α-gustducin and activates phospholipase C-β2 and the calcium-sensitive transient receptor potential cation channel subfamily M member 5 (TRPM5) that induce cell depolarization, which in the intestine have been related to GLP1 secretion [137]. Until now, the presence of sweet receptors has been confirmed in L-cells [138]. In humans, when sweet taste receptors are pharmacologically blocked, GLP1 and PYY secretion are attenuated [138]. However, the direct involvement of sweet taste receptors in enterohormone secretion is controversial since studies in rats revealed that glucose-induced incretin secretion (GLP1 and GIP) is totally independent of sweet taste receptors and TRPM induced calcium influx[139]. Nevertheless, an indirect link regarding sweet taste receptors and enterohormone secretion has been suggested as sweet tasting molecules directly stimulate the translocation of SGLT1 to the apical membrane of enterocytes[26].

The involvement of K^+ ATP channels in glucose sensing in EECs is unclear, since inhibitors perfused in rat small intestine elevated GLP1 levels, although in *ex vitro* human intestinal biopsies this effect was lost [132].

3.1.2 Proteins activate secretion of CCK and GLP-1

Although it is known that EECs respond to amino acids and peptides, what are the receptors or transporters that stimulate these cells is still largely unknown. Nevertheless, several GPCRs including Calcium sensing receptor (CasR), the umami receptor, GPRC6A, GPR92 and sodium-coupled transporters have been identified to play an endocrine role in the gut, with larger evidence for GLP1 stimulation, as recently reviewed by our group [140].

CasR, which is coupled to the G_q protein, is expressed in P/D_1 G, D, I and L-cells and detects aromatic and basic amino acids [141], [142]. *In vitro* studies showed that, I-cells, which express CasR, show intracellular calcium increase in response to amino acids. Moreover, when CasR is pharmacologically inhibited, CCK and GLP1 response to L-amino acids like L-phenylalanine and L-tryptophan is impaired, suggesting an important role of CasR in the sensing of proteins in I-cells and L-cells. However, CasR-knockout did not have an effect *in vivo* [143], [144]). Accordingly, CasR activation in P/D_1-cells, inhibits ghrelin secretion [141].

Another *in vitro* study with GLUTag cells revealed that GPRC6A, which senses basic and neural aminoacids, is responsible for sensing luminal L-ornithine that results in GLP1 secretion [145]. Not only GLP1 secretion has been observed in response to protein I *in vitro* studies, but also CCK. GPR92 senses protein hydrolysates and peptones and is expressed in G and D-cells [146], [147] and is able to stimulate CCK but not GLP1 secretion in murine STC-1 cells [148].

Other *in vitro* studies revealed that sodium-coupled transporters SLC38A2, SLC6A19, and SLC15A1 (PEPT1) are activated by amino acids and peptides which results in membrane depolarization [149], [150]. While SLC38A2 and SLC6A19 are responsive to neutral amino acids, SLC15A1 (PEPT1) senses di- and tripeptides in L-cell [151], [152]).

Umami taste receptors include mGluR1, mGluR4 and TAS1R1/TAS1R3, which belongs to the same family as sweet taste receptors, TAS1R, but they are formed by a different combination [136]. Only mGluR1 and TAS1R1/TAS1R3 are expressed in the GI tract [153]and, while mGluR1 is specific for sensing glutamate, TAS1R1/TAS1R3 heterodimer senses a large range of L-amino acids including glutamate and aspartate [136]. Although the expression of these receptors in EECs has not yet been confirmed, it is known that L-glutamate induces CCK secretion from STC1 cells and porcine duodenal tissue through activation of phospholipase C (PLC) by T1R1/T1R3 heterodimer [154], [155].

3.1.3 Absorbed fatty acids stimulate a broad range of enterohormones

Lipids are a stimulus for GIP, CCK, PYY and GLP1 secretion. As it happens for carbohydrates, digestion and absorption are required to stimulate enterohormone secretions. In fact, stimulation of EECs by lipids is dependent on luminal lipase activity that hydrolyses ingested triacylglycerols (TAGs). In contrary to what happens with carbohydrates, risen plasma levels of TAGs and non-esterified fatty acids (NEFAs) do

not stimulate EECs [156]. GIP and CCK release after oil ingestion is disrupted when digestion is impaired or when absorption is inhibited by pharmacologically inhibition and knockout of fatty acid transporters, like CD36, DGAT1, MGAT2 and FABP5, which are involved in the monoacylglycerides and long chain fatty acids (LCFA) uptake, and formation of chylomicrons [157].

Different infusion rates are required to stimulate the different enterohormones. While a low amount of lipids is effective to stimulate CCK secretion, higher infusion rates are required for PYY secretion, probably due to the more distal location of L-cells [130], [131]. More studies are available evaluating the response of fatty acid uptake inhibition by GIP secretion than by CCK secretion. GIP and CCK release are reduced when fatty acid uptake is inhibited, while PYY and GLP1 secretion are paradoxically increased. Thus, whereas EECs in the proximal gut appear to respond to fatty acids when they are digested and absorbed by epithelial cells, PYY and GLP1 secretion may rise due to the increased presence of fatty acids in the colon when they are not metabolized in the small intestine. The free fatty acid receptors FFA1 and FFA4, are highly expressed in K and L-cells [158], couple $G_{\alpha q}$ and they recognise long-chain fatty acids. Moreover, FFA4 seems to be involved in the linoleic acid-induced secretion of CCK from STC1 cells [158]. Another relevant GPCR in the sensing of lipids by EECs is GPR119, which recognises endogenous lipids substrates, products from intestinal triglyceride digestion. GPR119 is coupled to Gαs and has been identified in L-cells [158]. Proximal EECs differ from distal EECs in that EECs in the small intestine express these GPCRs mainly in the basolateral membrane, indicating the responsiveness of these cells to the absorbed and metabolized lipids in this region of the GI tract, while they are found apically in distal EECs. However, GPR119 is also expressed in the basolateral membrane in the distal gut. These differences are also found within the L-cells from different locations. In contrast to proximal L-cells, distal L-cells respond preferably to SCFA [58], which goes in agreement with the hight density of microbiota in colon. SCFA are therefore produced because of the fermentation of dietary fibres and non-absorbed carbohydrates. They are sensed by L-cells though FFAR2 and FFAR, GPCR linked to $G_{i/o}$ and G_q, respectably [145].

Finally, bile acids play an important role in the sensing of lipids by the enteroendocrine system because they are fundamental for emulsification, digestion and make lipids available for the intestinal epithelium.

3.2 Endogenous enterohormones stimulators: bile acids, microbiota and the proximal-distal loop

In the previous section, how the macronutrients acquired from the diet are responsible for EE stimulation has been reviewed. However, diet also plays an indirect role in the modulation of the enteroendocrine system since there are several endogenous components of the GI tract susceptible to the diet stimulus involved in the regulation of EE secretion. Diet-induced bile acid secretion, in its turn, activates distal L-cells, which express the bile acid receptor TGR5 (GPBAR), predominantly coupled to the Gs protein. Through this receptor, luminal bile acids activate GLP1 secretion and have a positive effect on glucose homeostasis [159]. In this direction, the increased GLP1 secretion after a bariatric surgery is partly explained by an also increased bile acid production, which is shown in their increased concentration in plasma [160].

Moreover, diet influences the composition of gut microbiota and, therefore, the production of microbial metabolites [161]. In the colon, microbiota produces a large variety of metabolites among which SCFA [162], secondary bile acids [159], indole [163] and lipopolysaccharide (LPS) [164] are known to be bioactive in the enteroendocrine system. Not only these microbial metabolites stimulate enterohormone biosynthesis and secretion, but also EECs growth and proliferation. The importance of these metabolites was shown in studies with germ-free (GF) mice, which exert increased GLP1 plasma levels and L-cell number [165]. In these studies, microbial colonization of GF mice reduced GLP1. Moreover, the number of EECs was reduced when SCFA-producing bacteria were introduced and when fed with a high-fat diet. Contrastingly, *in vitro* studies in mouse reveal that treatment with SCFA increases the number of GLP1 and PYY producing cells, while in human cell lines, butyrate, a SCFA, increased PYY gene expression. This finding suggests another mechanism by which fermentable fibres can induce satiety, by inducing enterohormone secretion when they reach the colon and are metabolized into SCFA. Several GPCRs expressed in the colon epithelium are associated with the stimulation of EECs by microbial SCFAs (GPR41–FFA3, GPR43–FFA2 and OLFR558) [162], [166], secondary bile acids (GPBAR1) [159], indole (K_v channels) and LPS (toll-like receptors, TLRs) [167]. However, in the case of GPBAR1 and TLR, their activation requires the crossing of the intestinal barrier of bile acids and LPS, respectively, since their expression in the colon is only in the basolateral membrane of the epithelial cells. Thus, this finding points to that the activation of EECs by these microbial metabolites only takes place when intestinal barrier integrity is compromised.

In this case, although GLP1 and PYY induced secretion may contribute to nausea and anorexia, the stimulation of GLP2 by microbial compounds could have an important role in activating epithelial repair pathways and maintaining intestinal integrity [28].

Finally, increasing evidence suggests that a proximal-distal loop exists that stimulates the enteroendocrine secretions of the distal gut. Selective blood sampling from segments of the intestine revealed that treating the duodenum with oil and protein hydrolysates stimulated GLP1 secretion in the distal gut, probably through the intermediate stimulation of gastrin-releasing peptide[168]. Glucose-induced GIP secretion in the proximal gut was first believed to be an appropriate candidate for GLP1 stimulation in the distal gut. In rats, GIP-induced GLP1 secretion was blocked after supradiaphragmic vagotomy, revealing that GIP stimulates GLP1 secretion in this species through the vagus nerve [169]. However, studies in which GIP was infused in the duodenum of pigs revealed that GLP1 does not respond to GIP, indicating, at least, that GIP-induced GLP1 secretion is highly species-specific [170]. In fact, elevated GIP concentrations typically found in T2D humans do not lead to induced GLP1 secretion [171]. Lipid-induced secretion of CCK in the duodenum leads to a small GLP1 response that is blocked with a CCK1 receptor antagonist, thus revealing a modulation of distal GLP1 secretion by CCK. However, since there is no evidence of the expression of CCK receptors in L-cells, the modulation of GLP1 by CCK could indirect, by CCK-induced secretion of bile acids that are stimulators of GLP1 secretion [172]. CCK intravenous infusions in humans also stimulate PYY secretion [173]. In contrast, while infusion of GLP1 decreased PYY secretion, GLP1 blocking with exendin(9-39) increased it [174], revealing an autoregulatory loop of GLP1/PYY secreting L-cells. A recent finding from Stevens *et al.* [175] arises the possibility of GLP1 and PYY autocrine/paracrine regulation in colon, since they detected for the first-time luminal secretion of these enterohormones. This finding contrasts sharply with the well-established idea of basolateral secretion of enterohormones into the bloodstream and activating the vagal afferent nerves. Somatostatin inhibits GIP and GLP1 secretion through Gi-coupled somatostatin receptors, that are known to be expressed in colonic cells. This regulatory mechanism is overcompensated with enhanced somatostatin production in patients treated with GLP1 mimetics [176], [177]. Gi protein seems to be linked to enterohormone release inhibition since the Gi-coupled endocannabinoid receptor Cnr1 is expressed in K and L-cells. Cnr1 is more expressed in K-cells than in L-cells, indicating a preferential inhibition of GIP rather than GLP1 secretion [176]. Moreover, insulin is also implicated in the stimulation of GLP1 secretion, which seems to act as a positive feedback loop on

improving β-cell function [178]. Studies in rats revealed that leptin receptors are also expressed in L-cells and that leptin is able to induce GLP1 secretion [178]. The fact that, in conditions of leptin and insulin resistance, GLP1 response to oral glucose is impaired, reveals the importance of their regulatory loop and that L-cell function is dysregulated in disrupted nutrient homeostasis [179].

3.3 Bitter tastants as enterohormones modulators through bitter taste receptors.

As happens with sweet and umami taste receptors, bitter taste receptors which belong to the taste receptor type 2 family (TAS2R) have been recently found expressed in the GI tract. Increasing evidence indicates that physiological bitter tastants and other pharmacological bitter molecules exert a role in the enteroendocrine system through the modulation of these intestinal bitter taste receptors. Unlike sweet taste or umami taste receptors, which both belong to the TAS1R family, bitter taste receptors are from the TAS2R family. In the case of humans, the TAS2R family consists of 25 subtypes, of which only a subset of all TAS2R subtypes is expressed in the GI tract: so far, 3 and 18 subtypes have been found to be expressed in the small intestine and colon of humans, respectively [180], [181].

Expression of TAS2R subtypes has been found to co-localise in GLP1 producing cells, evidencing the direct link of bitter tastants in the modulation of the enteroendocrine system. Moreover, our research group [182] showed that the flavanol epicatechin gallate (ECG), stimulates ghrelin secretion in MGN3-1, a murine ghrelinoma cell-line through activating a TAS2R, mTas2r39. Blocking mTas2r39 with the specific antagonist 4'-fluoro-6-methoxyflavanone prevented the ECG-induced ghrelin secretion. Beside the few studies linking bitter taste receptors activation with enterohormone secretion, many preclinical and clinical studies evaluate the effect of bitter molecules on the enteroendocrine secretions. Kim *et al.* [183] have shown that human NCI-H716 enterohormone-secretory cell-line respond to denatonium benzoate (DB) and quinine, two known ligands of several TAS2R. They argue that, since TAS2R are coupled to the G- α-gustducin, the activation of the α-gustducin downstream pathway that leads to GLP1 release in the case of quinine and GLP1 and PYY in the case of DB, is due to these two molecules stimulate TAS2R. In addition, intragastric infusion of DB in healthy individuals slows gastric motility and induces satiation [184]. Long-term (4 weeks)

intragastric infusion with DB in obese mice increases meal-induced GLP1 levels and reduces body weight [185]. GLP1 secretion from several secretory cell-lines has been related to the activation of the TAS2R subtype TSA2R38. In NCI-H716 and STC-1, a murine secretory cell-line, berberine-induced stimulation of GLP1 secretion is blocked when silencing the expression of TAS2R38 with siRNA [186], [187]. Phenylthiourea (PTU), a known agonist of TAS2R38, stimulates GLP1 secretion from HuTu-80 cells, an effect that is lost in TAS2R39-KO HuTu-80 cells [180]. In fresh human duodenal and ileal intestinal samples, the stimulation of the TAS2R5 subtype by a known agonist, 1,10-phenanthroline, lead to GLP1 secretion, which was verified by the co-localization of GLP1 with TAS2R5 [188]. Treatments with DB and PTU in STC-1 cells have shown the ability to stimulate CCK secretion [189]. PTU also stimulated TAS2R38-dependent CCK secretion in Caco-2 cells, since secretion was abolished with TAS2R38 specific siRNA [190]. In HuTu-80 cells, *Hoodia gordonii* (H.g.) extract, a known natural appetite suppressant [191] stimulated CCK secretion, which after transfection of TAS2R subtypes in HEK cells and subsequential analysis of cell depolarization, was related to the specific stimulation of TAS2R7 and TAS2R14 [192]. CCK release was also related to increased satiety and reduced intake after intraduodenal quinine treatment in healthy individuals [193]. Accordingly, plasma ghrelin levels were lower in a similar study with intragastric quinine treatment of young women, that also exhibited reduced food intake [194]. In contrast, in a comparable study with intragastric DB treatment of young women ghrelin plasma levels were not affected [195]. Ghrelin secretion has been seen to be increased by intragastric infusion of a mix of bitter molecules, including DB, PTU, quinine and D-[-] salicin, with no effect in CCK and GLP1 secretion [196].

Bitterness is a common sensory attribute of polyphenol-rich food and beverages [197]. A few remarkable studies performed over the last decade have identified most polyphenols as TAS2R ligands, indicating a possible mechanism of interaction with the GI tract. The first evidence of polyphenols interacting with TAS2R was provided by Meyerhof *et al.* [198], that screened the 25 TAS2R subtypes by transfecting HEK-cells with the several TAS2R subtypes, tracing calcium imaging and recording the half-maximal concentration (EC50) to identify bitter compounds, including flavonoids, with the ability to bind TAS2R. Roland *et al.* [199] followed the same methodology in order to find which TAS2R would bind genistein, a polyphenol of the isoflavone type present in soy. They identified that genistein binds TAS2R14 and TAS2R39. Later, they screened a subset of 68 commercially available polyphenol to observe which would bind TAS2R14 and TAS2R39 [200]. Similarly, Soares *et al.* [201] studied the commercially available and

most frequently found polyphenols in red wine, beer, tea, and chocolate: the hydrolysable tannin pentagalloylglucose (PGG), the flavanols (-)-epicatechin (EC), procyanidin dimer B3 and trimer C2, and the anthocyanins malvidin-3-glucoside and cyanidin-3-glucoside. They identified that (-)-epicatechin binds TAS2R4, TAS2R5, and TAS2R39, while PGG only binds TAS2R5 and TAS2R39. Malvidin-3-glucoside binds TAS2R7 and procyanidin trimer C2 binds TAS2R5. Later, they studied subset of 16 polyphenols extracted from grape seeds and oak chips, which included phenolic acids (such as protocatechuic, ferulic, and vanillic acid ethyl esters), flavanols (epigallocatechin gallate (EGCG) and procyanidins dimers) and hydrolysable tannins (punicalagin, castalagin, and vescalagin). They identified that the 12 of the tested polyphenols were able to bind 7 TAS2R subtypes: TAS2R4, TAS2R5, TAS2R7, TAS2R14, TAS2R30, TAS2R39 and TAS2R43 [202] (summarized in table 1).

Table 1. Summary of the human bitter taste receptors (TAS2Rs) activated by polyphenols tested in the study of Soares *et al.* [202].

Compound	TAS2Rs						
	R4	R5	R7	R14	R39	R43	R30
Condensed Tannins							
Procyanidin B1	−	+	+	−	−	−	−
Procyanidin B2	−	−	−	−	−	−	−
Procyanidin B3	−	−	−	−	−	−	−
Procyanidin B4	−	+	−	−	−	−	−
Procyanidin B6	−	−	−	−	−	−	−
Procyanidin B7	−	+	−	−	−	−	−
Procyanidin C1	−	−	−	−	−	−	−
Procyanidin B2g	−	+	−	−	+	−	−
EGCG	+	+	−	−	+	+	+
Hydrolyzable Tannins							
Vescalagin	−	−	+	−	−	−	−
Castalagin	−	−	+	−	−	−	−
Grandinin	−	−	+	−	−	−	−
Punicalagin	−	+	+	−	−	−	−
Ethyl Esters							
Ferulic acid	−	−	−	+	−	−	−
Protocatechuic acid	−	−	−	+	−	−	+
Vannillic acid	−	−	−	+	−	−	−

"+" indicates activation while "−" indicates a lack-of activation. Only the activated TAS2Rs are presented. EGCG: epigallocatechin gallate.

All these studies suggest the binding of flavonoids with TAS2R, but whether this interaction leads to EE release remains largely obscure. Further evidence on whether the effects of polyphenols at stimulating EE release are mediated through TAS2R is required. Nevertheless, although the mechanisms involved are still unknown, the increasing knowledge of how polyphenols modulate EE secretion will be discussed in the next section.

3.4 Enterohormone-stimulating bioactive compounds: Polyphenols

As above mentioned, plant-based diet influxes polyphenols that are bioactive in the enteroendocrine system. Polyphenols are plant secondary metabolites which, unlike nutrients, are not essential dietary molecules. They are classified into flavonoids and non-flavonoids, depending on their chemical structure [203]. Flavonoids are characterized by having two aromatic rings connected by three-carbon atoms that form an oxygenated heterocycle. In their turn, depending on the hydroxylation pattern, flavonoids are classified into flavones, flavonols, isoflavones, flavanones, anthocyanidins and flavanols [204]. Amongst all flavonoids, flavanols, are the most structurally complex subclass and represent one of the main phenolic components of the human diet. They can be found in beans, nuts, apples, grapes, cocoa, tea and wine. Unlike other classes of flavonoids, which are usually found in nature in glycoside forms, flavanols are usually present in the aglycone form or esterified with Gallic acid, the most common non-flavonoid. Flavanols exist in the monomer form and the oligomer and polymer form (proanthocyanidins), of which procyanidins are the most common [205].

3.4.1 Grape-seed proanthocyanidin extract modulates the enterohormone system

A grape-seed proanthocyanidin-rich extract (GSPE) has been widely studied by our research group and others as a source of polyphenols. In rat *ex vitro* intestinal segments, GSPE administered with 10mM glucose stimulates GLP1 secretion in the ileum and the microbial metabolites derived from GSPE digestion stimulate GLP1 secretion in the colon. In contrast, GSPE decreases CCK secretion, an effect reproduced with gallic acid, present in GSPE [206]. Similarly, GLP1 plasma levels in rats are increased after an oral glucose load followed by an acute dose of 1g/kg of GSPE [207]. Accordingly, GLP1 plasma levels are also increased after a subchronic treatment of 8-days with dose of 500 mg/kg of GSPE, that has previously shown to reduce food intake [208]. Moreover,

while GLP1 levels were decreased in insulin resistant rats after 12 weeks of being fed with cafeteria diet, rats that received a cafeteria diet simultaneously to a dose of 25 mg/kg of GSPE, GLP1 production in colonic cells was higher. In addition, GSPE prevented the decline on intestinal DDP4 activity, upregulated the colonic expression of GLP1, PYY and chromogranin A, a marker of EECs, while downregulating the hypothalamic expression of GLP1R [209]. Contrastingly, another study showed that GSPE decreased the DDP4 expression in genetically obese rats [210]. The effects on enterohormone secretions of simultaneous versus corrective treatments of GSPE in cafeteria diet-fed rats were compared in a study [211]. It was shown that both GSPE treatments limited CCK and secretion from the duodenum. Differences were found in the case of GLP1 and PYY. While the corrective treatment with GSPE increased GLP1 levels and decreased PYY levels, the simultaneous treatment had no effect. In addition, the health benefits of a preventive treatment of GSPE, which consisted of a dose of 500 mg/kg administered for 10-days followed by a 17-week cafeteria diet, were related to the epigenetic modulation of the GLP1R expression in the ileum [212]. In regards of ghrelin, while an acute high dose of GSPE increases ghrelin plasma levels in rats, a chronic treatment of GSPE reduces ghrelin plasma levels and ghrelin secretion production in intestinal segments [182]. Similarly, the effects of the GSPE corrective and simultaneous treatments to cafeteria diet revealed a reduced ghrelin production in the stomach [211]. However, when administered simultaneously with a high-fat diet (16.6g/kg of ω-3 polyunsaturated fatty acids) for 24 weeks, GSPE (0.8 g/kg) increases ghrelin production in comparison to the rats only fed with high-fat diet [213].

3.4.2 Other polyphenol-rich extracts may also regulate EE release

Green tea is another rich source of polyphenols with many beneficial health effects. An intervention in T2D humans revealed that a consumption of 1.5 g/day of green tea extract for 16 weeks, significantly increased their GLP1 plasma levels [214]. Contrastingly, a metanalysis has revealed that green tea consumption for long periods is associated with increased ghrelin levels [215]. Several interventional studies with other polyphenol-rich extracts have been recently performed in healthy subjects. Polyphenol-enriched biscuits resulted in an increase in PYY levels [216], blueberry extract is able to increase GLP1 and PYY levels [217], bergamot juice reduces ghrelin secretion and improves insulin resistance and homeostasis [218] and polyphenol-rich cocoa after an oral glucose tolerance test promotes GLP1-induced insulin secretion [219]. Accordingly, a proanthocyanidin-rich extract from cacao has been shown to stimulate

GP1-induced insulin secretion in mice, which alters the clock gene expression [220]. Decaffeinated coffee, with a high content of phenolic acids such as chlorogenic acid [221], is able to increase PYY and prevent high-fat diet-induced non-alcoholic fatty liver disease (NAFLD) in mice [222]. In addition, a black soybean seed coat polyphenolic extract modulates the synthesis of NO through GLP1 stimulation [223]. Extracts have also been tested *in vitro*. NCI-H716 cells treated with polyphenol extracted from sprouted quinoa yogurts exert increased GLP1 secretion [224].

3.4.3 Pure polyphenols as enterohormone secretagogues

Pure polyphenols have also been studied as enterohormone secretagogues. An 8-week treatment with isoflavones is able to increase PYY plasma levels in healthy postmenopausal women [225]. In ovariectomized and high-fat fed rats, a 4-weeks treatment with isoflavones decreases ghrelin and increases CCK plasma levels, while reducing body weight, abdominal fat and food intake [226]. In rats with induced diabetis, genistein, an isoflavone, is able to induce GLP1 secretion alone and in combination with metformin [227]. Epigallocatechin gallate (EGCG) stimulates GLP1 and CCK secretion from murine *ex vitro* intestinal segments [228]. In a subchronic treatment of 8 weeks, 100 µg/kg of EGCG is able to increase GLP1 and PYY secretion in high-fat-fedmice [229]. In contrast to that observed with GSPE, an oral glucose load is not needed in the case of cinnamtannin A2, a tetrameric procyanidin, when administered in an acute dose of 10 mg/kg to increase GLP1 and insulin levels [230]. Moreover, myricetin has been shown to enhances the levels of circulating GLP1 through a potent inhibition of DPP4 [231]. In contrast, while resveratrol increases ghrelin in diet induced obese rats [232], it reduces GLP1 secretion in genetically induced diabetic rats [233]. Other pure polyphenols have been assayed in *in vitro* studies. In murine STC1 cells, curcumin and tiadenol A, a polyphenol derived from microbial fermentation of tea, induce GLP1 secretion [234], [235]. In addition, naringenin and hesperetin induce CCK secretion through transient receptor potential (TRP) channels [236], [237]. Quercetin, kaempferol and apigenin are also able to induce CCK secretion *in vitro* [238]. Moreover, procyanidins B2, B2 gallate and C1 are able to inhibit ghrelin in MGN3-1 cells [182].

4. Beneficial health effects of polyphenols derived from the interaction with the GI tract

The GI tract is the first contact of the ingested polyphenols with the body. This, along with the fact that most of them are poorly absorbed, suggests that the various health beneficial effects of polyphenols are due to this first interaction with the gastrointestinal tract. Besides the activation of the enteroendocrine system, above detailed, they have been identified to modulate several GI tract related functions, such as the barrier function through the control of tight junction and inflammation responses, and antibacterial ability via the modulation of the composition of gut microbiota [239]. Thus, reviewing how do flavonoids interact with the GI tract will shed light about which health benefices result from this interaction.

4.1 Effects of polyphenols in the GI tract

4.1.1 Reduced digestibility induced by polyphenols may contribute to an improved nutrient homeostasis

In regard to the effect of polyphenols in carbohydrates digestion, it has been observed that they influence this process at several levels, ultimately reducing their absorption and increasing their faecal excretion [240], [241]. Carbohydrates are broken down into glucose by two main enzymes: α-amylase and α-glucosidase. Several *in vivo* studies have revealed the ability if polyphenols in the inhibition of α-amylase α-glucosidase activities. Those inhibitory polyphenols are catechins, isoflavones, anthocyanins, flavonols, flavanones, flavones, ellagitannins, and proanthocyanins [242]. Moreover, an *in vivo* study evaluating the effect of a phenolic extract from chokeberry on glycaemia showed that it was able to reduce blood glucose, in addition to cholesterol and glycated haemoglobin levels, by means of reducing α-glucosidase activity [243]. Accordingly, a study analysing tannins extracted from grape seeds revealed that not only did inhibit α-amylase and α-glucosidase, but they also inhibit the enzymes involved in the glycation of proteins [244]. Besides, the effect of the degree of polymerisation of polyphenols on the inhibitory activities has been evaluated by Yamashita *et al.* [245], who revealed that highly polymerised polyphenols have a stronger inhibition of α-glucosidase than less polymerised ones. In addition, polyphenols have an impact in glucose absorption. Intestinal glucose is mainly absorbed by sodium-dependent glucose transporter 1 (SGLT-1), and GLUT-5. *In vitro* experiments have shown that ECG inhbits SGLT-1 [246]. *In vivo* experiments revealed that an acute administration of a green tea decoction (GTD),

rich in EGCG and EGC, to fasted rats, inhibited their SGLT-1 activity while increasing their GLUT-2 activity and expression in jejunum. Besides the modulation of the SGLT-1/GLUT-2 ratio, a hallmark of glucose absorption regulation, GTD also improved glucose tolerance [247]. Similarly, an acute administration of EGCG+EGC inhibited SGLT1 activity. Besides, *in vivo* administration of resveratrol to rats increases glucose uptake through the increased expression of GLUT4 [248]. Thus, these findings suggest that, while polyphenols with low degree of polymerization are able to modulate glucose uptake, highly polymerized polyphenols regulate the enzymatic breakdown of carbohydrates.

Polyphenols reduce the digestibility of proteins by interacting with co-ingested dietary proteins and also interacting with luminal endogenous proteins of the GI tract, such as digestive proteases and proteins of saliva and mucus. In regards of salivary proteins, it has been observed that condensed tannins, which reduce protein digestibility of diets and increase proline-rich proteins in the salivary glands, have high affinity for proteins of saliva. A prevalent hypothesis is that proline-rich proteins of saliva neutralize and precipitate tannins to reduce their anti-nutritive effects [249]. In this direction, Georgiades *et al.* and Davies *et al.* [250], [251] have shown that EGCG, and EC to a lesser extent, can cause the aggregation of gel-forming and non-gel-forming saliva and intestinal mucins, therefore influencing digestibility. Several *in vitro* studies have revealed that pepsin activity is modulated by polyphenols, offering controversial results. While Tagliazucchi *et al.* [252] showed that polyphenol-rich beverages such as dealcoholized red wine and green tea and the pure flavanols within increase pepsin activity, He *et al.* [253] showed that tea, a rich source of flavanols such as catechin, EGCG, ECG, EGC and EC, inhibits α-amylase, pepsin, trypsin and lipase. Similarly, Naz *et al.* [254] reported that EGCG inhibits α-amylase, trypsin and chymotrypsin but not pepsin. Samuels *et al.* [255] showed that curcumin and a black raspberry extract inhibit pepsin activity. These contrasting findings could be explained by the use of different pepsin sources and protein substrates to evaluate the modulation of the protease activity of pepsin by polyphenols. In contrast, *in vitro* studies evaluating the effect of polyphenols on trypsin, chymotrypsin and elastase offer more consistent results, since condensed and hydrolysable tannins, procyanidin oligomers, phenolic acids and polyphenol-rich extracts and beverages, at different affinities, are able to inhibit their protease activities [256]–[258]. While tannic acid has been shown to be the most effective in inhibiting trypsin, procyanidin oligomers have the higher inhibitory activity of pancreatic elastase. Despite this evidence *in vitro*, there is poor evidence of protease modulation by

polyphenols *in vivo*. However, the increased reactivity of polyphenols at higher pH indicates that these inhibitions occur more likely in the duodenum than in stomach. Nevertheless, *in vivo* experiments in rats have revealed that the protein digestibility is reduced while endogenous nitrogen excretion is increased when diets are supplemented with polyphenols, as seen for high-polyphenol buckwheat [259], green and black tea [260], catechin [261], chologenic acid [262] and quercetin [262].

Digestion of fat consists of several steps including fat emulsification, hydrolysation and absorption involving enzymes lipid transporters expressed in the intestinal lumen. Once fatty acids and cholesterol are transferred into enterocytes, they are packaged into chylomicrons that are released into the lymphatic system. Several of these steps have been modulated by polyphenols. As reviewed by Salvadó *et al.*, procyanidins from different sources, in *in vitro* and in animal studies, have been shown to reduce lipase activity, therefore reducing the lipid absorption. In these studies, it has been established the negative correlation of the degree of polymerisation of procyanidins with their lipase-inhibitory activity; less polymerised procyanidins inhibit lipase more effectively than the highly polymerised ones. Besides, a human interventional study consistently revealed that consumption of polyphenols from red wine delays lipid absorption [263]. Polyphenols also have an impact in the regulation of the transport of absorbed lipids. In *in vivo* studies, GSPE has been shown able to improve the chylomicrons profile by reducing the very low-density lipoprotein (VLDL)-rich levels and limiting the lipoprotein secretion in rats [264]. Accordingly, an interventional study in humans at high cardiometabolic risk given a polyphenol-rich diet revealed that VLDL were reduced by the diet in addition to reduced blood cholesterol and triglycerides levels. Moreover, while low-density lipoprotein (LDL) were enriched with TAGs, TAG content in high-density lipoprotein (HDL) was reduced [265].

4.1.2 Polyphenols-induced anti-inflammatory profile improves the intestinal barrier function

In a recent work of our research group, we reviewed the increasing evidence of the beneficial effects of proanthocyanidins (PACs) on the intestinal barrier [239]. At *in vitro* level, PACs are able to reduce the proinflammatory cytokine production of TNFα, IL-6, and IL-8 promoted by inflammatory agents such as LPS or the cytokines themselves [266]–[269]. In addition, PACs promote the increased expression of antioxidant enzymes like glutathione peroxidase (GPx), superoxidase dismutase (SOD), and hemeoxygenase 1 (HO-1), has also been reported [266]. As revealed by the permeability markers of

transepithelial electrical resistance (TEER) and the transepithelial transport marker lucifer yellow-labelled dextrans, PACs reduce permeability, which can partly be explained by increased expression of tight junctions such as claudins and occludins [270]. Accordingly, studies with GSPE treatments in cafeteria diet-induced obese rats revealed the ability of GSPE to reverse the intestinal dysfunction induced by the diet, shown as an increase in intestinal permeability and proinflammatory markers [271]. In rats with chemically induced intestinal dysfunction with trinitrobenzene sulphonic acid (TNBS) and dextran sodium sulphate (DSS), GSPE and procyanidin B2 have also been shown able to prevent the intestinal damage, as revealed by a reduction of oxidative stress and macrophage infiltration [272], [273]. When it comes to humans, few studies are done to evaluate the effect of PACs in intestinal health. A study evaluated the effect of procyanidin-rich bark extract from the French maritime pine on pediatric Crohn's disease. After 10 weeks of treatment, no differences were found in inflammatory markers, but the treatment increased the activity of antioxidant enzymes [274]. Another study evaluating the effect of GSPE intake after a high-fat meal revealed that it was able to reduce plasma LPS, which is associated with the low-grade inflammation of the metabolic syndrome [275]. Finally, epidemiological studies have connected the consumption of PACs-rich food with a lower risk of colorectal cancer [276].

4.1.3 Polyphenols promote a healthy composition of the gut microbiota

The microbiota colonizing the GI tract contributes to its correct functioning and health by exerting several functions such as antibacterial against pathogens, contribution to vitamins K and B production, maintainment of the intestinal barrier through anti-inflammatory signalling, and production of metabolites that stimulate the enteroendocrine system [277]. On the contrary, dysregulation on intestinal microbiota can have negative health effects such as neoplasm growth promoted by mutagens produced by *Bacteroides*, *E. coli*, and *Faecalis* [278]. Dietary components can modulate the composition of microbiota and its functions. Although the mechanisms microbiota metabolizes polyphenols is well defined [279], how polyphenols impact on the functions of microbiota is still a matter of study. Furthermore, the effects that the food matrix has in which polyphenols reach the gut on their later bioavailability have been widely studied [280]. Nevertheless, several studies have reported the antimicrobial activity of polyphenols. In several *in vitro* studies, catechin has been shown to inhibit growth of pathogenic microbiota, such as *Helicobacter pylori* [281], *Staphylococcus aureus*, *E. coli*

[282], and *Pseudomonas aeruginosa* [283]. Similarly, in an *in vitro* human digestive model, catechin has shown to improve the growth of E. coli and members of the *Clostridium coccoides-Eubacterium rectale* group, while inhibiting the growth of *Clostridium histolyticum*. The growth rate of Lactobacillus spp. and Bifidobacterium remained unaltered by the catechin treatment [284]. Moreover, phenolic compounds extracted from berries have shown to reduce the growth rate of lactic and gram-negative bacteria, without an effect on salmonella. The benefices of polyphenols in the gut microbiota are not limited to reducing pathogenic microbes, but also promoting a healthy composition. Flavanols derived from coca have been shown to modulate human gut microbiota increasing the presence of *Bifidobacteria* and *Lactobacilly* [285]. In our research group, Casanova *et al.* showed that GSPE modulates rat gut microbiota by increasing the presence of *Bacteroidetes* and reducing that of *Firmicutes*, which is related to the increased caecal content of SCFA, improved adiposity and modulation of the enteroendocrine system [208]. Accordingly, the simultaneous administration of tea polyphenols and high fat diet to mice resulted in an improved glycaemic and lipidemic profile that correlated with an increase of microbial production of butyric and acetic acids [241]. Similarly, the authors of a study with tannin-rich diet have suggested that beneficial health effects promoted by the increase of *Bacteroidetes* and ruction of *Clostridium leptum* induced by diet function as an overcoming mechanism for the tannin antinutritive properties [286].

4.2 Effects of polyphenols in the endocrine pancreatic function

Polyphenols have been shown modulate the pancreatic β-cell function. In particular, GSPE is able to modulate insulin synthesis, secretion and degradation in both *in vivo* studies with rats and pancreatic β-cell lines. In *in vivo* studies, a corrective administration of GSPE to diet-induced insulin resistant rats (which had been fed with cafeteria diet for 13 weeks) reduced insulin production at a gene expression level, while reducing the pancreatic TAG content and downregulating lipogenesis-related genes. This finding suggests a counteraction of GSPE to the hyperinsulinemia of these insulin resistant rats [287]. However, other studies with genetically obese rats, revealed that GSPE was unable to improve hyperinsulinemia, suggesting that procyanidins are only able to correct β-cell damage at early stages of insulin resistance [288], [289]. Besides, GSPE has been shown to promote pancreatic glucose uptake through miRNA modulation [290]. Not only hyperinsulinemia is a hallmark of insulin resistance, but also increased β-cell mass. Pancreatic β-cells, in hyperglycaemic conditions, reduce apoptosis and increase

proliferation to counteract the glucose excess. GSPE has been reported to have controversial effects in the modulation of β-cell proliferation. While a corrective treatment with 25 mg/kg GSPE for 21 days after 7 weeks of diet enhanced the cafeteria-induced anti-proliferative effects on β-cell of male rats [291], previous results in female rats of a 10 or 30-day treatment of GSPE at similar doses counteracted the cafeteria-induced apoptosis by downregulating Bcl2 protein [292]. These contrasting results have been attributed to the different gender, dose, and time of administration. Nevertheless, *in vitro* studies revealed a clear pro-apoptotic and anti-proliferative under pathogenic conditions. The pancreatic cell line INS-1E under hyperglycemia and hyperinsulinemia conditions exhibited increased pro-apoptosis and anti-proliferation gene expression when treated with GSPE, while GSPE had none of these effects in this cell line under physiologic conditions [292]. The proliferative profile of β-cells is also a hallmark of pancreatic cancer. In the pancreatic adenocarcinoma cell line MIA PaCa-2, GSPE was also able to promote this antiproliferative profile. Besides, gallic acid and EGCG have been related to some of these beneficial anti-proliferative effects of GSPE [293]. Finally, several studies have reported that flavanols are able to inhibit amylin fibre formation, an event occurring during T2DM which induces pancreatic cell death [294]–[296].

Although these findings reveal a clear effect of flavonoids in the regulation of pancreas physiology and function, the mechanism by which these molecules reach the pancreas remains to be elucidated. Nevertheless, it is well-established that enterohormones, GLP1 in particular, preserve pancreatic β-cell mass through stimulating proliferation and inhibiting apoptosis [297]. Several studies have evidenced the benefices on β-cell of GLP1-based therapies occur through the activation of GLP1-R [298]. Thus, given that flavonoids are clear stimulators of the enteroendocrine system, including GLP1[299], flavonoid-induced enteroendocrine secretions could be the mechanism by which they positively impact on pancreatic function. In this direction, recent studies point at the DPP4-inhibitory activity, and therefore increased GLP1 circulating levels, of tea flavonoids and bioactive peptides to be related to the reduced pancreatic apoptosis [300]. However, further research is needed to study the mechanism of GSPE action on the pancreas and whether is dependent on the enteroendocrine secretions.

4.3 Food intake regulation by dietary polyphenols

Whilst the ability of polyphenols to modulate the enteroendocrine system have been thoroughly studied (Section 3.4. Enterohormone-stimulating bioactive compounds: Polyphenols), there are few studies actually showing that such EE modulation leads to an altered food intake. As mentioned before, the supplementation with soy isoflavones in ovariectomized and HFD-fed rats, manages to reduce their body weight gain and abdominal fat by reducing ghrelin and increased CCK secretion. Concordantly to this enteroendocrine modulation and limited weight gain, rats exhibited reduced food intake [226]. Similarly, plasma ghrelin levels together with food intake were also shown reduced after an 8-week treatment with genistein, an isoflavone, in female mice but not in males [301]. Polyphenols have also been suggested to induce a quicker release than normal of enterohormones. Specifically, a polyphenol-rich spinach extract has been shown to reduce food intake in rats when administered previous to a meal and steadily reduce body weight through an induced advanced release of CCK [302]. In studies from our research group, the minimal dose of phenolics present in GSPE and to acutely reduce food intake in rats has been defined and related to a GLP1R-dependent pathway, since the treatments lead to an increase in GLP1 but decrease in CCK plasma levels [303] and the food intake inhibition was lost when exendin9, a GLP1R blocker, was used. Moreover, this limiting effect on food intake of an acute dose of GSPE was maintained for 8 days after the dose in rats fed with standard diet [182]. The effectiveness of simultaneous or corrective GSPE treatments in the prevention or correction of cafeteria diet-induced obesity was assessed in rats, which showed that, while both treatments effectively limited CCK and ghrelin secretion while stimulating GLP1 and PYY expression, only the simultaneous GSPE treatment effectively reduced food intake and prevented body weight gain [211]. Similarly, a preventive treatment of 10 days followed by a cafeteria diet, only manage to limit food intake during the duration of the treatment but successfully prevented a body weigh increase until 14 weeks of cafeteria diet after the treatment [304]. Although several cohort studies have been conducted to study the effect of polyphenol on body weight, the ability of polyphenols to regulate appetite has been seldomly studied in humans [305]. In healthy individuals, the consumption of polyphenol-enriched cookies resulted in enteroendocrine modulation accompanied with an increased satiety feeling, although it was not followed by a substantially reduced food [216]. Although food intake was not quantified, increased satiety induced by polyphenolic rich extract from lemons and hibiscus was reported in another clinical trial, together with increased GLP1 and reduced ghrelin levels in plasma [306]. The effect of

extracts administered within bread as a food matrix was evaluated in another study and, although green-tea and baobab supplemented bread showed improved insulinemia in healthy humans, they did not have an effect in appetite nor glycaemia [307]. Altogether, these findings show that polyphenols can modulate intake and that it is accompanied by enteroendocrine modulation. However, their mechanisms have not been fully studied and no attempt has been made to combine polyphenols in the right measure to obtain a specific enteroendocrine response to reduce food intake. Thus, further research is needed to understand the mechanisms of enteroendocrine regulation of polyphenols that affect food intake to improve antidiabetic therapies.

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HYPOTHESIS & OBJECTIVES

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Enterohormones are satiety-modulating hormones produced in the gastrointestinal tract by the enteroendocrine cells, and they control different metabolic functions, including glycaemia, gut motility and nutrient uptake and pancreatic function. Previous research from our group has proved that grape-seed procyanidin extract (GSPE) prevents the prediabetic state in rats under obesogenic diet. The antidiabetic effect of GSPE is partly explained by its ability to modulate the enteroendocrine system. Understanding the mechanisms through which specific bioactive compounds exert their effects is crucial to be able to promote their use for a better health. We previously proved that GSPE acts by increasing energy expenditure, reducing body weight and targeting pancreatic function through sustained GLP1 production and correct functioning of β-cells. However, despite the relevance of the pancreatic GLP-1 receptor on glucagon production, how GLP-1 modulation by GSPE targets glucagon production has not yet been elucidated.

Our group has also described that GSPE acts as a satiating agent, modulating the secretion of the enterohormones GLP1, GIP, PYY, CCK and ghrelin. But GSPE is a complex mixture of molecules. The different flavanols within might act through different mechanisms to modulate the enterohormone system, being the effects observed for GSPE the interaction of all these molecules with the system. Moreover, recent findings controvert what was established regarding the stimulation and response of the enteroendocrine system, indicating basolateral stimulation by some bioactive compounds and apical secretion in some conditions, among other new regulatory mechanisms. In this regard, flavanols have been shown to differently modulate TAS2Rs, a family of bitter taste receptors which in humans comprise 25 subtypes, and whose presence in the gastrointestinal tract has been linked to enterohormone release. Thus, relating stimulation of specific TAS2Rs with the effects on food intake derived from their enteroendocrine regulating function would be of interest for human health.

Considering this**, we hypothesised that GSPE-induced enterohormone secretions modulate pancreatic glucagon production, and that the enterohormone secretions are regulated differently by the specific flavanols, in part through the stimulation of bitter taste receptors, leading to a differential control food intake by these flavanols.**

To demonstrate our hypothesis, we have defined several specific objectives:

Objective 1: To determine whether modulation of GLP-1 by flavanol-rich grapeseed-procyanidin extract (GSPE) affects glucagon production.

GSPE is known to reduce lower blood glucose levels in cafeteria diet-fed rats by preventing a GLP1 decrease, thus, maintaining insulin production. In acute treatments, GSPE has been shown to produce an increase in plasma GLP1. This effect has been clearly related improving b-cell functionality. Hence, we aimed to determine whether GSPE could also affected glucagon production in cafeteria diet-fed rats.

Objective 2: To identify the mechanisms through which flavanols and other secretagogues stimulate the enteroendocrine system

The enteroendocrine system is a complex mix of different chemosensory receptors and secretory endocrine cell types and its regulation by food components it is still far from being fully understood. Moreover, recent findings on apical PYY and GLP1 secretion and alternative receptors for GLP1 point at novel functions of enterohormones within the gastrointestinal tract. Hence, using several *ex vivo* experimental methods, model species and secretagogues, we aimed to identify which specific stimulations of the enteroendocrine system by pure flavanols occurs, how enteroendocrine secretions are produced and explore the role of apical enteroendocrine secretions in the gastrointestinal tract.

Objective 3: To associate the enteroendocrine stimulation by flavanols, specifically targeting bitter taste receptors in the gastrointestinal tract, with the control of food intake.

Most flavanols have been identified as agonists for bitter taste receptors (TAS2Rs). TAS2Rs have been identified in the gastrointestinal tract and their specific stimulation has been linked to the control of enterohormone secretion. Thus, we aimed to determine whether stimulation of TAS2Rs by flavanols and could modulate enteroendocrine secretions and thus regulate food intake in rats.

HIPÒTESIS I OBJECTIUS

Les enterohormones són hormones moduladores de la sacietat produïdes al tracte gastrointestinal per les cèl·lules enteroendocrines i controlen diferents funcions metabòliques, inclosa la glucèmia, la motilitat intestinal i la captació de nutrients i la funció pancreàtica. Resultats previs del nostre grup de recerca han demostrat que l'extracte de procianidina de llavors de raïm (GSPE) impedeix l'estat prediabètic en rates amb dieta obesogènica. L'efecte antidiabètic de GSPE s'explica en part per la seva capacitat de modular el sistema enteroendocrí. La comprensió dels mecanismes mitjançant els quals els compostos bioactius específics exerceixen els seus efectes és crucial per poder promoure el seu ús per a assolir una millor salut. Prèviament, s'ha demostrat que el GSPE actua augmentant la despesa energètica, reduint el pes corporal i modulant la funció pancreàtica mitjançant una producció sostinguda de GLP1 i mantenint un funcionament correcte de les cèl·lules β. Tanmateix, malgrat la rellevància del receptor pancreàtic GLP-1 en la producció de glucagó, encara no s'ha aclarit com la modulació del GLP-1 per GSPE s'orienta a la producció de glucagó.

El nostre grup també ha descrit que el GSPE actua com un agent saciant, modulant la secreció de les enterohormones GLP1, GIP, PYY, CCK i grelina. Però el GSPE és una barreja complexa de molècules. Els diferents flavanols que el componen poden actuar mitjançant diferents mecanismes per modular el sistema enterohormonal, sent els efectes observats per GSPE la interacció de totes aquestes molècules amb el sistema. A més, descobriments recents sobre el sistema enteroendocrí controverteixen el que s'havia prèviament establert sobre els seus mecanismes d'estimulació i de resposta, com ara l'estimulació basolateral per part d'alguns compostos bioactius i la secreció apical en algunes condicions, entre altres nous mecanismes. En aquest sentit, s'ha demostrat que els flavanols modulen de manera diferent els TAS2R, una família de receptors del gust amarg que en humans comprenen 25 subtipus i la presència del qual en el tracte gastrointestinal s'ha relacionat amb l'alliberament d'enterohormones. Per tant, relacionar l'estimulació de TAS2R específics amb els efectes sobre la ingesta d'aliments derivats de la seva funció reguladora enteroendocrina seria d'interès per a la salut humana.

Tenint en compte això, **s'ha plantejat la hipòtesi que les secrecions enterohormonals induïdes per GSPE modulen la producció de glucagó pancreàtic i que les secrecions**

enterohormonals estan regulades de manera diferent pels flavanols específics, en part mitjançant l'estimulació dels receptors del gust amarg, que condueixen a un control diferencial de la ingesta d'aliments per part d'aquests flavanols.

Per demostrar la nostra hipòtesi, hem definit diversos objectius específics:

Objectiu 1: determinar si la modulació de GLP-1 per l'extracte de procianidina (GSPE) ric en flavanol afecta la producció de glucagó.

Se sap que el GSPE redueix els nivells de glucosa en sang en rates alimentades amb dieta de cafeteria evitant una disminució de GLP1, mantenint així la producció d'insulina. En tractaments aguts, s'ha demostrat que GSPE produeix un augment del GLP1 plasmàtic. Aquest efecte s'ha relacionat clarament amb la millora de la funcionalitat de les cèl·lules β. Per tant, pretenem determinar si el GSPE també pot afectar la producció de glucagó en rates alimentades amb dieta de cafeteria.

Objectiu 2: identificar els mecanismes mitjançant els quals els flavanols i altres compostos bioactius estimulen el sistema enteroendocrí.

El sistema enteroendocrí és una barreja complexa de diferents receptors quimiosensorials i tipus de cèl·lules endocrines secretores i la seva regulació per components alimentaris encara està lluny d'entendre's completament. A més, les troballes recents sobre la secreció apical de PYY i GLP1 i els receptors alternatius per a GLP1 apunten a noves funcions de les enterohormones dins del tracte gastrointestinal. Per tant, utilitzant diversos mètodes experimentals *ex vivo*, espècies model i compostos bioactius bioactius, intentem identificar quines estimulacions específiques del sistema enteroendocrí per flavanols purs es produeixen, com es produeixen les secrecions enteroendocrines i explorar el paper de les secrecions enteroendocrines apicals al tracte gastrointestinal.

Objectiu 3: Associar l'estimulació enteroendocrina per flavanols, específicament dirigits als receptors del gust amarg del tracte gastrointestinal, amb el control de la ingesta d'aliments.

La majoria dels flavanols s'han identificat com a agonistes dels receptors del gust amarg (TAS2Rs). S'han identificat TAS2R al tracte gastrointestinal i la seva estimulació específica s'ha relacionat amb el control de la secreció d'enterohormones. Per tant, ens proposem determinar si l'estimulació de TAS2Rs per part de flavanols podria modular les secrecions enteroendocrines i regular així la ingesta d'aliments en rates.

UNIVERSITAT ROVIRA I VIRGILI
REGULATION OF ENTEROENDOCRINE FUNCTION BY BIOACTIVE COMPONENTS THROUGH THEIR INTERACTION WITH BITTER TASTE RECEPTORS
Carme Grau Bové

RESULTS

RESULTS: Part 1

Determination of whether modulation of GLP-1 by flavanol-rich grapeseed-proanthocyanidin extract (GSPE) affects glucagon production

Manuscript 1:

Glucagon Shows Higher Sensitivity than Insulin to Grapeseed Proanthocyanidin Extract (GSPE) Treatment in Cafeteria-Fed Rat

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Article

Glucagon Shows Higher Sensitivity than Insulin to Grapeseed Proanthocyanidin Extract (GSPE) Treatment in Cafeteria-Fed Rats

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Abstract: The endocrine pancreas plays a key role in metabolism. Procyanidins (GSPE) targets β-cells and glucagon-like peptide-1 (GLP-1)-producing cells; however, there is no information on the effects of GSPE on glucagon. We performed GSPE preventive treatments administered to Wistar rats before or at the same time as they were fed a cafeteria diet during 12 or 17 weeks. We then measured the pancreatic function and GLP-1 production. We found that glucagonemia remains modified by GSPE pre-treatment several weeks after the treatment has finished. The animals showed a higher GLP-1 response to glucose stimulation, together with a trend towards a higher GLP-1 receptor expression in the pancreas. When the GSPE treatment was administered every second week, the endocrine pancreas behaved differently. We show here that glucagon is a more sensitive parameter than insulin to GSPE treatments, with a secretion that is highly linked to GLP-1 ileal functionality and dependent on the type of treatment.

Keywords: glucagon; GLP1; GLP-1 receptor; procyanidin; insulin/glucagon; cafeteria diet

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1. Introduction

The proper functioning of the endocrine pancreas plays a key role in the whole-body energy homeostasis. The concerted actions of insulin and glucagon warrant that plasma glucose levels are kept within a relatively narrow healthy range [1]. An insufficient release of insulin combined with impaired regulation of glucagon secretion is a hallmark of type-2 diabetes [2]. However, there is controversy regarding the relative importance of insulin deficiency and glucagon excess to the hyperglycemia observed in diabetes pathology. Studies with glucagon receptor knockout mice (GlcR−/−) together with streptozotocin-induced destruction of the β-cells indicate that hyperglucagonaemia may be far more important than previously recognized [3]. These results highlight the role played by glucagon in diabetes and indicate that chemical modulation of glucagon release may represent a way of achieving improved glycemic control in diabetes. Indeed, the introduction of treatments based on glucagon-like peptide-1 (GLP-1), which affects both insulin and glucagon secretion [4], illustrates the potential of the α-cells as a pharmacological target.

Glucagon-like peptide-1 (GLP-1) secreted from L-cells of the intestinal tract and from specific cells of the central nervous system exerts pleiotropic biological actions, including the stimulation of glucose-dependent insulin secretion and biosynthesis, inhibition of glucagon secretion, gastric emptying, and inhibition of food intake [5]. GLP-1 shows dysfunction in obesity-related pathologies, such as type 2 diabetes, due to defects in intestinal GLP-1 secretion and β-cell responsiveness [6]. However, α-cells retain near normal responsiveness to GLP-1 infusion, since diabetic and nondiabetic subjects showed similar inhibition of glucagon secretion [7]. The disrupted coordination of glucagon and insulin

secretion observed in type 2 diabetes is characterized by impaired and delayed insulin secretion as well as basal hyperglucagonemia and non-suppressed glucagon secretion in response to glucose [8]. It must be highlighted that glucagon receptor knockout mice fed a high-fat diet (HFD) showed better glycemic control and reduced hyperinsulinemia [9]. Furthermore, GLP-1R agonist treatment of HFD mice reversed obesity and insulin resistance [10]. These data highlight that glucagon and GLP-1 have a critical position in the development of hyperglycemia in obese rodents.

It has been shown that an acute dose of grapeseed-derived procyanidin extract (GSPE) can increase plasma GLP-1 [11] and prevent the decrease in GLP-1 associated with a cafeteria diet [12]. Furthermore, this effect is still maintained 17 weeks after GSPE is administered before a cafeteria diet [13]. Some of these effects could be related to the corrective effects of GSPE on β-cells that are disturbed by a cafeteria diet [14]; however, there is no information regarding the effects of GSPE on glucagon production. Here, we show that different GSPE treatments, administered as a preventive treatment against a cafeteria diet according to two different experimental designs, had a stronger effect on glucagon production than on insulin production, altering the insulin/glucagon ratio in different ways depending on the GSPE treatment.

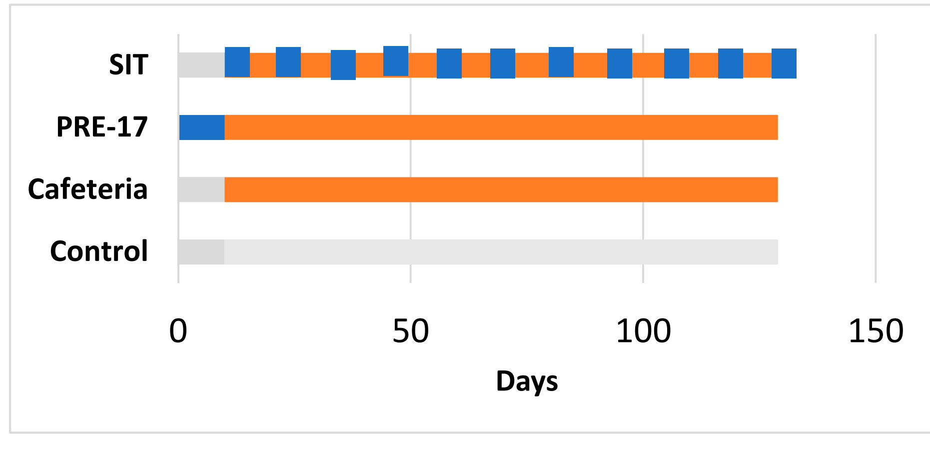
2. Materials and Methods

2.1. Proanthocyanidin Extract

The grapeseed extracts enriched in proanthocyanidins (GSPE) were kindly provided by Les Dérivés Résiniques et Terpéniques (Dax, France). We used batch numbers 124,029 [13] and 174,860 (containing 21.6% flavan-3-ol monomers, 41.6% dimers+trimers) for the 17-week cafeteria study and the 12-week cafeteria study, respectively.

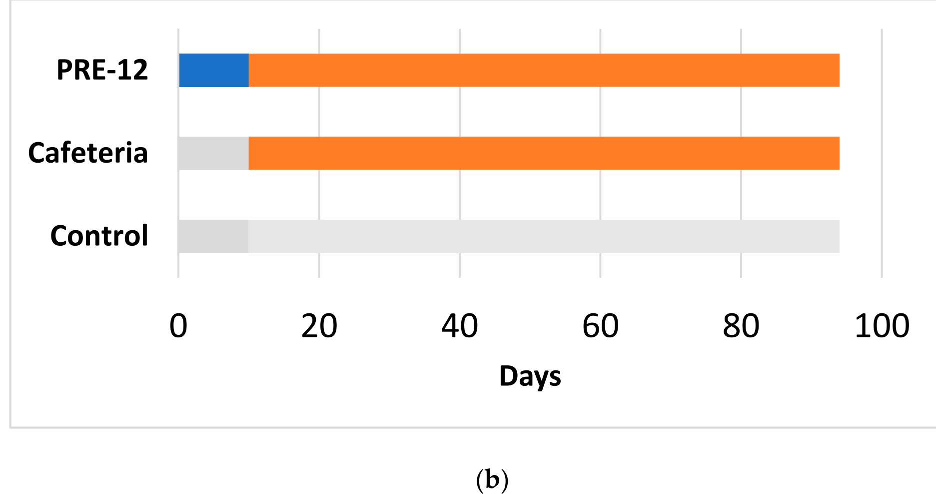
2.2. Animal Experiments

The animals were kept in animal quarters at 22 °C with a 12-h light/12-h dark cycle and fed ad libitum with a standard chow diet and tap water. We ran two similar experiments (detailed in Figure 1) on female Wistar rats, approved by the Animal Ethics Committee of the Generalitat de Catalunya (respective codes: 0152S/4655/2015 and 10183).



(**a**)

Figure 1. *Cont.*



(**b**)

Figure 1. Experimental design for the 17-week (**a**) and 12-week (**b**) cafeteria studies. Control groups received a standard chow diet (grey bar). The other groups received a cafeteria diet (orange bar) for 17 weeks (**a**) or 12 weeks (**b**). The preventive treatment groups (PRE-17) and (PRE-12) received a dose of 500 mg GSPE/Kg (blue bar) for 10 days before starting the cafeteria diet. The simultaneous intermittent treatment-CAF (SIT) group received a five-day dose of 500 mg GSPE/Kg every second week at the same time that they were fed a cafeteria diet.

The 17-week cafeteria study: Rats weighing 240 to 270 g were purchased from Charles River Laboratories (Barcelona, Spain). After one week of adaptation, they were individually caged and randomly distributed into experimental groups (n = 7–10/group), as detailed in Figure 1a. The control group received a standard chow diet (Panlab 04, Barcelona, Spain). The other groups received a cafeteria diet for 17 weeks [15] (for detailed composition, see Table S1). At week 14, the food was withdrawn at 10 p.m. The next morning, at 9 a.m., tail blood samples were collected before oral glucose load (2 g of glucose per kg of BW) and 15 min after it. At the end of the study, the animals fasted for 1 to 4 h, were anaesthetized with sodic pentobarbital (70 mg/kg body weight) (Fagron Iberica, Barcelona, Spain), and exsanguinated from the abdominal aorta. Most of the tissues from the animals were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

The 12-week cafeteria study: Rats weighing 200 to 225 g were purchased from Envigo (Barcelona, Spain). After one week of acclimation, rats were individually caged and were handled so that they became used to manipulation and oral gavage (twice, with vehicle) during one more week. The animals were separated into three experimental groups (n = 10/group), as detailed in Figure 1b. The control group received a standard chow diet (2014-Teklad, Envigo, Barcelona, Spain). The cafeteria group received a cafeteria diet [15] supplemented with 0.5 mL of condensed milk three times a week until the eighth week. After 12 weeks of cafeteria diet, all the animals were overnight fasted and sacrificed by beheading. Most of the animals' tissues were obtained and immediately frozen in liquid nitrogen and stored at −80 °C until processing.

2.3. Plasma and Tissue Hormone Analysis

The plasma was obtained as previously defined [16]. Pancreatic insulin and glucagon contents were extracted as previously described [17]. Plasma glucose was analyzed using an enzymatic colorimetric kit (GOD-PAP method from QCA, Tarragona, Spain). ELISA kits were used to analyze active GLP-1 and total GLP-1 7–37 amide (EGLP-35K; EZGLP1T-36K, Millipore, Madrid, Spain), insulin and glucagon (10-1251-01; SE-754 50, Mercodia, Uppsala, Sweden), and Amylin (CEA812Ra, Cloud-Clone Corp. Katy, TX, USA).

The homeostatic model assessment for insulin resistance (HOMA-IR) and the HOMA-β index were calculated using the fasting values of glucose and insulin with the following formulas:

$$\text{HOMA} - \text{IR} = \frac{\text{insulin}\ (\mu\text{U/mL}) \times \text{glucose}\ (\text{mM})}{22.5}$$

$$\text{HOMA} - \beta = \frac{20 \times \text{insulin}\ (\mu\text{U/mL})}{\text{glucose}\ (\text{mM}) - 3.5}$$

2.4. Quantitative Real-Time RT-PCR Analysis

Total RNA and cDNA were obtained as previously defined [16]. Quantitative PCR amplification was performed using a specific TaqMan probe (Applied Biosystems, Waltham, USA) for the GLP-1 receptor (Rn00562406_m1), proglucagon (Gcg) and the gene encoding for GLP-1 (Rn00562293_m1), and insulin (Rn01774648_g1). The relative expression of each gene was compared with the control group using the 2-ΔΔCt method, with PPIA (Rn00690933_m1) as a reference.

2.5. Statistical Analysis

The results are expressed as the mean ± SEM. A Student's *t*-test was used to compare the treatments with the CAF group. *p*-values < 0.05 were statistically significant. These calculations were performed using the XL-Stat 2017 software (Addinsoft, Paris, France).

3. Results and Discussion

3.1. Glucagonemia Remains Sensitive to GSPE Treatment Several Weeks after the Treatment Has Finished

Rats that have had a cafeteria diet for 12 weeks have increased glucose and insulin, resulting in increased HOMA-IR and increased HOMA-β compared to the control group (Table 1). These results are in agreement with previous literature that states that the cafeteria diet causes peripheral insulin resistance, and the pancreas compensates for this by producing more insulin [18]. In addition, there is a trend towards higher glucagonemia in a fasting situation (Table 1), as previously described [19].

Table 1. Fasting plasma parameters at the sacrifice of rats treated for 12 weeks with a cafeteria diet with/without a 10-day pre-treatment with grapeseed proanthocyanidin extract (GSPE).

	Control	Cafeteria	PRE-12
Glucose (mM)	6.6 ± 0.2 *	7.56 ± 0.3	7.31 ± 0.4
Insulin (pM)	252.1 ± 27.1 *	589.1 ± 83.0	558.3 ± 111.4
HOMA-IR	9.99 ± 1.2 *	31.89 ± 3.3	22.23 ± 4.2 #
HOMA-β	225.5 ± 27.7 *	387.05 ± 54.88	330.67 ± 48.25
Glucagon (pM)	4.08 ± 1.03 #	9.3 ± 2.44	11.89 ± 2.51 §
Glucagon/insulin	0.024 ± 0.007	0.013 ± 0.0033	0.03 ± 0.007 *

All the data are mean ± SEM of 5–7 animals per group. *t*-tests were applied (* $p \leq 0.05$ versus cafeteria, # $p \leq 0.1$ versus cafeteria; § $p \leq 0.05$ versus control). HOMA-IR, homeostatic model assessment for insulin resistance; PRE, preventive treatment groups.

Treatment with 500 mg GSPE /kg BW (PRE-12) for 10 days prior to the 12-week cafeteria diet prevented some of these effects. Table 1 shows that GSPE-treated animals tended to have reduced HOMA-IR, an indicator of peripheral resistance to insulin. The GSPE effects that ameliorate peripheral insulin resistance have been previously described [20]. What is new in this work is the effect of GSPE on glucagon. Under fasting conditions, GSPE-treated animals showed significantly higher plasma glucagon levels than the control group. This trend in glucagon produced a significantly higher glucagon/insulin ratio than in the cafeteria group (Table 1).

Another group of rats was given the same treatment with GSPE (500 mg/kg BW for 10 days before feeding them a cafeteria diet), but the cafeteria diet was extended to 17 weeks (PRE-17) (Table 2). In this case, these animals were sacrificed under conditions of light fasting, only three hours without food from when the lights were turned on. Under these conditions, neither the cafeteria group nor the GSPE group showed significant differences in plasma glucose or insulin [15]. However, the pre-treatment with GSPE (PRE-17) led to a lower glucagonemia than in the cafeteria group. Furthermore, while the cafeteria group showed a decrease in the insulin/glucagon ratio compared to the standard-fed group, the GSPE pre-treatment normalized this ratio (Table 2). That is, under partially fed conditions at week 17, GSPE pre-treated rats were exposed to an insulin/glucagon signal closer to that of the control group.

Table 2. Plasma and tissue parameters at the sacrifice of rats treated for 17 weeks with cafeteria diet with/without a 10-day pre-treatment with GSPE or a synchronic treatment with GSPE. Animals were sacrificed under a light fast of 3 h.

	Control	Cafeteria	PRE-17	SIT
Glucagon (pM)	7.24 ± 2.28	9.81 ± 1.43	5.83 ± 0.92 *	1.24 ± 1.05 *
Amylin (pg/mL)	8.79 ± 1.10	12.62 ± 2.08	12.91 ± 0.61	9.28 ± 1.86
Insulin/glucagon	0.15 ± 0.02 *	0.07 ± 0.02	0.16 ± 0.03 *	0.10 ± 0.02 #

All the data are mean ± SEM of five to seven animals per group. *t*-tests were applied and are indicated versus the cafeteria group (* $p \leq 0.05$, # $p \leq 0.1$). SIT, simultaneous intermittent treatment.

3.2. Pre-Treatment with GSPE Might Increase GLP-1 Sensitivity in the Pancreas

We then analyzed the pancreas of the animals. Table 3 shows that 17 weeks of cafeteria diet only produced a trend towards an induced expression of insulin in the pancreas. There were no changes in pancreatic insulin or glucagon. There was a similar situation in the animals treated for 12 weeks with a cafeteria diet. Despite the higher plasma glucagon levels exhibited by the rats that received a pre-treatment with GSPE before the cafeteria diet (PRE-12), these animals did not show statistically significant differences in glucagon content in the pancreas (3846.9 ± 1210, 3433.8 ± 1264, 6239.2 ± 1163 pg glucagon/mg tissue for the control, cafeteria and PRE-12 groups, respectively).

Table 3. Pancreas parameters for rats treated for 17 weeks with a cafeteria diet after a 10-day GSPE pre-treatment (PRE-17) or concomitant to a GSPE treatment every two weeks (SIT).

	Control	Cafeteria	PRE-17	SIT
Contents in tissue				
Insulin (ng/g tissue)	59.43 ± 14.88	83.54 ± 15.63	82.48 ± 13.90	51.57 ± 25.44
Glucagon (nmol/g tissue)	787.53 ± 241	917.91 ± 167	958.67 ± 166	304.08 ± 212 *
mRNA (A.U. vs. Control)				
Insulin	1.65 ± 0.58 #	6.03 ± 2.35	2.81 ± 0.88	2.17 ± 0.48 #
Glucagon	1.17 ± 0.27	2.88 ± 1.07	0.86 ± 0.48	0.92 ± 0.19 #
GLP-1 Receptor	2.96 ± 1.72	0.86 ± 0.24	2.02 ± 0.47 #	2.71 ± 0.99

Gene expression results are relative to the cafeteria group. All the data are mean ± SEM of five to seven animals per group. *t*-tests were applied and are indicated versus the cafeteria group (* $p \leq 0.05$, # $p \leq 0.1$).

We analyzed the endocrine pancreas functionality because it is highly influenced by the GLP-1 produced at the intestinal level. At week 17, there were no effects due to the GSPE pre-treatment on plasma-active GLP-1 at sacrifice (5.17 ± 1.98 vs. 3.41 ± 0.46 pM for the PRE-17 and cafeteria groups, respectively). However, we found a different situation in response to an oral glucose load at week 14. The total GLP-1 was measured at time 0 (fasting conditions) and 15 min after oral glucose loading. The change observed between these

two points in time was indicative of the new production of GLP-1 due to this glucose load. The cafeteria diet did not show differences in the total GLP-1 ratio (Figure 2). The present results, together with previous data showing no effects of GLP-1 on the ileum or colon of cafeteria-fed rats [13], do not evidence higher intestinal GLP-1 production due to cafeteria treatment. At the pancreatic level, no significant changes were observed in GLP-1R, similar to what was observed in the ileum of the same animals [13] and in the hypothalamus of rats after 12 weeks of cafeteria diet [12]. However, we found a statistically significant increase in total GLP-1 time 15 compared to time zero after a glucose load in the animals that received the GSPE pre-treatment (PRE-17), compared to the cafeteria animals, suggesting a higher ability to secrete GLP-1 after stimulus (Figure 2), although there were no effects on food intake after GSPE treatment at this time point (Table S2 and [21]). This result agrees with the higher GLP-1 expression found in the ileum of these rats [13]. These animals might generate a higher GLP-1 signal after stimulation. This greater GLP-1 signaling was accompanied by a tendency towards an increase in the mRNA expression of GLP-1R in the pancreas, compared to the cafeteria group (Table 3). Therefore, the pancreatic cells of GSPE pre-treated rats are receiving and detecting a higher GLP-1 signal than the cafeteria group when opportunely stimulated. A higher GLP-1 stimulatory signal maintains better pancreatic functioning, together with a higher sensitivity to GLP-1 [22]. The relative presence of GLP-1R in α-cells is very low (70% in β-cells, 60% in δ-cells, and less than 0.5% in α-cells) [22]; however, as shown by Zhang et al. [23], GLP-1R regulation is much more important for glucagon than for insulin. These authors showed that islets of α-cell-specific GLP-1R knockout (αGLP-1R-/-) failed to inhibit glucagon secretion at high glucose levels and failed to stimulate glucagon secretion under very low glucose conditions, with no effects on insulin secretion. Consequently, the trend towards higher GLP-1R maintained 17 weeks after GSPE treatment could be an explanation for the rats' higher glucagon secretion in fasting situations and their lower secretion in a fed state.

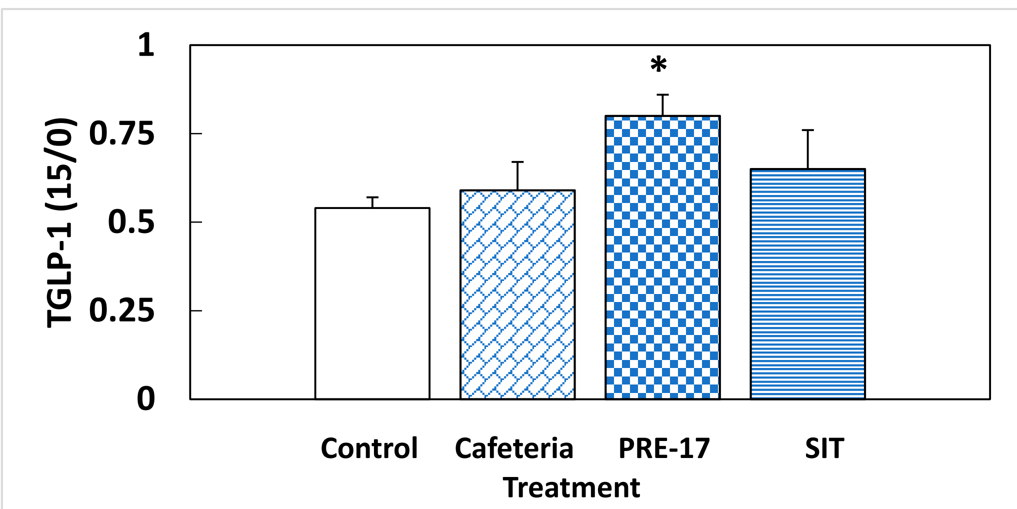


Figure 2. Relative total GLP-1 secretion in a fasting situation. Rats were treated with 0.5 g/Kg BW for the first 10 days, and then they were put on a cafeteria diet for 14 weeks (PRE-17), or a GSPE dose was administered, simultaneously with the cafeteria diet, every second week (SIT). After o/n fasting, a tail blood sample was obtained at time 0 and 15 min after an oral glucose load (2 g/kg BW). At both times, the total GLP-1 was measured, and their ratio was calculated. The data are the mean ± standard error (S.E.M.) (n = 7). Statistical differences identified by Student's t-test are defined by * when $p < 0.05$ between treatments.

3.3. Administration of GSPE Simultaneously to a Cafeteria Diet Produces Effects on the Endocrine Pancreas That Are Different to the Preventive Approach

Simultaneously to the animals that were fed a cafeteria diet for 17 weeks, we ran an experiment on a group of rats that received treatment with GSPE every second week throughout the cafeteria diet (SIT).

A different pattern was found in these rats. Under fasting conditions, at week 14 of treatment, there was a trend towards a lower presence of plasma insulin compared to the cafeteria group, with no changes in plasma glucose. This lower insulinemia produced a lower HOMA-IR, although it cannot be read as a lower peripheral resistance due to the high glucose in the fasting situation, which, together with the HOMA-β, evidenced a difficulty to secrete enough insulin to maintain normalized glycaemia (Table 4).

Table 4. Fasting plasma samples obtained at week 14 of the rats treated with GSPE simultaneously to a cafeteria diet (SIT).

	Control	Cafeteria	SIT
Glucose (mM)	5.83 ± 0.28 *	7.12 ± 0.12	7.16 ± 0.30
Insulin (μg/L)	0.24 ± 0.03 #	0.39 ± 0.10	0.16 ± 0.002 #
HOMA-IR	1.34 ± 0.12 *	2.01 ± 0.25	1.24 ± 0.05 *
HOMA-β	47.17 ± 1.54	38.99 ± 3.72	21.95 ± 1.86 *

All the data are mean ± SEM of 5-7 animals per group. *t*-tests were applied and are indicated versus the cafeteria group (* $p \leq 0.05$, # $p \leq 0.1$).

Three weeks later, at the time of death, and in non-fasting conditions, these animals had lower plasma glucagon levels than the cafeteria group. The insulin/glucagon ratio of these animals showed a tendency towards a certain normalization with respect to the cafeteria group (Table 2). They showed more marked effects at the pancreatic level because they showed a tendency to have a lower expression of insulin and glucagon than the cafeteria group, reinforced by a lower amount of glucagon protein contents in this tissue (Table 3).

We also found that several hours post-stimulation, SIT rats tended to have lower levels of active GLP-1 (2.09 ± 0.46 vs. 3.41 ± 0.46 for the cafeteria group; pM; $p < 0.1$) together with a GLP-1 receptor mRNA expression in the pancreas that was not statistically different from the cafeteria group. The two parameters suggest that the pancreas of the SIT group received lower GLP-1 signaling, which could be an explanation for the limited development found in the endocrine function. This is suggested by the insulin and glucagon mRNA abundance and glucagon contents as well as their limited HOMA-β.

Considering the results at week 14, Figure 2 shows that, at this time, SIT animals had GLP-1 signaling similar to the cafeteria group. We have reported previously that this treatment leads to increased GLP-1 expression in the ileum and colon at week 17. Thus, this lower amount of active GLP-1 in plasma could be a sign of impaired GLP-1 secretion after week 14. Another aspect to be considered is that SIT animals received a similar amount of sucrose as the cafeteria group from the diet, but probably a limited entrance of lipids. GSPE has been shown to be effective for limiting intestinal lipid absorption [24] and these rats showed, at the time of death, lower plasma triglycerides and cholesterol than the cafeteria group [15]. In fact, the food intake of SIT animals was 56% energy derived from sucrose, assuming a total absorption of the of the diet ingested. These animals reproduce a dietary pattern that resembles a high-sucrose diet more than a high-sucrose, high-fat diet, as expected for a cafeteria diet. It has been found that mice receiving a diet with 38.5% sucrose for five weeks showed impairment in GLP-1 secretion [25].

4. Conclusions

Working with different experimental groups of animals, we show that glucagon is more sensitive than insulin to GSPE. The moment at which the GSPE is administered seems

to be a key point in modulating the endocrine pancreas, since preventive and simultaneous treatments induce different endocrine regulations. Without ruling out the possible direct effects of GSPE on the pancreas, the data presented suggest that GSPE has a long-lasting effect on the endocrine pancreas, which is related to the effects of GSPE on GLP-1 ileal production and not related to its effects on food intake.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu13041084/s1, Table S1: Cafeteria diet offered to each rat, Table S2: Mean food intake during the five initial days on cafeteria diet.

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RESULTS: Part 2

Identification of the mechanisms through which flavanols and other secretagogues stimulate the enteroendocrine system

Manuscript 2:

Effects of Flavanols on Enteroendocrine Secretion

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Manuscript 3:

GLP1 exerts paracrine activity in the intestinal lumen of human colon

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Article

Effects of Flavanols on Enteroendocrine Secretion

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Abstract: Some beneficial effects of grape seed proanthocyanidin extract (GSPE) can be explained by the modulation of enterohormone secretion. As GSPE comprises a combination of different molecules, the pure compounds that cause these effects need to be elucidated. The enterohormones and chemoreceptors present in the gastrointestinal tract differ between species, so if humans are to gain beneficial effects, species closer to humans—and humans themselves—must be used. We demonstrate that 100 mg/L of GSPE stimulates peptide YY (PYY) release, but not glucagon-like peptide 1 (GLP-1) release in the human colon. We used a pig ex vivo system that differentiates between apical and basolateral intestinal sides to analyse how apical stimulation with GSPE and its pure compounds affects the gastrointestinal tract. In pigs, apical GSPE treatment stimulates the basolateral release of PYY in the duodenum and colon and that of GLP-1 in the ascending, but not the descending colon. In the duodenum, luminal stimulation with procyanidin dimer B2 increased PYY secretion, but not CCK secretion, while catechin monomers (catechin/epicatechin) significantly increased CCK release, but not PYY release. The differential effects of GSPE and its pure compounds on enterohormone release at the same intestinal segment suggest that they act through chemosensors located apically and unevenly distributed along the gastrointestinal tract.

Keywords: GLP-1; PYY; CCK; intestine; flavonoids

1. Introduction

The gastrointestinal tract is in charge of nutrient digestion and absorption. In addition, it is one of the bigger hormonal tissues. It is a source of various regulatory peptide hormones, secreted along the gastrointestinal tract by different enteroendocrine cells, which are involved in the coordination of digestive processes within the gastrointestinal system via autocrine and paracrine effects. Gut peptides, acting as both hormones and neurotransmitters, allow signalling between the periphery and central nervous system to coordinate systemic changes in our physiology [1]. The presence of enterohormones along the gastrointestinal tract is uneven and differs between species [2]. Among these enterohormones are found cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), and peptide YY (PYY). 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 [3,4]. PYY is often co-expressed and secreted with GLP-1 into the circulation in response to food intake, and it has been involved in energy homeostasis by regulating food intake and

suppressing excessive consumption [5–7]. GLP-1 increases glucose-dependent insulin release; reduces glucagon secretion, thereby contributing to limiting postprandial glucose excursions; and decreases gastric emptying [8,9]. Altogether, considering the importance of these hormones on regulation of body homeostasis, modulation of the enteroendocrine system has become a target for treatment of obesity and type 2 diabetes [10,11].

Grape seed proanthocyanidin extract (GSPE) is a source of flavan-3-ols compounds including catechin and epicatechin monomers and their respective oligomers. GSPE has been shown to be beneficial against obesity and the metabolic syndrome [12,13]. Suggested mechanisms used by GSPE to exert this effect include modulation of the enteroendocrine system [14]. GSPE is a mixture of compounds, some of which are not absorbed and pass through the intestine to reach the colon, where they undergo metabolization [15]. In rats, a model in which polyphenols can be more easily measured, catechin, epicatechin, and dimmer B2 were found along the intestine after GSPE administration [16]. Unmetabolyzed flavan-3-ols (catechin, epicatechin or oligomers) have been detected in feces in pigs [17] and humans [18]. Thus, not only the polyphenol's metabolites, but also some of the original compounds found in GSPE may interact with the enteroendocrine cells that are distributed along the gastrointestinal tract. In rats, acute GSPE treatment inhibits food intake (this is partly mediated by an increase in GLP-1 levels [19,20]), while ex vivo studies in rat intestinal segments have shown that GSPE directly stimulates GLP-1 and PYY release [21]. GSPE contains a mixture of different molecules and it has not yet been explained which of these are responsible for the effects of GSPE in stimulating enterohormone release. Studies in a ghrelin-secretory cell line have shown that the molecules that make up the grape extract have different effects on ghrelin secretion, for example, monomeric molecules stimulate ghrelin secretion through interaction with bitter taste receptors, while polymeric forms inhibit ghrelin secretion [22]. Several polyphenols have been shown to bind and activate bitter taste receptors, but those studies were conducted in silico [23] or were based on Ca^{2+} release in HEK293 cells that express human bitter taste receptors [24]. Therefore, although bitter taste receptors activate enterohormone release [25], the direct effects of polyphenols have not been tested for most of them. In vitro assays with STC-1 cell line have shown that flavonoids such as naringenin and hesperetin are able to stimulate CCK release [26,27]. Some other flavonoids as quercetin, kaempferol, and apigenin have also resulted in an increase in CCK levels in vitro, while others such as rutin and baicalein have not [28]. Moreover, in the STC-1 cell line, no stimulatory effect of epicatechin monomers, epicatechin gallate, procyanidin B2 dimer, or B2 gallate on GLP-1 or CCK was found [21]. In an ex vivo assay of murine intestines, epigallocatechin gallate stimulates CCK secretions in the duodenum [29], as well as GLP-1 secretions in the ileum [29]. Altogether, these results did not shed light on which molecules of those found in GSPE might contribute to its enterohormone releasing effects.

The above-mentioned studies were performed in STC-1 cells and ex vivo in intestinal tissue. The STC-1 cell line is shown to be suitable for studying enterohormone response to nutrients, though it has certain weaknesses [30], as cells are cultivated as monolayers on plastic surfaces and lack a normal cellular environment and polarization. Ex vivo studies maintain cellular environment. Furthermore, the distribution of enterohoromones and chemoreceptors that sense nutrients and activate the release of the hormones also differs along the intestine [31,32], and ex vivo studies offer the advantage over cell lines that the different intestinal segments can be tested. However, neither the cell lines nor the ex vivo studies enable luminal stimulation to be conducted. To overcome this problem, various ex-vivo approaches have been developed, including everted sacs, perfused intestinal loops, Using chambers [33], intestinal punches, precision-cut intestinal slices (PCIS) [34], organoids [35], and gut-on-a-chip [36].

In this paper, we used a specifically developed approach based on a porcine ex vivo system [37] to conduct a vectorial study of the effects of apically added bioactive compounds on the basolateral secretion of enterohormones. We used this system to investigate the effect of GSPE on PYY, GLP-1, and CCK secretion, and to identify some of the molecules responsible for this effect. To compare with

our results in pig, in parallel, we also studied how treating human colon explants (a common ex vivo system) with GSPE affected the secretion of the same enterohormones.

2. Materials and Methods

2.1. Materials

GSPE was obtained from Les Dérivés Résiniques et Terpéniques (Dax, France). The same batch (#124029) was used in all studies. According to the manufacturer, the extract contains monomers of flavan-3-ols (21.3%), and dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%), and oligomeric (5–13 U; 31.7%) proanthocyanidins. Catechin, (-)-Epicatechin, gallic acid (GA), 3-hydroxyphenyl acetic acid (3-HPAA), and protocatechuic acid (PCA) were obtained from Sigma (St. Louis, MO, USA), while procyanidin dimer B2 (B2) was obtained from Extrasynthese (Genay, France). For all studies, stocks were prepared in dimethyl sulfoxide (DMSO) and further diluted in the specific buffer required for each experiment. To transport and treat the intestinal samples, we used Krebs–Ringer bicarbonate (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) pH 7.4, prepared with either 10 mM D-Glucose (KRB-D-Glucose buffer) or 10 mM D-Mannitol (KRB-D-Mannitol buffer). For the enterohormone secretion studies, KRB-D-Glucose was supplemented with protease inhibitors: 10 µM amastatin (Enzo Life Sciences, Madrid, Spain), 500 KIU aprotinin (Sigma, Madrid, Spain), and 0.1% fatty acid free-bovine serum albumin.

2.2. Ex Vivo Pig Experiments

Pig intestines were obtained and mounted in the Ap-to-Bas ex vivo system as previously described [37]. Briefly, intestinal tissues were obtained from female pigs (*Sus scrofa domesticus*) sacrificed for meat production at a local slaughterhouse. The duodenum, ascending colon, and descending colon were collected for the experiments; transported to the laboratory in ice-cold KRB-D-Mannitol buffer saturated with 95% oxygen and 5% CO_2; and immediately used for the ex vivo experiments. The time between excision and the beginning of the experiments was approximately 30 min.

Serosal and outer muscular layers were removed and circles of tissue with a diameter of 14 mm were punched out using a biopsy punch. The whole process was maintained at a low temperature with cold buffer and an ice-cold bath. Pieces of 1.5 cm in length were cut up and the apical side of the intestinal segment was glued (with 3M Vetbond, Cat No.: 1469SB, St. Paul, MN, USA) to a silicon tube, which was then placed inside a cell culture insert without a bottom membrane (Cat No.: MCRP12H48, 12-well hanging inserts). The entire insert containing the tissue segment and the piece of tube was placed in the well of a 12-well plate prefilled with 1 mL of KRB-D-Glucose buffer. Apically, the tube was filled with 400 µL of KRB-D-Mannitol buffer. The tissues were then pre-incubated at 37 °C for 15 min in a humidified incubator (5% (*v*/*v*) CO_2).

Treatments were initiated by replacing basolateral KRB-D-Glucose with new KRB-D-Glucose with protease inhibitors and the apical KRB-D-Mannitol buffer solution with 400 µL of pre-warmed (37 °C) KRB-D-Glucose buffer containing the test compounds. KRB-D-Glucose buffer was used as a control. After 30 min of treatment, an aliquot of 200 µL was picked from the basolateral side of the Ap-to-Bas system. Finally, 60 min (for colon) or 90 min (for duodenum) after the beginning of the experiment, the entire volume of the apical and basolateral sides was frozen and stored at −80 °C for later analysis of the enterohormones.

2.3. Ex Vivo Human Colon Experiments

Human colon samples were obtained and processed as previously described [38] using an ex vivo system of human colon derived from the healthy margin of the mucosae near the fragment that is excised from colon cancer patients who require colectomy. These procedures were conducted at the Hospital Universitari Joan XXIII in Tarragona, Spain. The subjects were aged between 45 and 85. The exclusion criteria were as follows: alcohol intake above 30 g/day; body mass index above 40 kg/m^2;

use of drugs unrelated to metabolic syndrome treatment; the presence of intestinal malabsorptive or inflammatory bowel diseases; the presence of acute or chronic inflammatory or infectious disease; and the presence of neoplastic disease at advanced stages or requiring pharmacological treatment. Table 1 shows the characteristics of the subjects used for the study.

Table 1. Patient characteristics.

Clinical Characteristics	Number of Patients	Percentage (%)
Gender		
Male	12	70.6
Female	5	29.4
Colon segment		
Ascending	8	47.1
Descending	9	52.9
Hypertension	11	64.7
under treatment	5	29.4
Dyslipidemia	5	29.4
under treatment	4	23.5
Diabetes mellitus type II	4	23.5
under treatment	4	23.5
Clinical Characteristics	**Mean ± SEM**	
Age	63.4 ± 3	
Body mass index	25.6 ± 0.8	
Blood glucose (mM)	5.8 ± 0.3	
Blood cholesterol (mg/dL)	186.6 ± 12.8	

The samples were obtained during surgical treatment. Immediately after their extraction in the operating room, the samples were inserted into an ice-cold KRB-D-Mannitol buffer saturated with 95% oxygen and 5% CO_2 and transferred to the laboratory within 20 min. There, the tissues were rinsed and the serosal and outer muscular layers were removed with a scalpel. After a 10 min washing period, tissue segments were placed in pre-warmed (37 °C) KRB-D-Glucose buffer 0.1% DMSO with protease inhibitors and either GSPE or vehicle. The samples were treated for 30 min in a humidified incubator at 37 °C and 5% CO_2. Media were collected and frozen at −80 °C for enterohormone analysis. This experimental procedure was approved by the Clinical Research Ethics Committee (CEIC) of the Hospital Universitari Joan XXIII in Tarragona (CEIm 101/2017).

2.4. Enterohormone Quantification

The enterohormones were assayed using commercial ELISA kits in accordance with the manufacturer's instructions: PYY from human and pig intestinal segments were measured using fluorescent immunoassay kits (Cat No.: FEK-059-02 and Cat No.: FEK-059-03, respectively, Phoenix Pharmaceuticals, Burlingame, CA, USA). CCK from pig samples was measured using an ELISA kit (Cat No.: EKE-069-04, Phoenix Pharmaceuticals, Burlingame, CA, USA). GLP-1 from both pig and human colon samples was measured using an ELISA kit (Cat No.: EZGLPT1-36k, Millipore, Burlington, MA, USA).

2.5. Statistical Analysis

The results are presented as mean ± SEM. Data were analysed using XLSTAT 2020.1 (Addinsoft, Barcelona, Spain) statistical software. Statistical differences were assessed by Student's *t*-tests between the means of each treatment and the control. Significance was accepted over 5%.

3. Results

3.1. Apical Treatment with GSPE Stimulates Basolateral GLP-1 and PYY Secretion from Pig Intestinal Segments

Our methodology was based on intestinal pig samples mounted on a system that enables apical treatments and measures the basolateral secretion of enterohormones [37]. Figure 1a shows the effects of GSPE on pig duodenum, where 50 mg/L of GSPE stimulated PYY secretion. No significant effects were found on CCK secretion, but a tendency to an increase at the highest concentrations. In the duodenum, there was not a dose response effect of GSPE (analysis of variance (ANOVA) test, data not shown). In the ascending colon, an increase in basolateral GLP-1 secretion was observed with 50 and 100 mg/L GSPE (Figure 1b), again without a dose-response effect (ANOVA test, data not shown). In the descending colon, 100 mg/L GSPE did not modify GLP-1 secretion, but a strong increase in PYY was observed (Figure 1c). It has previously been shown in rat intestinal explants that higher doses do not affect tissue viability [21] or membrane permeability [38].

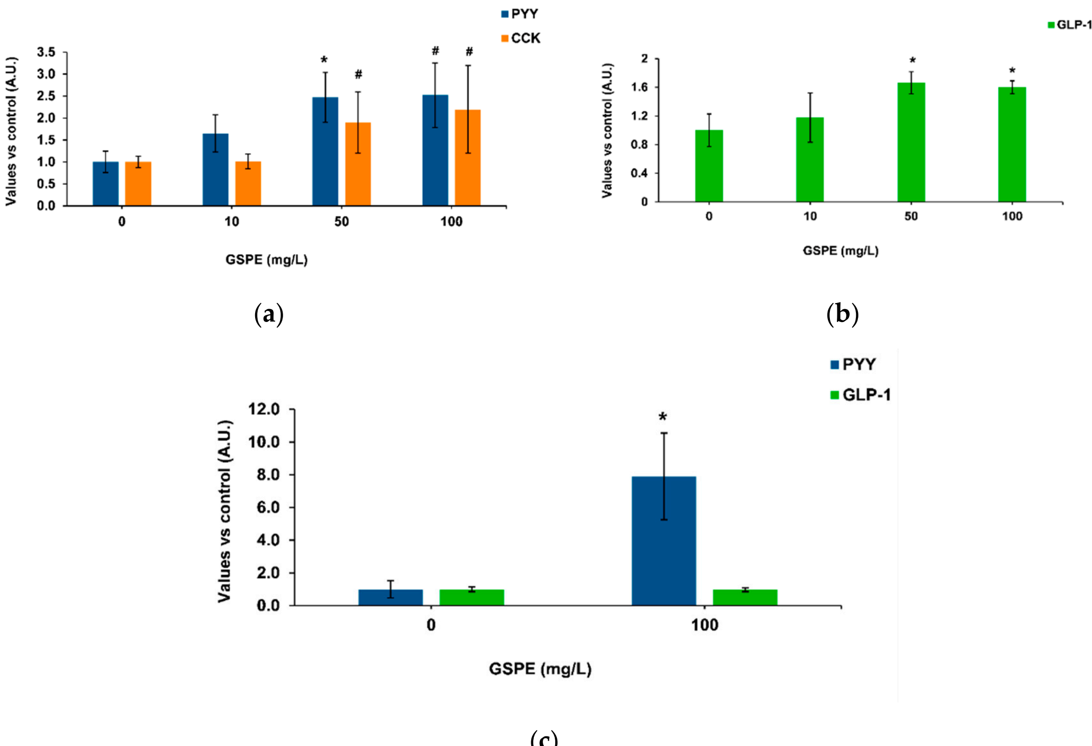
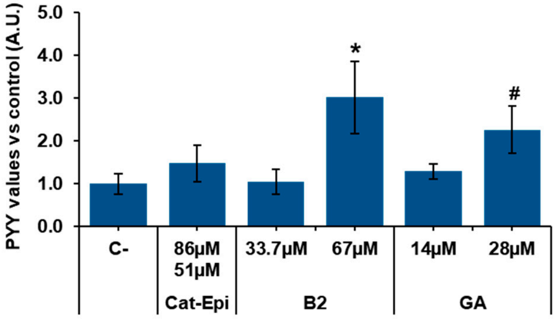


Figure 1. Enterohomone secretion levels in the basolateral medium after apical stimulation of pig intestinal segments with grape seed proanthocyanidin extract (GSPE). (**a**) Cholecystokinin (CCK) (orange columns) and peptide YY (PYY) (blue columns) secretion in duodenum. (**b**) Glucagon-like peptide 1 (GLP-1) secretion in ascending colon. (**c**) PYY (blue columns) and GLP-1 (green columns) secretion in descending colon. Results are expressed in arbitrary units (A.U.), which are values normalized versus the control and represent mean ± SEM. * $p < 0.05$ vs. control, # $p < 0.1$ *t*-test, n = 7–15.

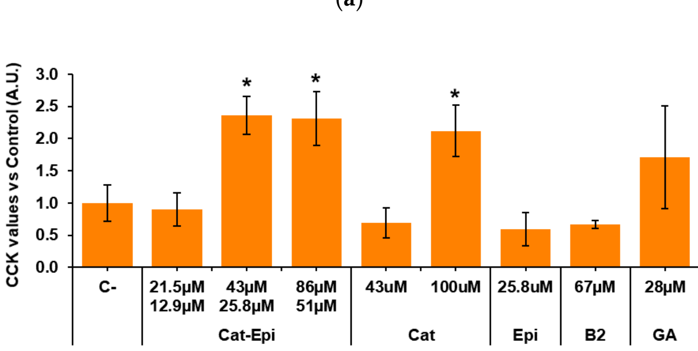
3.2. Apical B2 and Catechin/Epicatechin Specifically Stimulate PYY and CCK Secetion, Resectively

We then analysed whether purified molecules contained in GSPE stimulate enterohormone release. To do so, we first tested, in the duodenum, the most available and abundant pure compounds of GSPE. According to the manufacturer, monomeric flavanols account for 21.3% of the phenolics in GSPE. A mixture of the monomers catechin plus epicatechin in the proportion in which it is found in GPSE [39] did not modify PYY secretion (Figure 2a). Gallic acid, a different monomeric form of phenolic compound that is highly abundant in GSPE, showed no effect at 14 µM. However, a tendency to increase PYY secretion was found at double the concentration. Dimeric structures represent 17.4% of

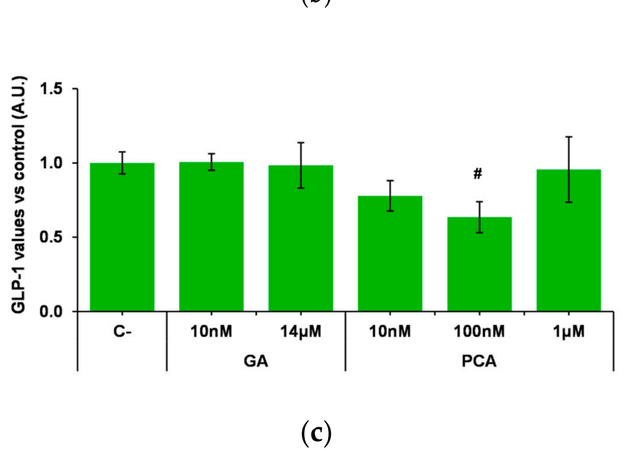
the GSPE and, to represent them, we assayed dimer B2. When tested at a concentration equivalent to the content of dimers in 100 mg/L GSPE, B2 did not modify PYY secretion. However, doubling the dose to 67 µM significantly increased PYY secretion. In the descending colon, B2 also significantly increased PYY secretion (at 1.000 ± 0.04 and 1.300 ± 0.14 in control and B2, respectively; values normalized to control levels, $p = 0.038$, n = 11–13).



(**a**)



(**b**)



(**c**)

Figure 2. Enterohormone levels in the basolateral side after apical stimulation of pig intestinal samples with different purified compounds, for 60 min. (**a**) PYY secretion in the duodenum, (**b**) CCK secretion in the duodenum, (**c**) GLP-1 secretion in the ascending colon. Results are expressed in arbitrary units (A.U.), which are values normalized versus the control and represent mean ± SEM. * $p < 0.05$ vs. control, # $p < 0.1$, *t*-test. n = 7–9. C-, negative control vehicle-treated; Cat, catechin; Epi, epicatechin; B2, B2 procyanidin dimer; GA, gallic acid; PCA, protocatechuic acid.

The effects of purified phenolic forms were also tested for CCK secretion in pig duodenum. We found that catechin/epicatechin (43/25.8 µM, respectively) induced CCK secretion. A twofold stimulation appeared to be the maximum achievable because, when double the dose (86/51.1 µM) was tested, the same level of stimulation was observed. Instead, a half-reduction in concentration did not induce CCK release (Figure 2b). The stimulatory effect of the mixture was not the result of just one of the monomers, because, when catechin and epicatechin were tested alone at the same concentration at which the mixture was effective, they did not induce CCK release. On the other hand, when only one monomer was tested at a concentration similar to the sum of both monomers in the mixture (e.g., catechin at 100 µM), CCK release significantly increased to an equivalent level (Figure 2b). Figure 2b also shows that gallic acid and B2 had no significant effects on pig duodenal CCK secretion.

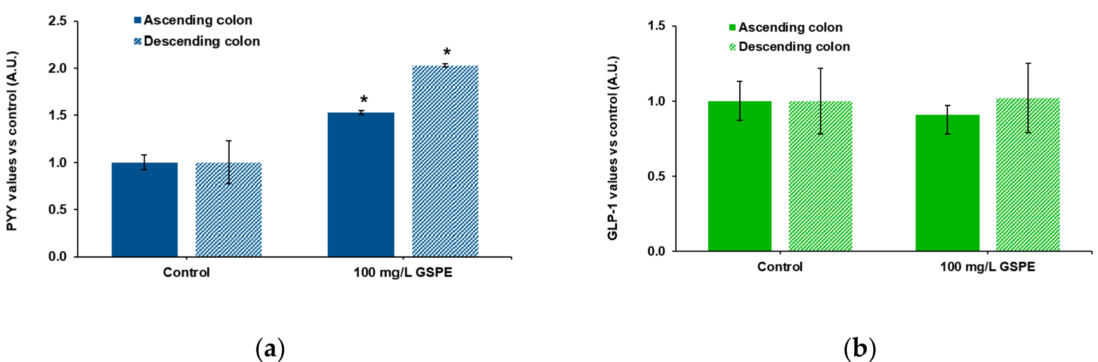
Finally, samples from the descending colon were used to test the effects of gallic acid and protocatechuic acid (PCA), which is found in GSPE, but is also one major GSPE metabolite produced in the colon [40]. Figure 2c shows no effects of gallic acid or PCA on GLP-1, but a tendency towards a reduction by 100 nM PCA.

3.3. The Stimulation of Enterohormone Secretion by Purified Molecules Takes Place Apically

To confirm that the stimulatory effects of B2 on PYY and of catechins on CCK were not the result of the interaction between possibly absorbed forms and factors of the basolateral side of the intestine, we treated duodenal samples with B2 or epicatechin/catechin, in this case, administered at the basolateral side, and measured enterohormone release to the medium. We found no increase in basolateral PYY after 33.7 µM B2 treatment (1.000 ± 0.12 and 0.769 ± 0.25 in control and B2, respectively; values normalized to control levels, $p = 0.39$, n = 7–8). Similarly, basolateral administration of 43/25.8 µM epicatechin/catechin showed no significant effect on basolateral CCK concentration (1.000 ± 0.13, 0.774 ± 0.07 in control and catechin mixture, respectively; values normalized to control levels, $p = 0.189$, n = 6).

3.4. GSPE Stimlates PYY Secretion in Human Colon

Finally, we checked the effects of GSPE on human intestine. These experiments were performed in explants because tissue availability did not enable us to mount the Ap-to-Bas system. Ascending and descending human colon explants were used to test the effects of GSPE on PYY and total GLP-1 secretion. Figure 3a shows that stimulation with 100 mg/L of GSPE led to a significant increase in PYY release to the medium in both intestinal segments. On the other hand, GSPE did not modify GLP-1 levels in the medium of either the ascending or the descending colon (Figure 3b).



(**a**) (**b**)

Figure 3. Enterohomone secretion in the medium after stimulation of human ascending (dark columns) and descending (light columns) colon explants with grape seed proanthocyanidin extract (GSPE, 100 mg/L). (**a**) PYY values, (**b**) total GLP-1 values. Results are expressed in arbitrary units (A.U.), which are values normalized versus the control and represent mean ± SEM. * $p < 0.05$ vs. control, *t*-test. n = 13–15 explants from 4–10 humans.

4. Discussion

We have previously shown that a grape seed proanthocyanidin extract modulates enterohormone secretion in rat explants [21]. We now refine our system using an ex vivo model to separate the apical and basolateral side and use pig as a more similar model to human intestine than rat [41] in order to clarify the effects of the extract and some of its compounds along the gastrointestinal tract.

We first show that GSPE stimulates basolateral PYY and GLP-1 release in pig explants. In rats, the effects of GPSE on PYY were tested in the ileum [21]. As pigs express PYY in the duodenum and the descending colon [2], we tested PYY release in those tissues. Our results show that GSPE stimulates PYY release in the descending colon more than in the duodenum. Our data suggest that basal PYY secretion is lower in the colon than in the duodenum (Supplementary Materials, Table S1). According to Albrechtsen et al., pigs express PYY in the duodenum and the descending colon at the same level [2]. However, other studies show a greater gene expression of PYY in the colon than in the duodenum [31,42]. Similarly, we found significant GLP-1 release induced by GSPE in the ascending colon, but not in the descending colon; in the latter, however, we observed a higher GLP-1 basal secretion (Supplementary Materials, Table S1). Previous studies showed similar GLP-1 expression in the ascending and descending colon [2,42]. The basal enterohormone secretion we found may thus not reflect tissue content. In any case, GSPE does not act more effectively where there is a major expression. It is likely, therefore, that the various intestinal segments respond differently to GSPE stimulus not because of the amount of enterohormone in each tissue, but because of different chemosensory machinery. Nutrient receptors along the pig gastrointestinal tract are differently expressed [31,42]. A higher gene expression in the colon has been found for certain nutrient and non-nutrient receptors related to enterohormone release, such as the fatty acid transporter GPR120 [31,42,43], as well as for the cannabinoid receptor GPR119 [42]. It has been suggested that phenolic compounds bind bitter taste receptors (TAS2R) [44,45], but information on the expression of this family of receptors along the gastrointestinal tract is scarce [46].

The above results also showed that PYY secretion and GLP-1 secretion were not always equally stimulated. In the pig ascending colon, we found significant GLP-1 release, but, in agreement with a previously reported lack of PYY expression in the pig ascending colon [2], we were unable to detect stimulation of PYY release. In the descending colon, on the other hand, we found only stimulated PYY release. This could be because the proportion of cells containing each enterohormone is different. It has been shown in mice that roughly 15% of descending colon enteroendocrine cells contain GLP-1 alone and 7% contain PYY alone [47]. This is in line with our previous results in rat explants, where we found a stronger effect of digested GSPE on GLP-1 secretion [21]. In the pig colon, 40% of GLP-1/PYY containing cells contain GLP-1 alone and 7% contain PYY alone [47], though the above paper does not specify whether this is in the ascending or descending colon. In the human colon, almost all cells with GLP-1 also contain PYY and 25% of them contain PYY without GLP-1 [48]. In the present paper, we show that GSPE stimulated PYY release in the human colon. In this case, the effect is stronger where basal PYY secretion is higher, in the descending colon (Supplementary Materials, Table S1). However, we found no effects on GLP-1 at any of these locations. A lower number of only GLP-1-containing cells found in the human colon may thus explain why we did not detect any effects on GLP-1 secretion, but GSPE acts on PYY. However, in pig, we do not know enough about enterohormone co-expression to confirm this. Another possibility is that secretion may somehow be regulated so that a cell that produces and stores both hormones may still secrete mainly one of the products [6]. This hypothesis is supported by the observation that GLP-1 and PYY are stored separately in different vesicles in L cells [49,50]. Most studies that have analysed the secretion of GLP-1 and PYY show that of both these hormones are similarly stimulated by bioactive molecules [6,51]. However, there are exceptions. For example, in an pig ex vivo ileum model, sucrose was shown to stimulate GLP-1 secretion, but not PYY secretion, while other compounds stimulated the secretion of both hormones [51]. Perhaps GSPE differentially activates PYY and GLP-1.

One reason for the differential stimulation of co-stored enterohormones by GSPE could be that the various compounds in the mixture act differently in the hormone secretion.

We thus tested the effects of pure compounds that are abundant in GSPE. Our results show that, in pig duodenum, dimer B2 activates PYY release. Our ex vivo system enables us to study the effect on secretion in a more physiological situation than with explants [37]. It has been suggested that the absorption and metabolism of flavonoids, and particularly B2, are rare [52]. However, most studies suggest that flavonoids can be absorbed in the upper gastrointestinal tract [15]. In any case, in this paper, we show that B2 interacts with some pathway that is initiated on the apical side and rule out that the effects are mediated through direct interaction with enterohormone-releasing sensory systems on the basolateral side, such as calcium-sensing receptors [53]. To our knowledge, the in vivo enterohormone-secretory effect of B2 has not previously been demonstrated. This finding could help to explain previously found B2 effects in vivo, such as reduction in food intake [54], but this suggestion requires confirmation. The concentration of B2 that is needed to observe PYY-releasing effects is higher than the concentration found in the extract. Similarly, gallic acid tended to increase PYY at a higher concentration than in GSPE, but not at that found in the active GSPE concentration. These results suggest that, in GSPE, some molecules act additively or synergically to stimulate enterohormone release. Interestingly, a mixture of catechin and epicatechin did not affect PYY duodenal secretion, but did increase CCK secretion. This, plus the fact that B2 and gallic acid did not stimulate CCK release, supports the hypothesis that a sensory machinery is activated differently by the different flavanol structures, but is also responsible for the differential secretion of enterohormones. Moreover, both catechin alone and a mixture of catechin and epicatechin increased CCK secretion. On the other hand, GSPE did not, in agreement with previous results in which GSPE at a higher concentration inhibits CCK secretion in rats [21]. In fact, we did not observe plasma CCK release after GSPE [55]. These observations suggest that compounds found in the extract antagonize the effects of catechin. We have not defined the molecules responsible for the GLP-1 stimulatory effects of GSPE, as the tested forms showed no significant effects, but a tendency towards inhibition by protocatechuic acid. It was not possible to test the effects of higher polymeric forms that may be involved in differential effects on enterohormone release. In a ghrelinoma cell line, for example, trimer C1 inhibited ghrelin secretion, which is the opposite effect to that of epicatechin gallate [22]. It has been shown that a compound can act as an agonist towards one subset of bitter receptors, but as an antagonist towards a different subset of receptors [56]. Different flavanones have been shown to antagonize epicatechin gallate response on bitter taste receptors [57] in studies that monitored calcium release in HEK293 cells expressing human bitter taste receptors. However, whether such agonism/antagonism led to differential enterohormone secretion has not been tested, though we have observed differential enterohormone secretion after stimulation with TAS2R agonists (results not shown). With ex vivo models containing not only enteroendocrine cells, but all the intestinal epithelia, we cannot ascertain the molecular mechanisms that explain this observation or discern whether there are differential mechanisms within one cell or whether there are effects on different enteroendocrine cells (Figure 4). Cellular models are used to test molecular events, though our previous results with STC-1 do not agree with enterohormone-activating secretion by GSPE or pure compounds [21,58]. Future studies will have to overcome these challenges to find out the exact mechanisms used by GSPE. Furthermore, in future studies, if the specific receptor/signalling pathway is demonstrated in pig, it will have to be established whether it is also found in humans and in which intestinal segment, as our results do not fully match between these species. In any case, these results highlight both the importance of the effects of extracts that comprise mixtures of polyphenols and the complexity involved in attributing these effects to individual molecules.

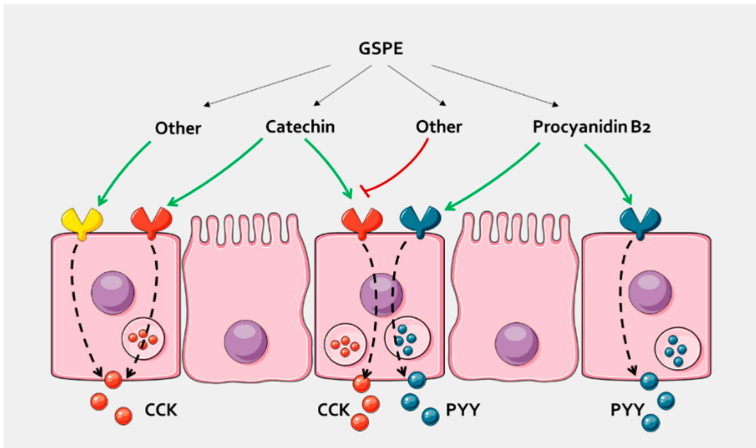


Figure 4. In pig duodenum, the different compounds of GSPE cause a selective enterohormone secretion. A possibility is that they act through different receptors that are related to a specific enterohormone secretion, either because they are expressed in cells containing only this hormone, or because they activate intracellular mechanisms that distinguish between hormones. Other compounds of the extract might antagonize the effects, or increase secretion through the same or other receptors. A similar pathway could take place in the colon, where differential GLP-1 and PYY secretion has been found.

5. Conclusions

We have shown that GSPE stimulates basolateral enterohormone release in pig and human intestine and defined the effects of some of its components. Different flavanol structures exert different effects. B2 stimulates PYY release and catechin increases CCK, both through interaction with the apical intestinal side. These enterohormone-releasing effects are altered when flavanols are administered in combination, as occurs in natural plant extracts.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/10/6/844/s1, Table S1: Basal enterohormone levels in the basolateral side of the controls in the different intestinal segments of pig samples.

Author Contributions: Conceptualization, C.G.-B., M.P., and A.A.; methodology, M.P., A.A., X.T., R.B.-D., R.J.-M., B.E., and M.T.B.; validation, C.G.-B.; formal analysis, C.G.-B.; investigation, C.G.-B. and C.G.-Q.; writing—original draft preparation, M.P.; writing—review and editing, C.G.-B., M.P., and A.A.; supervision, A.A. and M.P.; visualization, M.P.; project administration, A.A.; funding acquisition, A.A. and M.P. All authors have read and agreed to the published version of the manuscript.

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GLP1 exerts paracrine activity in the intestinal lumen of human colon

Short tittle: GLP1 activity in the colonic intestinal lumen

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Abstract

Objective: The GLP1 produced in the upper part of the gut is released after food intake and acts activating insulin secretion. The mechanism of action and function of GLP1 in the colon, where it is predominantly produced, remains unknown. Since our understanding of GLP1 and PYY has changed considerably in recent years, here we characterized the apical versus basolateral secretion of GLP1 and PYY and the paracrine mechanisms of action of these enterohormones in the human colon.

Methods: We stimulated human colon tissue in different *ex vivo* models with meat peptone and we use immunofluorescence to study the presence of canonical and non-canonical receptors of GLP1.

Results: We found that peptone stimulates both PYY and GLP1 secretion mainly at the gut lumen; as occurs under unstimulated conditions. We also found that GLP1R is widely expressed in the epithelium of human colon mucosa, and not only in the lamina propria. Moreover, we detected GLP1R expression in human colon cell culture models with Caco2 cells and infiltrating immune cells. We detected DPP4 activity and we found the presence of GCGR in the colon epithelium. Unlike GLP1R, GCGR is not expressed in the lamina propria but it is located in the crypts of Lieberkühn.

Conclusion: We show that the apical secretion of PYY and GLP1 occurs in humans and we provide evidence that GLP1 has a potential direct paracrine function through the expression of its receptors in the colon epithelium, opening new therapeutic perspectives in the use of enterohormones analogues in metabolic pathologies.

1. Introduction

Gut hormones are responsible for regulating such important metabolic pathways in the body as nutrient uptake, energy homeostasis, gut motility, food intake regulation and cell differentiation [1]. Among other signals, GLP1 and PYY are released when nutrients reach the intestinal lumen and act as satiety hormones through vagal afferent nerves activated in the intestinal mucosa. Moreover, both GLP1 and PYY slow gut motility and delay gastric emptying [2]. Unlike PYY, GLP1 secretion in the proximal intestine controls glycaemia because of its incretin effect. L-cells – i.e. the glucagon like peptide 1 (GLP1) and peptide YY (PYY) producing cells are increasingly abundant towards the distal intestine, and concentrations are highest in the colon [3]. It has been hypothesised that the function of L-cells in the colon is different from the function of those in the proximal intestine [4]. It is believed that the main function of GLP1 secretion by colonic L-cells is to slow gut motility [5, 6]. While PYY is known to slow colon motility through the Y2 receptors, it is not known whether the GLP1 mechanism of action in the colon is different from the mechanism in the proximal intestine.

In recent years, our understanding of GLP1's action and functions has changed considerably. Many non-insulinotropic functions of GLP1 have been found, most of which have been attributed to its cleaved form, GLP1 (9-36) [7 - 9]; initially believed to be inactive. It seems to function independently of GLP1R, the canonical receptor of the full form GLP1 (7-36), as its binding affinity is extremely low [10]. It has been postulated that several receptors recognise GLP1 (9-36), including CD36/fatty acid transporter [11] and the receptors for the members of the proglucagon gene family to which GLP1R belongs, such as the glucagon receptor (GCGR) [8]. In a recent study by Guida *et al.* [12], GLP1 (9-36) was shown for the first time to act through GCGR to inhibit glucagon secretion from pancreatic α-cells, while GLP1 (7-36) had the same effect through GLP1R.

Stevens *et al.* [13] recently identified luminal GLP1 and PYY secretion. This finding contrasts sharply with the widely accepted idea that enteroendocrine cells are activated apically and react by secreting enterohormones from the basolateral membrane and making them available for the vagal and afferent nerves in the lamina propria [2, 14]. Along the same lines, exogenous GLP1 has also been identified in the lumen of rat intestines. It was very recently shown to be released by Lactobacillus paracasei, present in the microbiota, and postulated to act via GLP1R expressed on some L-cells with a paracrine role [15].

In this paper, we aimed to provide further insight into apical versus basolateral secretion of GLP1 and PYY in the human colon and to explore the presence of apical canonical and non-canonical GLP1 receptors in the colon epithelium to understand the importance of apical enterohormone secretion in humans. We stimulated various *ex vivo* experimental models to test human colon samples with a meat peptone treatment, a common stimulus for enteroendocrine secretions such as GLP1 [16, 17] and PYY [18].

2. Methodologies

2.1 Materials

Peptone from bovine meat, enzymatically digested (Cat. No: 70175, Sigma-Aldrich, Madrid, Spain) was used as a treatment and protease inhibitors were used in the media in the enterohormone secretion studies. The specific inhibitors and their working concentrations were: 10 μM amastatin (Enzo Life Sciences, Madrid, Spain), 100 KIU aprotinin (Sigma, Madrid, Spain) and 0.1 % bovine serum albumin (BSA) fatty acid free. For cell culture experiments, DMEM (with 4.5 g L-1 glucose), L glutamine solution (200 mmol L-1 in 0.85% NaCl), penicillin-streptomycin mixture (10,000 U mL-1) and trypsin–EDTA solution (500 mg L-1 trypsin and 200 mg L-1 EDTA in Hank's Balanced Salt Solution) were purchased from Lonza Verviers SPRL (Verviers, Belgium). Foetal bovine serum (FBS) was provided by Sigma–Aldrich Chemie (Steinheim, Germany). RPMI 1640 medium and HEPES buffer solution (1 mol L-1) were from GIBCO (Grand Island, NY, USA). Phorbol-12-myristate-13-acetate (PMA) was provided by Quimigrancel (Barcelona, Spain). Different primary and secondary antibodies, listed in Supplementary table 1, were used for Western Blot and immunofluorescence.

2.2 Obtaining intestinal mucosa samples

Samples were obtained from the human ascending and descending colon and processed as previously described [19], using the healthy margin of the mucosae near the fragment that is excised from colon cancer patients who require colectomy. These procedures were conducted at the Hospital Universitari Joan XXIII in Tarragona, Spain. The subjects were aged between 52 and 78. The exclusion criteria were: alcohol intake above 30 g/day; body mass index above 40 kg/m2; use of drugs unrelated to metabolic syndrome treatment; the presence of intestinal malabsorptive or inflammatory bowel diseases; the presence of acute or chronic inflammatory or infectious disease; and the presence of neoplastic disease that was advanced or required pharmacological treatment. Supplementary table 2 shows the characteristics of the subjects who took

part in the study. The samples were obtained during surgical treatment. Written consent has been obtained from each patient or subject after full explanation of the purpose and nature of all procedures used. This experimental procedure was approved by the Clinical Research Ethics Committee (CEIC) of the Hospital Universitari Joan XXIII in Tarragona (CEIm 101/2017).

2.3 Enterohormone secretion study in explants

Human colon mucosa was cut into sections using a biopsy punch 6 mm of diameter. After being washed for 15 min in KRB-D-Mannitol buffer saturated with 95% oxygen and 5% CO2, tissue segments were placed in pre-warmed (37 °C) KRB-d-Glucose buffer 0.1% DMSO with protease inhibitors and either meat peptone or vehicle. The samples were treated for 30 min in a humidified incubator at 37 °C, 95% O2 and 5% CO2. After 30 min of treatment, the whole volume was frozen and stored at −80 °C for enterohormone quantification.

2.4 Enterohormone secretion study in an Ussing chamber

Human colon mucosa was cut into 1 cm x 1 cm sections. These sections were placed in an Ussing chamber with a 6 mm aperture (Dipl.-Ing. Muβler Scientific Instruments, Aachen, Germany). The mucosal preparations were stabilized for 15 min in a bath of 1.5 mL KRB-D-Mannitol in the apical chamber and 1.5 mL KRB-D-Glucose in the basolateral chamber. Next, baths in both chambers were replaced with KRB-D-Glucose with protease inhibitors containing either meat peptone or vehicle. Bathing solutions were oxygenated and circulated in water-jacketed reservoirs maintained at 37 °C. After 30 minutes of treatment, the entire volumes of the apical and basolateral sides were frozen at -80ºC for subsequent analysis of the enterohormones. Transepithelial electrical resistance (TEER) (ohm × cm2) was measured through short-circuit current using Ag-AgCl electrodes.

2.5 Enterohormone quantification

The enterohormones in the intestinal media collected during the enterohormone secretion studies were assayed using commercial ELISA kits in accordance with the manufacturer's instructions: Human PYY(3-36) was measured with fluorescence immunoassay (FEK-059-02, Phoenix Pharmaceuticals, Burlingame, CA, USA). Human total GLP-1 was measured using an ELISA kit (Cat No.: EZGLPT1-36k, Millipore, Burlington, MA, USA).

2.6 DPP4 activity measurements

DPP4 activity from human colon mucosa lysates was measured with a fluorimetric assay, using H-Gly-Pro-AMC HBr (Bachem, Bubendorf, Germany) as substrate. DPP-4 activity was normalized to total tissue weight.

2.7 Cell culture

Caco-2 (HTB-37) and THP-1 (TIB-202) cells were obtained from the ATCC (American Tissue Culture Collection) and used between passages 19–23 and 2–24, respectively. Raji-B cells were kindly provided by Dr Juan Otero from the Immunology department of the Hospital Clínic (Barcelona, Spain). For details on cell culture, see Supplementary materials.

2.8 Immunofluorescence staining

Immunofluorescence staining was performed in human mucosa samples and fixed human colonic cell culture models grown on porous membranes: Caco2 cells monoculture, Caco2 + THP1 macrophages co-culture and Caco2 + THP1 + Raji B lymphocytes tri-culture. Samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) with a pH of 7.4 for 24 h and stored in 70% ethanol. After gradient dehydration, samples were embedded transversally to the cutting direction in paraffin blocks in order to ensure good visualization of the mucosa. Embedded tissues were cut into sections 5 μm thick that were fixed to polarized glass slides. Samples were rehydrated and stained, following the previously used method [20], with a few modifications. Antigens were retrieved from rehydrated samples with a basic antigen retrieval solution (10 mM Tris base, 1 mM EDTA solution, pH 9), at 90 °C for 20. The blocking solution used was 0.1% bovine serum albumin (BSA) in PBS for 20 min at RT. The anti-GLPR, anti-GCGR and anti-actin primary antibodies and the appropriate fluorochrome-conjugated secondary antibodies were diluted in BSA 0.01% PBS and are specified in Supplementary Table 1. The double labelling was performed as follows: GLP1R and actin.

2.9 Statistical analyses

The results are presented as mean ± SEM. Data were analysed using XLSTAT 2020.1 (Addinsoft, Barcelona, Spain) statistical software. Statistical differences were assessed by subjecting the means of each treatment and the control to Student’s t-tests. Significance was accepted over 5%.

3. Results

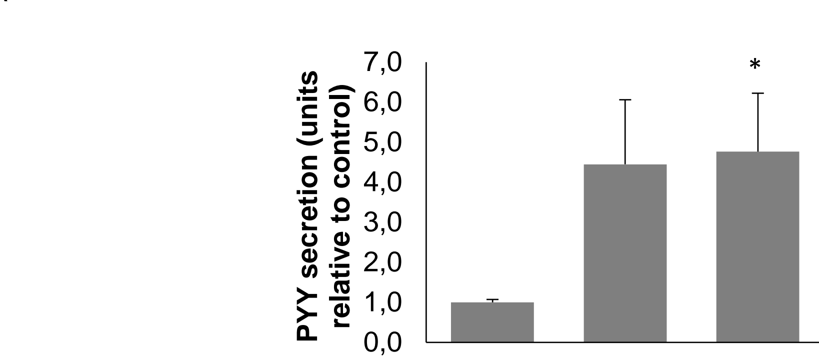
3.1 Meat peptone stimulates PYY and GLP1 secretion in human colon

The first approach to assess if an enterohormone secretory stimulus such as meat peptone can stimulate the secretome in *ex vivo* experimental models was to analyse the conditioned media after treatment. Figure 1A and 1B shows that meat peptone treatment significantly stimulated the secretion of peptide YY (PYY) and glucagon-like peptide 1 (GLP1) from human colon.

In order to dissect the compartment in which the secretion occurred, we used Ussing chambers, which compartmentalize the apical and basolateral sides of the intestinal samples treated *ex vivo*. Apical treatment of samples with 50 mg/ml meat peptone resulted in GLP1- but not PYY-stimulated secretion in the basolateral compartment of human colon samples (Fig. 1C).

To discard tissue damage by meat peptone treatment, we monitored the effect of different concentrations of meat peptone on the transepithelial electric resistance (TEER) of human colon, which is an index of tissue integrity. We observed that 50 but not 15 mg/ml meat peptone decreased human colon TEER by 30% after a 30-minute treatment in the *ex vivo* model (Supplementary Fig. 1). Therefore, we used the low concentration to subsequently evaluate the effects of meat peptone on apical secretion of enterohormones in Ussing chambers model with human samples.

A



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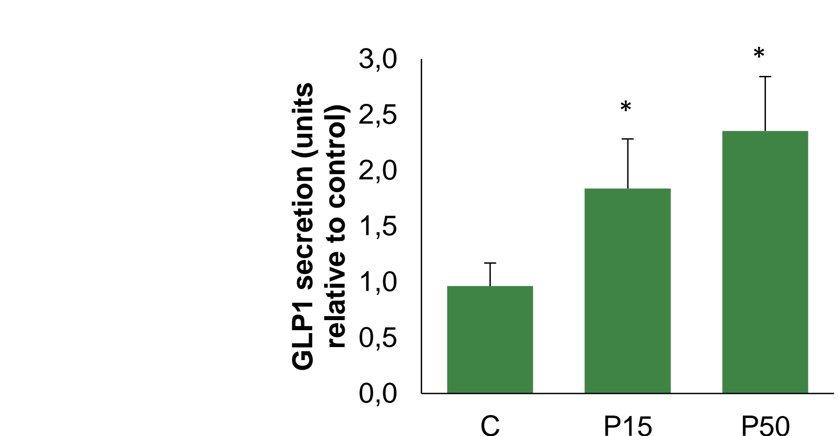

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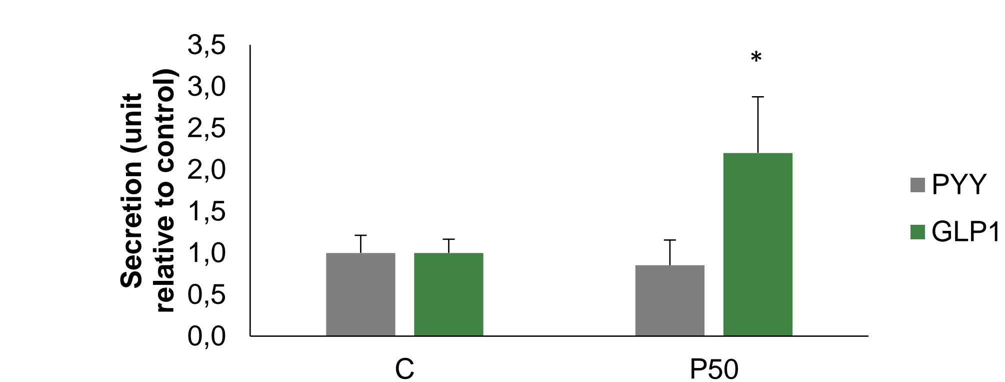


Figure 1. Enterohormone secretions from *ex vivo* intestinal segments of human colon. PYY (1A) and GLP1 (1B) secretion from free explants in untreated Control (C) after treatment with 15 and 50 mg/ml meat peptone (P15 and P50). 1C. PYY and GLP1 secretion in Ussing chambers in response to P50. Data are expressed as mean fold increase over basal secretion ± SEM in n= 9-13 experiments; * indicates significant differences ($p<0.05$) between control and treated conditions.

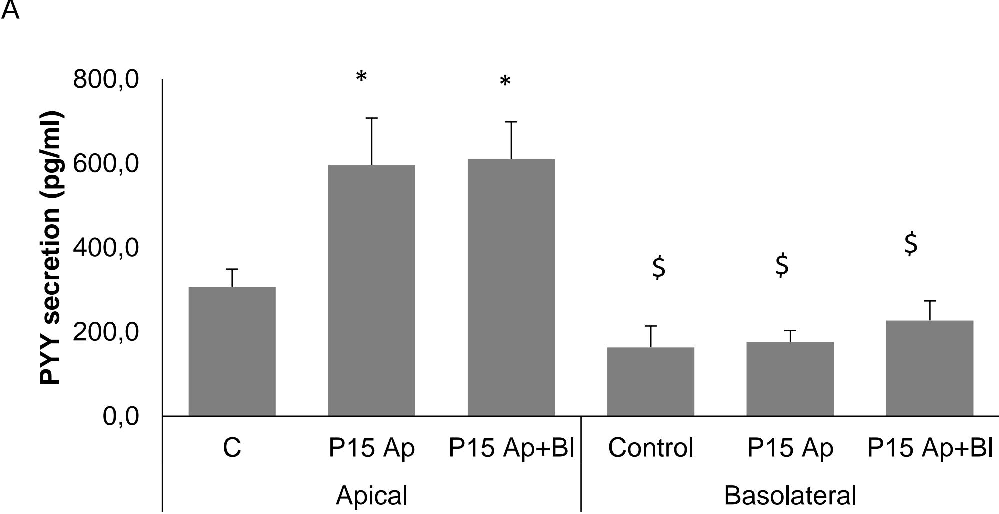
3.2. GLP1 and PYY are secreted into the intestinal lumen of human colon

We then used Ussing chambers to dissect the compartment of secretion and study how apical+basolateral treatment affects GLP1 and PYY secretion from the human colon mucosa.

Now, peptone has no effect on PYY secretion in explants on the basolateral side. But meat peptone significantly increased PYY secretion into the apical side (independently of the side of stimulation) (Fig. 2A).

Similarly, 15 mg/ml meat peptone stimulated GLP1 secretion on the apical side of human colon mucosa (Fig. 2B). Interestingly, the basal secretion on the apical side was higher than that on the basolateral side for both GLP1 and PPY (Fig. 2A and B).

A



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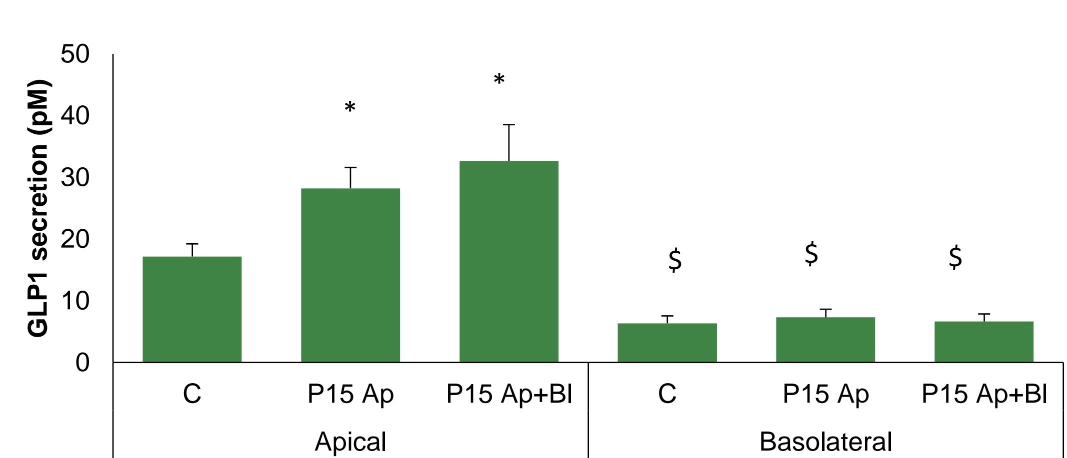

Figure 2. Apical and basolateral secretion of PYY and GLP1 in human colon. 2A. PYY secretion from human colon in the apical and basolateral compartments of Ussing chambers in untreated control (C) and after apical (P15 Ap) or apical+basolateral (P15

Ap+Bl) treatment with 15 mg/ml meat peptone. 2B. GLP1 secretion from human colon in apical and basolateral compartments of Ussing chambers in untreated control (C) and after apical (P15 Ap) or apical+basolateral (P15 Ap+Bl) treatment with 15 mg/ml meat peptone. Data are expressed as mean ± SEM hormone secretion in n=9-13 experiments; * indicates statistically significant differences ($p<0.05$) between control and treated conditions; $ indicates significant differences ($p<0.05$) between apical and basolateral secretion levels for the same condition (control and treated).

3.3. GLP1R is expressed in the intestinal lumen of human colon

The first step in determining whether enterohormones exert a function in the apical lumen is to investigate whether their receptors are present on the apical membrane. Focusing on GLP1, we evaluated whether we could detect GLP1R in the apical side of mucosa.

Initially, Western Blot analysis of protein extracts from ascending and descending human colon mucosa showed that they both expressed similar amounts of GLP1R (Supplementary Fig. 2). Immunofluorescence analyses showed an intense GLP1R signal throughout the epithelial layer of the mucosa (Fig. 3A). This layer is composed predominantly of colonocytes, but also of goblet cells, which are the mucin-producing cells, and enteroendocrine cells, which are mainly L-cells in the colon. Immunofluorescence microscopy suggested that GLP1R positivity is restricted to colonocytes in the colon epithelium, and is not expressed in L-cells, stem cells or goblet cells (marked with an arrow, Fig. 3A). We also detected intense positivity for GLP1R in some cells of the lamina propria (marked with a circle, Fig. 3A). Beneath the lamina propria are found the crypts of LieberKühn or intestinal glands. The negativity of the GLP1R signal in the intestinal glands indicates that the cell types that are also in the epithelium do not express GLP1R. A similar result was obtained working with *in vitro* models of intestine: 1) a monoculture of Caco2, 2) a co-culture of Caco2 cells with THP1 macrophages, and, finally 3) a tri-culture of Caco2 with THP1 macrophages and Raji B lymphocytes. All these different *in vitro* models were grown in cell inserts with membranes cut transversally to resemble mucosal sections with polarized Caco2 cells. We found that GLP1R was abundantly expressed in Caco2 cells in all culturing conditions but not in the infiltrating cells (Fig. 3C, 3D and 3E).

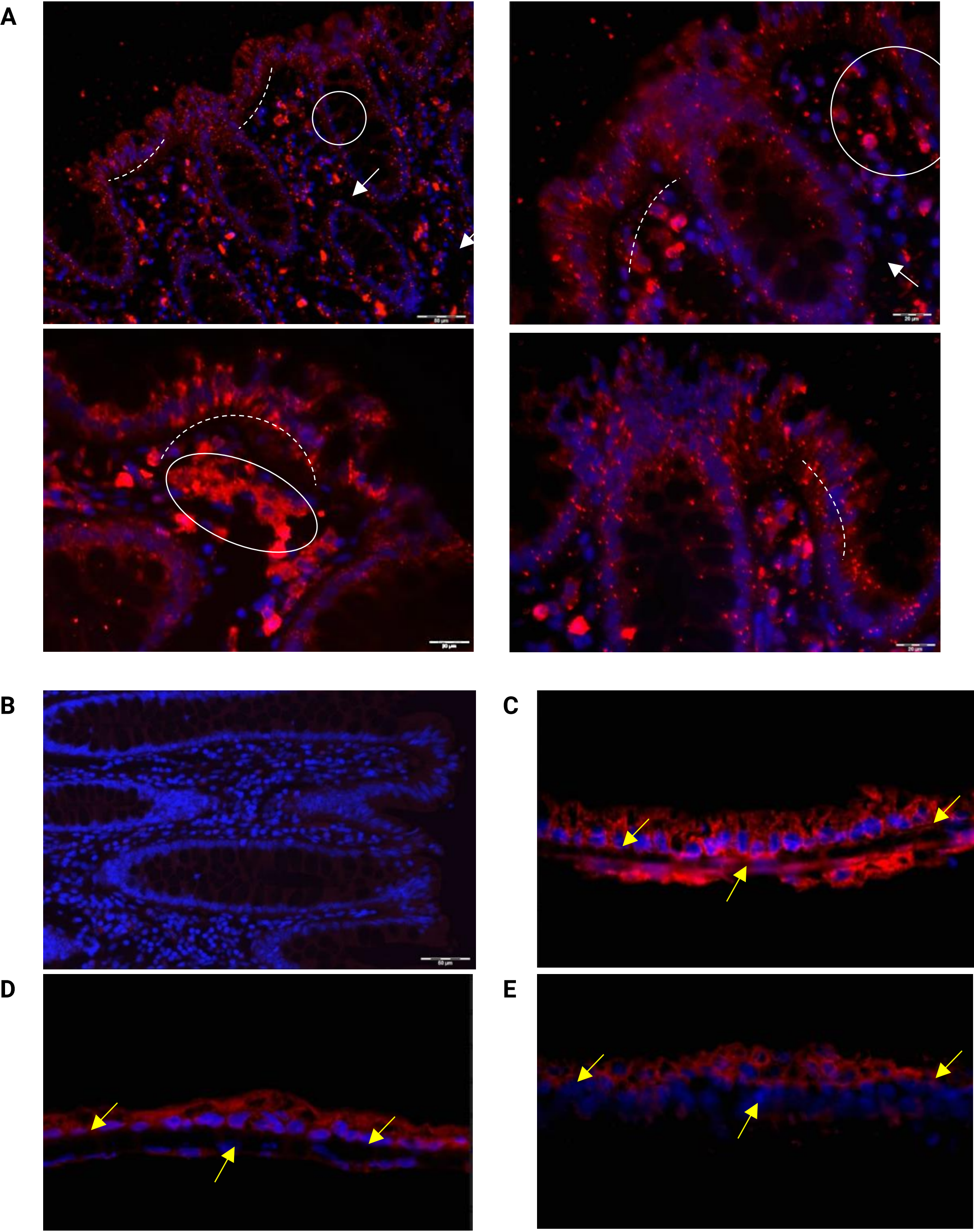

Figure 3. Immunofluorescence images of the human colon mucosa and colonic cell culture stained with GLP1R antibody. 3A. GLP1R (red) positive cells in the colon epithelium (dotted line) and in the colon lamina propria (circle), GLP1R negative cells in the crypts of Lieberkühn (arrow). Representative of n=4 preparations. 3B. Negative control of human colon mucosa, stained only with secondary antibody. 3C. GLP1R (red) positive Caco2 polarized colonocytes. 3D. GLP1R (red) positive Caco2 + THP1 co-

culture. 3E GLP1R (red) positive Caco2 + THP1 + Raji B tri-culture. Representative of n=4 preparations. Membranes where cell culture were grown are marked with yellow arrows (3C, 3D and 3E). Nuclei were stained with DAPI (blue).

The detection of GLP1R positivity in colonocytes raised the question of whether the GLP1R was expressed in their apical membrane where lumen stimuli (nutrients, hormones) act. To investigate this, we performed immunofluorescence of GLP1R together with specific staining of compartment markers using actin staining as a marker of the apical side of colonocytes [21]. Immunofluorescence staining of human colon with actin revealed that actin clearly marks the apical membrane of the colon epithelium and that it is also widely expressed in all cell types in this layer, the lamina propria and the crypts of Lieberkühn (Fig. 4A). Moreover, co-staining of human colon mucosa with GLP1R and actin showed that the GLP1R signal is located beneath the apical membrane in colonocytes, with no colocalization with actin. However, GLP1R co-localized with actin in some cells of the lamina propria (Fig. 4B). In addition, PAS staining of mucins revealed that our samples were not covered by mucus, which was present only inside goblet cells, which suggests a definite expression of GLPR on colonocyte surface with no unspecific diffusion of the signal by mucin (Fig. 4C).

Actin co-staining was also used as a marker of the apical surface in the *in vitro* intestinal models. In fact, although THP1 and Raji B express actin diffusely in the cytosol [22, 23] only polarized Caco2 cells are expected to clearly show organized actin at the apical edge. Actin staining at the apical surface of Caco2 cells confirmed their polarization in mono- and co-culture conditions. Moreover, GLP1R co-staining with actin filament in Caco2 cells suggests that the GLP1R signal is below the apical surface (Fig. 4D and 4E), as observed in colonocytes (Fig. 4B).

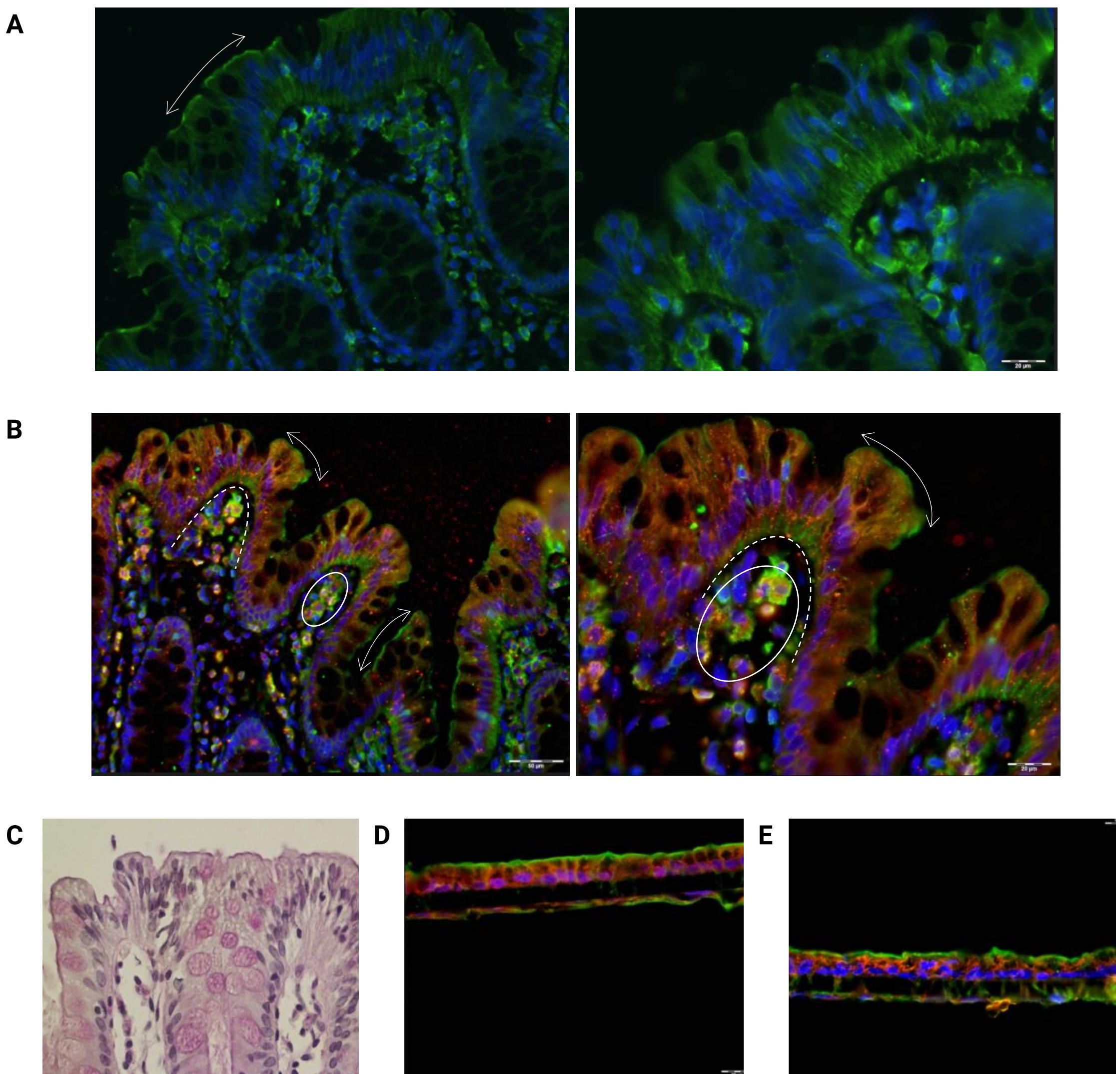

Figure 4. GLP1R localization in human colon mucosa and in *in vitro* coculture Caco2 cell models. 4A. Actin (green) staining of human colon mucosa. Apical actin filaments in the brush border are indicated with a two-sided arrow. 4B. Actin (green) and GLP1R (red) co-staining of the epithelium (dotted line) and lamina propria (circle) in human colon mucosa. Apical actin filaments in the brush border are indicated with a two-sided arrow. 4C. PAS (pink) staining of mucins in human colon mucosa. 4D. Actin (green) and GLP1R (red) co-staining of Caco2 + THP1 co-culture. 4E. Actin (green) and GLP1R (red) co-staining of Caco2 + THP1 + Raji B tri-culture. Representative of n=4 preparations. Nuclei were stained with DAPI (blue).

3.4. Alternative GLP1 receptors may be responsible for GLP1 (9-36) sensing in human colon lumen

To understand the role of GLP1 secretion into the intestinal lumen, it has to be taken into consideration that GLP1 may be cleaved by dipeptidyl peptidase IV (DPP4), a protease that cleaves enterohormones and, among others [24], can be located at the apical surface of colonocytes and the vascular endothelium of the capillaries of the lamina propria [25, 26]. Since we detected similar DPP4 activity in the human mucosa of the ascending and descending colon (Fig. 5A), we can assume that the GLP1 secreted into the lumen can be cleaved by DPP4 to GLP1 (9-36).

It has recently been suggested that the GLP1 (9-36) cleaved form possesses activity through binding to an alternative receptor [9]. It has been suggested that GCGR, the receptor for glucagon, mediates the activity of the DPP4-cleaved GLP1 form, at least in preadipocytes [9]. Immunofluorescence staining revealed that GCGR was widely expressed throughout the human colon mucosa. The GCGR signal was localized in the epithelium, like GLP1R. In contrast with GLP1R, however, GCGR was expressed in the crypts of Lieberkühn, but not in the lamina propria (Fig. 5B). GCGR and GLP1R immunofluorescence co-staining showed that although the signals did not co-localize, both receptors were expressed together in the same epithelial cells. While the GLP1R signal was localized at the apical edge of colonocytes, the GCGR signal was stronger at the basal pole of the cells.

Moreover, while only GLP1R was found in the lamina propria, GCGR can be expressed in other cell types in the crypts of Lieberkühn (Fig. 5C). Finally, we assessed GCGR expression in the *in vitro* intestinal co-culture models of Caco2 cells, where we observed widespread expression of GCGR in Caco2 but unclear expression in THP1 cells. When we performed double fluorescence immunostaining of GLP1R and GCGR in the co-culture model, we did not observe a clear localisation of these proteins in the same cells (Supplementary Fig. 3A and 3B).

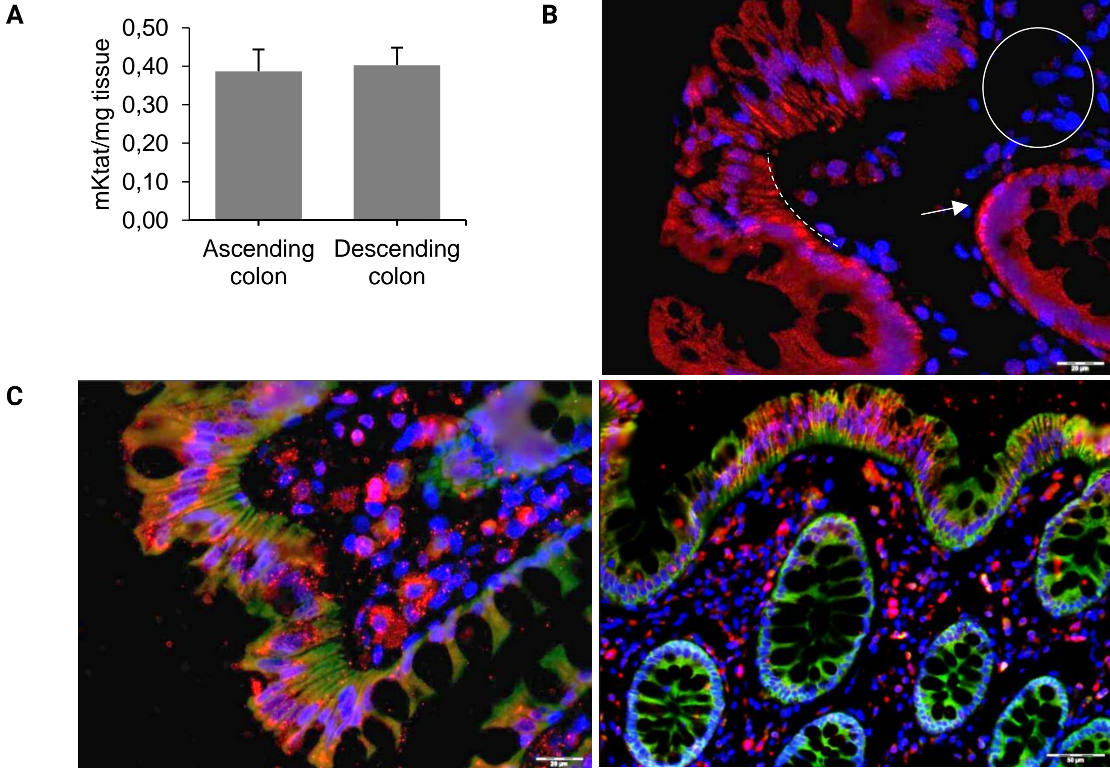

Figure 5. DPP-IV activity and presence of glucagon receptor in human colon mucosa. 5A. DPP-IV activity in lysates from human ascending and descending colon mucosa. Data are expressed as mean ± SEM of n= 4 preparations. 5B. Glucagon receptor (GCGR) positive (red) cells in human colon epithelium (dotted line) and crypts of Lieberkühn (arrow) and negative cells in the lamina propria (circle); representative of n=X preparations. 5C. GCGR (green) and GLP1R (red) co-staining of human colon mucosa. Representative of n=4 preparations from independent models. Nuclei were stained with DAPI (blue).

4. Discussion

In the present paper, we have described for the first time how treating colon mucosa with meat peptone affects the luminal secretion of PYY and GLP1, and whether the stimulation was more effective on the apical or the basolateral side of the intestine. In human colon, we observed that in basal conditions and after meat peptone treatment both PYY and GLP1 secretion into the luminal (apical) compartment was higher than into the basolateral compartment. We also found that, in comparison with control conditions, meat peptone only significantly increases PYY and GLP1 in the luminal compartment and that treatment other than just apical stimulation (apical+basolateral stimulation)

does not have a different outcome. Thus, meat peptone apically stimulates PYY and GLP1 secretion, which also occurs apically.

Luminal secretion of PYY and GLP1 from human colon was first reported by Stevens *et al.* [13], who used another *ex vivo* vector model known as InTESTine. They evaluated the response of human colon to apical treatment with 12.5 mM rebaudioside A or 2.5% casein. In response to the casein treatment, GLP1 was only stimulated into the lumen while PYY secretion was higher into the basolateral compartment than into the lumen. These results are similar to ours only in the case of GLP1, since in response to meat peptone GLP1 and PYY secretion occurred apically. Stevens et al showed similar results with the rebaudioside treatment. Unlike meat peptone, rebaudioside did stimulate GLP1 secretion in the basolateral side, although apical secretion was still higher in both control and treated conditions.

The activity of luminal secreted enterohormones may be influenced by the DPP4 present in the intestinal lumen. DPP4 activity in the intestinal lumen is not only due to intestinal epithelium transmembrane expression of the enzyme (24), but also to microbiota DPP4-like activity, as explained by Olivares *et al.* (27). This is confirmed by the fact that DPP4 activity is greater in caecal material in microbiota-colonized mice than in germ-free mice (GFM). Olivares *et al.* hypothesised that through DPP4 activity, microbiota could affect the host metabolism by protein digestion in the lumen and by regulation of enterohormone secretion in response to microbial DPP4 that has crossed the epithelial intestinal barrier. One finding that supports this hypothesis is that DPP4 KO mice showed blood DPP4 activity of unknown origin, which could be explained by microbial DPP4 that has crossed the epithelial intestinal barrier. Our findings provide novel biological relevance to this microbiota-host interaction through direct microbiota DPP4 activity on the luminal PYY and GLP1.

Cleaved GLP1 (9-36) has been generally been thought to be inactive since it has no action on the glucose metabolism and a very low binding activity on GLP1R (28). However, Cantini *et al.* [8] showed that GLP1 (9-36) has a role in non-pancreatic functions, particularly human preadipocytes, in which *in vitro* treatments induced inhibition of cell proliferation and differentiation [9]. Since GLP1 (9-39) effects seem to be independent of GLP1R activation, the GLP1 heterodimeric receptor consisting of GLP1R and other receptors such as glucagon receptor (GCGR) has been suggested as an alternative to the classic homodimer GLP1R. Accordingly, it has recently been confirmed that GCGR is the GLP1 (9-39) receptor in the pancreas [12]. Here, we have detected for the first time

the presence of GLP1R and GCGR on human colon mucosa: GLP1R is expressed in the epithelium and the lamina propria, while GCGR is expressed in the epithelium and the crypts of Lieberkühn. We have also detected GLP1R and GCGR expression in Caco2 colonocytes cocultured with THP1 macrophages and Raji B lymphocytes. The GLP1R and GCGR positivity in the human colon epithelium implies that colonocytes express GLP1R and GCGR. Although we did not observe a clear co-localisation of GLP1R and GCGR, they are undoubtedly co-expressed in the same cells and they might co-localise in stimulated conditions. Our intestinal preparations were not stimulated since they were prepared after being washed in KRB-D-Mannitol. Several studies have shown that GLP1R is located in vesicles in pancreatic-beta cells and translocates to the plasma membrane when stimulated (29, 30). Thus, further studies need to be made with human colon tissue in stimulated conditions to determine whether GLP1R and GCGR form a heterodimer that interacts with GLP1 (9-39). Notably, both GLP1R and GCGR antagonists in adipose precursors have been shown to reverse glucagon inhibitory effects [31].

In addition to colonocytes, the colon epithelium hosts other cell types: endocrine cells and goblet cells. Goblet cells do not express GLP1R because they are found in the crypts of Lieberkühn, which are clearly negative for GLP1R. However, GCGR is more probably expressed in goblet cells, as well as in other cell types in the crypts. The lamina propria is composed of a variety of structural cells and immune cells, nerve endings and vascular vessels. We observed that several cells of the lamina propria expressed GLP1R: neurons [32], blood vessels [33], intraepithelial lymphocytes [34] and smooth muscular cells [35]. However, we did not characterize. It is still to be clarified whether L-cells, the GLP1- and PYY-producing endocrine cells in the colon epithelium, express GLP1R and GCGR, thus indicating that luminal GLP1 has an autocrine role. However, the expression of GLP1R in the colon epithelium suggests that luminal GLP1 has a regulatory function in this tissue. Several paracrine loops, including GLP1, have been identified. In the small intestine of mice, enterochromaffin cells, endocrine cells which produce 5-HT, are not activated by nutrients in the lumen but by GLP1 secreted by the neighbouring cells [36]. In addition, GLP1 indirectly stimulates L-cell proliferation because the release of GLP1 triggered by bile acids leads to serotonin secretion which, in turn, increases the number of L-cells [37]. This also suggests that the GLP1 receptor is present in intestine endocrine cells. Another recent study showed that D-cells, neighbouring cells to L-cells in the mouse proximal small intestine, produce somatostatin which inhibits GLP1 secretion [38]. All these findings highlight the paracrine loops involving GLP1 in the proximal part of the intestine. However, in our study, we did not observe GLP1R signal in the crypts of

Lieberkühn suggesting that the paracrine role of GLP1 is not mediated by classical GLP1R in the colon. The expression of GCGR but not of GLP1R in some colon cell types reveals a possible novel role for this receptor in mediating cleaved GLP1 activity and for glucagon in human colon mucosa. In fact, it has just been reported that glucagon is released from ileal and colonic human explants [39]. According to this report, glucagon secretion in the intestine is part of normal physiology, and not a compensatory mechanism for disrupted pancreatic secretion, since all donors in the study had a healthy pancreas. Another recent study revealed a paracrine modulation of GCGR in L-cells, since GCGR antagonism increases the L-cell population [40].

Finally, PYY is also a substrate of DPP4 and, unlike other DPP4 substrates, its cleaved form, PYY (3-36), has higher affinity for one of its receptors, Y2, than its intact form, PYY (1-36). Conversely, PYY (1-36) binds with similar affinity to its four different receptors, Y1-Y4. The activation of Y2 is associated with the anorectic effects of PYY and delayed gastric emptying, while the activation of Y1 receptor increases intestinal motility and orexigenic effects (see Deacon *et al.* [26]). The fact that this dichotomic function of PYY depends on DPP4 activity is further confirmed by the effect of DPP4 inhibitors on body weight. While the inhibition of DPP4 increases the levels of GLP1 (7-39) and its anorectic effect, the increased levels of PYY (3-36) counteract weight loss through its orexigenic effect [41]. While both Y1 and Y2 receptors are known to be expressed in the epithelium of human colon mucosa, only Y2 is expressed in the intrinsic primary afferent neurons of the mucosa that control motility, mucus secretion and local blood flow [42, 43]. Since we have identified the luminal secretion of PYY (3-36) and DPP4 activity in human colon mucosa, we can assume that the cleaved PYY (3-36) form is predominant to PYY (1-36), and that Y2 receptors in the lumen are preferentially activated, thus reducing gastric emptying.

In conclusion, our findings demonstrate that, under stimulation with meat peptone, PYY and GLP1 are secreted in the luminal compartment where they may have a direct impact on the lumen of human colon mucosa. The expression of GCGR in colon suggests a possible recognition of GLP1 (9-36) or glucagon, to mediate the action of these hormones. Although PYY seems to be involved in the regulation of gastric emptying and GLP1 shows a diffuse expression of its classical receptor in many different cell types of the mucosa [44], further research is needed to shed light on the specific roles of these enterohormones in this organ. Fully understanding the role of enterohormones is crucial

for the treatment of obesity and diabetes since they are one of the targets of current therapeutic treatments of these diseases.

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No potential conflicts of interest are reported by the authors.

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Supplementary materials

Cell culture:

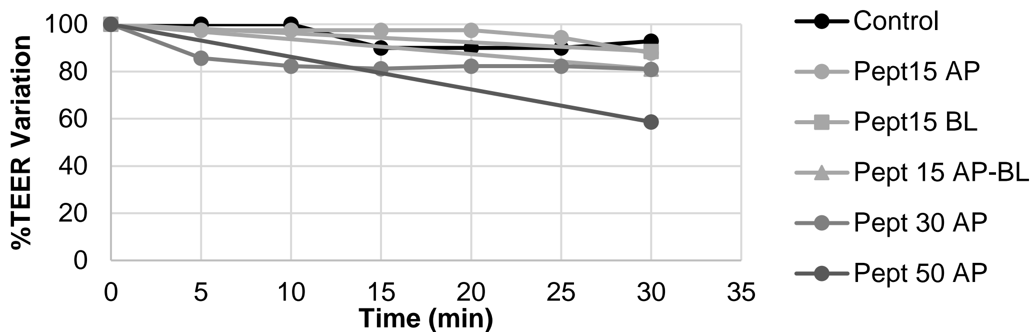
Cells were maintained in 75 cm^2 culture flasks (Greiner Bio-One, Frickenhausen, Germany) at 37°C in an atmosphere of 5% CO_2 with the medium being changed every 2–3 days. The growth medium for Caco-2 cells consisted of DMEM supplemented with 10% (v v 1) heat-inactivated FBS, 2 mmol L^{-1} L glutamine, 1 mol L^{-1} HEPES and 100 U mL^{-1} penicillin-streptomycin mixture. When confluence reached ~80%, Caco-2 cells were harvested by treatment with 0.25% Trypsin– 1 mol L 1 EDTA for 10 min, and then split and sub-cultured in fresh growth medium. The growth medium for Raji-B and THP-1 cells consisted of RPMI 1640 supplemented as described above. Both Raji-B and THP-1 cells were maintained at a density between 1×10^5 and 1×10^6 cell mL^{-1}. When density reached 1×10^6 cell mL-1, cells were split 1:10 and then sub-cultured in fresh growth medium.

We used three different culture-based models of gut epithelium: a Caco-2 cell monoculture, a Caco-2/THP-1 cell co-culture and a Caco-2/Raji-B/THP-1 tri-culture. The

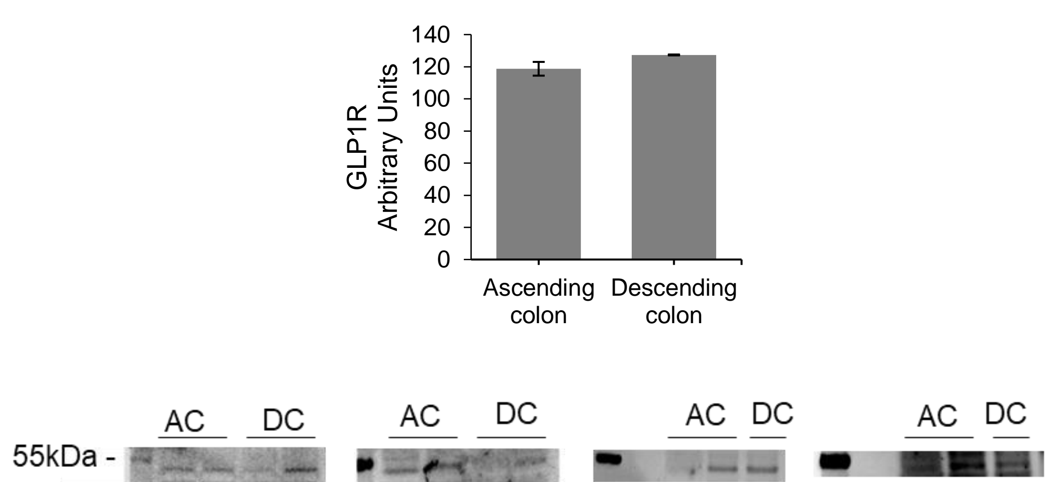
co- and tri-culture models were set up by adapting the protocol described by des Rieux *et al.* [308] for co-cultured Caco-2/Raji-B cells as follows: 0.5-mL aliquots of Caco-2 cell suspension (5×10^5 cells mL^{-1}) were added in permeable transwell inserts (1.1-cm^2 surface area and 3-µm pore size) in 12-well plates (Merck Millipore, Darmstadt, Germany). One mL of DMEM growth medium was added to the wells and both apical and basolateral media were changed every other day. On day 5, inserts were inverted over a culture dish (245 mm×245 mm×20 mm) filled with DMEM growth medium. Pieces of silicon tubes were then placed on the basolateral sides and filled with DMEM growth medium. Inverted inserts were cultivated for 11 days and the basolateral media were changed every other day. On day 16, 0.4-mL aliquots of Raji-B cell suspension (5×10^5 cells mL^{-1} in DMEM:RPMI (2:1) growth medium) were added to the basolateral compartments and cultured for five additional days. On day 21, the silicon tubes were removed, and the inserts were washed twice with HBSS. Inserts where then placed in 12-well plates with fully differentiated THP-1 in each well. THP-1 monocytes (5.5×10^5 cells per well) were differentiated into adherent macrophage-like cells by treatment with PMA (100 ng mL-1 in RPMI growth medium without FBS) 48 h prior to the experiments.

Western Blot procedure:

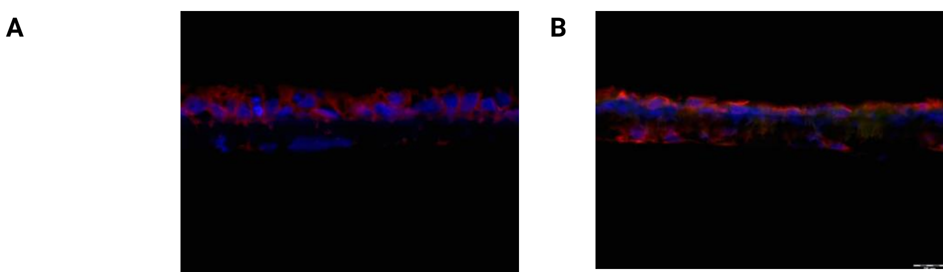
Proteins from human colon mucosa, adipose tissue and heart were extracted using RIPA buffer (20mM Tris, pH 7.4, 150mM NaCl, 0.5% Triton-100, 1mM Na3VO4, 1mM PMSF). Then, SDS-PAGE electrophoresis with stain-free gels (Bio-Rad) was performed to separate thirty micrograms of proteins per sample, which were then transferred to PVDF membranes with Trans-Blot® TurboTM (Bio-Rad). Membranes were incubated overnight at 4°C with primary antibodies (Table 1). Next, membranes were incubated for 2 hours at room temperature with peroxidase-conjugated secondary IgGs. Images were acquired and analysed with Image Lab software on a ChemiDoc TM Touch instrument (Bio-Rad), using fluorescence emission of protein bands separated on stain-free gels for total lane normalization.



Supplementary figure 1. Effect of 30 minutes meat apical peptone treatment in human descending colon. Effect of meat peptone at different concentrations (15, 30 or 50 mg/ml) on TEER (C) (n=4-7).



Supplementary figure 2. GLP1R expression in human ascending and descending colon detected by Western Blot



Supplementary figure 3. GCGR and GLP1 expression in Caco2+THP1 cell co-culture. (A) GCGR (red) expression. (B) GCGR (green) and GLP1 (red) expression in Caco2+THP1 cells. Representative of n=4 preparations from independent models. Nuclei were stained with DAPI (blue).

Supplementary table 1. Antibodies used for Western Blot (WB) and Immunofluorescence (IF) analyses

Antibody	Ref.	Dilution
1ary Anti-GLP1R (mouse)	sc-390773, Santa Cruz Biotechnology	1:1000 WB 1:100 IF
1ary Anti-Actin (goat)	sc-1615, Santa Cruz Biotechnology	1:100 IF
1ary Anti-GCGR (rabbit)	ab75240, Abcam	1:250 IF
2ary peroxidase-conjugated IgG	Southern Biotech #2010-05	1:2000 WB
2ary Alexa green 488 (anti-rabbit)	AffiniPure sheep, Jackson ImmunoResearch Europe	1:175 IF
2ary Alexa red 594 (anti-mouse)	AffiniPure sheep, Jackson ImmunoResearch Europe	1:175 IF
2ary Alexa red 594 (anti-rabbit)	AffiniPure sheep, Jackson ImmunoResearch Europe	1:175 IF

Supplementary table 2. Patient characteristics

Clinical characteristics	Number of patients	Percentage (%)
Gender		
Male	9	47.4
Female	10	52.6
Colon segment		
Ascending	11	57.9
Descending	8	42.1
Hypertension	10	52.6
under treatment	9	90.0
Dyslipidemia	6	31.6
under treatment	5	83.3
Diabetes Mellitus type II	5	26.3
under treatment	5	100.0
Clinical characteristics	**Mean ± SEM**	
Age (years)	66.3 ± 1.8	
BMI	26.9 ± 1	
Blood glucose (mM)	5.8 ± 0.4	
Blood cholesterol (mM)	3.1 ± 0.3	

RESULTS: Part 3

Association of flavanols-induced enteroendocrine stimulation by specifically targeting bitter taste receptors in the gastrointestinal tract, with the control of food intake

Manuscript 4:

Modulation of Food Intake by Differential TAS2R Stimulation in Rat

Published in: Nutrients (10/12/2021)

- Related patent

Manuscript 5:

Functional and genomic comparative study of the bitter taste receptor family TAS2R: insight into the role of human TAS2R5

Submitted to: FASEB Journal (01/08/2021)

Article

Modulation of Food Intake by Differential TAS2R Stimulation in Rat

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Abstract: Metabolic surgery modulates the enterohormone profile, which leads, among other effects, to changes in food intake. Bitter taste receptors (TAS2Rs) have been identified in the gastrointestinal tract and specific stimulation of these has been linked to the control of ghrelin secretion. We hypothesize that optimal stimulation of TAS2Rs could help to modulate enteroendocrine secretions and thus regulate food intake. To determine this, we have assayed the response to specific agonists for hTAS2R5, hTAS2R14 and hTAS2R39 on enteroendocrine secretions from intestinal segments and food intake in rats. We found that hTAS2R5 agonists stimulate glucagon-like peptide 1 (GLP-1) and cholecystokinin (CCK), and reduce food intake. hTAS2R14 agonists induce GLP1, while hTASR39 agonists tend to increase peptide YY (PYY) but fail to reduce food intake. The effect of simultaneously activating several receptors is heterogeneous depending on the relative affinity of the agonists for each receptor. Although detailed mechanisms are not clear, bitter compounds can stimulate differentially enteroendocrine secretions that modulate food intake in rats.

Keywords: TAS2R5; TAS2R39; TAS2R14; agonist; food intake; GLP1; CCK; PYY

1. Introduction

Controlling food intake is a complex process that requires the combination of signals with very different origins. In animals, the nervous and hormonal systems play a role but in humans, feelings and sensations due to other environmental factors are also involved [1]. To study and monitor the regulation of food intake, numerous approaches involving diet, physical activity, medical devices, pharmacotherapy and metabolic (bariatric) surgery have been applied.

One of the most effective treatments against obesity and associated metabolic disorders is metabolic surgery [2], which leads to a huge change in metabolism and modifies the gastrointestinal secretome of patients. Enterohormones reach several targets in the body, including the brain and other peripheral tissues (e.g., adipose tissue, muscle and the gastrointestinal tract) [3]. The most consolidated effect of bariatric surgery in gastrointestinal secretome is an increase in glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) [4]. Reproducing this enterohormone modulation without surgery could produce

some of the beneficial effects of surgery without being so invasive. Several nutritional approaches cause enterohormone profile regulation [5]. However, they are not always effective at controlling food intake, probably because there is little control over the composition of the food components.

The bitter taste helps us to protect ourselves against unhealthy natural products [6]. Nevertheless, not all toxins are bitter and not all bitter compounds are toxic [7]. In fact, many bitter compounds have health benefits. It has even been suggested that healthier diets contain a higher proportion of bitter-tasting ingredients, such as bitter vegetables [8]. Recently, bitter taste receptors (TAS2Rs) have been identified in locations other than the mouth—where taste perception occurs—but a clear role for them there has not yet been defined [9,10]. Meyerhof et al. studied the association between bitter ligands and specific TAS2Rs using in vitro assays and calcium imaging [11]. Together with Di Pizio [12], they showed that humans and mice [13], which have a relatively large number of bitter taste receptors (25 in humans and 35 in mice), contain different types of receptors depending on their selectivity. Some selective receptors (such as hTAS2R3) only bind 1–3 ligands. In contrast, less selective receptors (such as hTAS2R39) and highly promiscuous receptors bind several ligands. A promiscuous TAS2R is one that can be activated by several ligands. In turn, ligands can be specific or unspecific for a certain receptor [12]. Species with a more limited number of bitter taste receptors contain only promiscuous receptors. Research is currently being conducted into the role of these bitter taste receptors that are located away from the lingual papillae where taste is perceived. They have been found in several locations, including the lungs [14] or stomach. Our group and others have shown that stimulating them induces ghrelin secretion in the murine ghrelinoma cell line [15] and in human fundic cells [16].

In this paper, we test whether a profile of enterohormones that limits food intake can be obtained by stimulating bitter taste receptors differentially. To do so, we used specific ligands for bitter taste receptors with different ranges of specificity, according to Meyerhof's definitions [11,17]. We also tested the effects of these ligands on enterohormone secretions in various segments of the rat intestine. Finally, we associated their effects on rat gastrointestinal secretome with their ability to modulate food intake in the whole animal.

2. Materials and Methods

2.1. Chemicals and Reagents

1,10-Phenantroline, (-)-epicatechin, Thiamine, Flufenamic acid, Vanillic acid and Protocatechuic acid were purchased from Sigma (Barcelona, Spain). Procyanidin B2 gallate, Epigallocatechin gallate (EGCG) and epicatechin gallate were purchased from Extrasynthese (Genay, France) and Procyanidin B2 was purchased from Adooq-Bioscience (Irvine, CA, USA).

We used Krebs–Ringer bicarbonate (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) pH 7.4, supplemented with either 10 mM D-Glucose (KRB-D-Glucose buffer) or 10 mM D-Mannitol (KRB-D-Mannitol buffer). For enterohormone secretion studies, KRB-D-Glucose was supplemented with protease inhibitors: 10 µM amastatin (Enzo Life Sciences, Madrid, Spain), 100 KIU aprotinin (Sigma, Barcelona, Spain) and 0.1% fatty acid free-bovine serum albumin.

2.2. Animals

We used 26 male Fischer-344 rats (Charles River Laboratories, Barcelona, Spain) and 20 female Wistar rats (Envigo, Barcelona, Spain). Most of these animals were housed at the animal housing facility of the Universitat Rovira i Virgili. Ten female Wistar rats were bred and housed at the Faculty of Biology of the Universitat de Barcelona. All rats were housed under standard conditions, i.e., they were caged in pairs at a room temperature of 23 °C with a standard 12-h light-dark cycle (lights on at 7 am), with ventilation, ad libitum access to tap water and a standard chow diet. The Fischer-344 rats were fed with a standard chow diet by SAFE (Cat No: A04, SAFE, Augy, France) while the Wistar rats were fed with a standard chow diet by Teklad (Cat No: Teklad 2014, Envigo, Barcelona, Spain). All procedures

were approved by the Experimental Animal Ethics Committee of the autonomous government of Catalonia, Spain (Ministry of Territory and Sustainability, Directorate-General for Environmental Policy and the Natural Environment, project authorization code 10715) and the University of Barcelona (Ministry of Territory and Sustainability, Directorate-General for Environmental Policy and the Natural Environment, project authorization code 10769).

2.3. Ex Vivo Treatment of Intestinal Segments

We used 26 male Fischer-344 rats weighing 350–400 g. After a short fasting period (1–3 h), the rats were euthanized by decapitation and their intestines were excised. Samples were collected from the proximal duodenum and distal ileum. The tissue was rinsed with ice-cold KRB-D-Mannitol buffer and dissected into segments (0.5 cm diameter). After 15 min of washing, the tissue segments were placed in prewarmed (37 °C) KRB-D-Glucose buffer 0.1% Dimethyl Sulfoxide (DMSO) containing the compounds to be tested. Duodenal and ileal segments were treated with different compounds or a mix of compounds (Table S1) in a humidified incubator at 37 °C, 95% O_2 and 5% CO_2. After 30 min of treatment, the whole volume was frozen and stored at −80 °C for enterohormone quantification.

2.4. Studies of Food Intake

Ten female Wistar rats were housed in pairs for one week of adaptation. After this adaptation period, the animals were housed in single cages, introduced to daily 4 h food deprivation before light offset (3 p.m. to 7 p.m.) to habituate them to the experimental schedule, and trained for intragastric oral gavaging with tap water 1 h before dark onset (6 p.m.). One experiment per week was performed in a cross-over design for all food intake studies. For each experiment, the trained animals were treated with different compounds or a mix of compounds at defined concentrations (see Supplementary Table S1) intragastrically by oral gavage 1 h before dark onset (6 p.m.) using tap water as a vehicle. Parallel controls were performed by administering the vehicle intragastrically. Chow diet was administered at dark onset (7 p.m.) and chow intake was measured 3, 12 and 20 h later.

Determination of the effects of an acute dose of intragastric treatments in portal vein enterohormone secretion

Intragastric treatments were performed in two sets of animals. The first set comprised 10 female Wistar rats that received a specific intragastric dose of 1,10-Phenanthroline. The second set comprised 10 female Wistar rats that received an intragastric dose of (-)-Epicathechin. The same procedure (described earlier) was applied to both sets of rats. The animals were randomly divided into a control group and a treated group. The rats were fasted from 10 p.m. to 7 a.m. before treatment and anaesthetized 5 min later with either inhaled isoflurane (5% for induction, followed by 3% for maintenance) for the 1,10-Phenanthroline assay or pentobarbital (70 mg/kg) for the (-)-epicatechin assay. The abdominal cavity was incised through the midline and the portal vein was catheterized with a PE tube (Inner Diameter(I.D.) 0.28 mm, Outer Diameter (O.D.) 0.61 mm; Becton Dickinson, Sparks, MD, USA) following a standard procedure. The catheter was fixed with cyanoacrylate and the abdominal cavity was closed with surgical clamps. The body temperature was kept constant at 37 °C by a heated surgical table and overhead lamps. At time zero, 200 µL of blood were obtained and the catheter was refilled with saline. The specific treatment or tap water as the vehicle was punctured into the forestomach. Two portal blood samples (200 µL) were taken after treatment (described in the results) and each time the catheter was refilled with heparinized 0.9% NaCl. The blood was transferred to heparinized tubes containing a 1:100 volume of a 1:1 mix of commercial Dipeptidyl peptidase-4 inhibitor (DPPIV, Millipore, Madrid, Spain), to which a serine protease inhibitor (cOmplete™ ULTRA Tablets, Roche, Barcelona, Spain) was added. Plasma was collected by centrifugation at 1500× *g* over 15 min at 4 °C and frozen immediately at −80 °C for enterohormone quantification. The rats were sacrificed by bilateral thoracotomy.

2.5. Enterohormone Quantification

We measured enterohormone secretions from intestinal segments and plasma with commercial kits. Total and active GLP-1 were measured with ELISA kits from Millipore (Cat No: EZGLPT1-36k and EGLP-35K, respectively, Burlington, MA, USA). PYY was measured using a fluorescent immunoassay kit (Cat No: FEK-059-03, Phoenix Pharmaceuticals, Burlingame, CA, USA). Total CCK was measured with an ELISA kit (Cat No: EKE-069-04, Phoenix Pharmaceuticals, Burlingame, CA, USA).

2.6. Statistical Analysis

Our results are presented as mean ± SEM. Data were analyzed with XLSTAT 2020.1 (Addinsoft, Barcelona, Spain) statistical software. Statistical differences were assessed by Student t-tests, and $p < 0.05$ was considered statistically significant.

3. Results

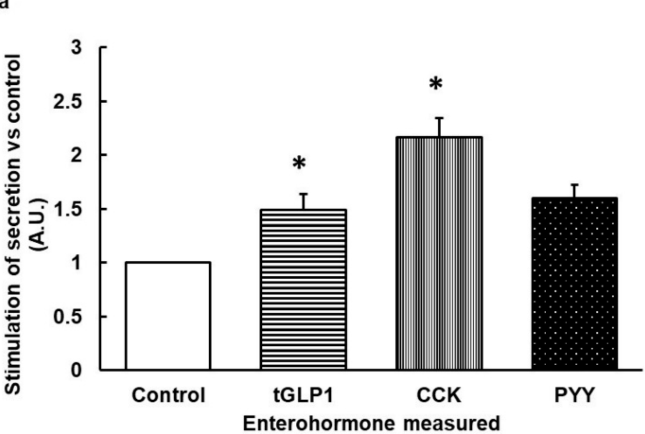
3.1. Stimulation with Specific Agonists of hTA2R5 Increases GLP1 and CCK Secretions, While Stimulation with Specific Agonists of hTA2R39 Tends to Increase PYY and Decrease CCK

We tested the stimulation of different receptors with different degrees of selectivity. In humans, the most selective bitter taste receptors are hTAS2R3, hTAS2R5, hTAS2R13 and hTAS2R8. 1,10-Phenantroline is the only selective agonist for the hTAS2R5 receptor [12]. That is, this compound does not bind to other receptors in human TAS2Rs. We assayed the extent to which 1,10-Phenantroline, at around its minimum effective dose for hTAS2R5 (defined in Table 1), stimulated explants of various segments of rat intestine. Figure 1a shows that 1,10-Phenantroline increased total GLP1 (tGLP1) and CCK secretion with no statistical differences on PYY secretions.

Table 1. Comparison of the doses of the agonists and ligands assayed in the study and the individual binding parameters defined for each.

Compound (hTAS2R)	EC_{50} [1] (µM)	Effective Concentration [2] (µM)	Dose Administered to Rats	Dose for Treatment of Intestine Explants
1,10-Phenantroline (hTAS2R5) [18]	Not defined	100	290 µM	150 µM
Thiamine(hTAS2R39) [11]	Not defined	1000	7.5 mM	1 mM
ECg (hTAS2R39) [19]	88.2	Not defined	31 µM	-
Epicatechin (hTAS2R5) [19]	3210	1000	0.84/1 mM	1 mM
Epicatechin (hTAS2R39) [19]	3800	-	-	-
B2 gallate(hTAS2R5) [17]	6.3	Not defined	-	20 µM
B2 gallate(hTAS2R39) [17]	9.11	Not defined	-	-
Epigallocatechin Gallate(EGCG) (hTAS2R5) [17]	12.3	-	-	-
EGCG(hTAS2R39) [17]	8.5	Not defined	21/43 µM	300 µM
EGCG(hTAS2R39) [20]	181.6	10	-	-
Flufenamic acid (hTAS2R14) [11]	Not defined	10	50 µM	-
Protocatecuhic acid(hTAS2R14) [17]	156	Not defined		300 µM
Vanillic acid (hTAS2R14) [17]	151	Not defined	1.5 mM	300 µM
Procyanidin B2 [21]	Not defined	485 µM [3]	0.11 mM	67/300 µM

[1] EC_{50}: half-maximum effective concentrations. [2] Effective concentration: minimal concentration that elicited response. [3] Sensorial umbral, not effective concentration.



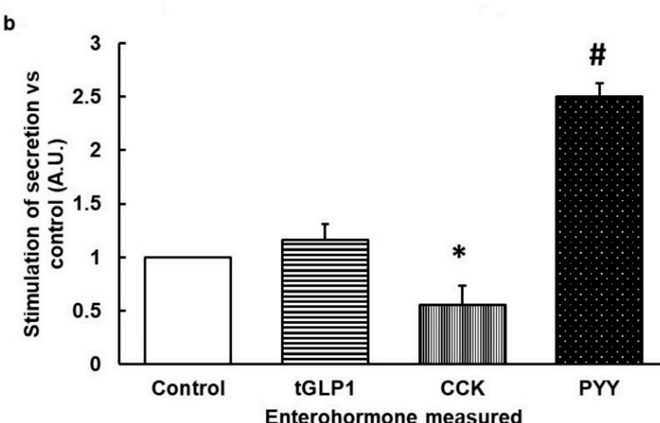


Figure 1. Ex vivo stimulation of enterohormone secretions induced by 1,10-Phenantroline (**a**) or Thiamine (**b**). Rat segments of duodenum (for cholecystokinin (CCK)) and ileum (for total glucagon-like peptide 1 (tGLP1) and peptide YY (PYY)) were treated with 1,10-Phenantroline 150 µM for 30 min (**a**) or Thiamine 1 mM for 30 min (**b**). Afterwards, medium was collected and respective enterohormones were quantified by ELISA (n = 6–10 segments). Results are calculated versus basal respective secretion in each hormone (Arbitrary Units, A.U.). Mean ± SEM. * denotes $p < 0.05$ vs. control; # indicate $p < 0.1$ vs. control.

According to Di Pizio et al. [12], hTAS2R39 is a less selective receptor. One of the selective agonists for it is Thiamine, though this compound also binds (h)TAS2R1 [11]. Treating rat intestine segments with the minimum effective dose of Thiamine for hTAS2R39 (Table 1) significantly reduced CCK secretion and tended to increase PYY secretion without affecting tGLP1 (Figure 1b).

3.2. When Bitter TAS2Rs Are Subjected to Simultaneous Stimulation, the Effect on Secretome Is Similar to the Effect on the Receptor with Lower EC50 Only

To understand responses closer to an in vivo situation, we assayed the simultaneous stimulation of hTAS2R5 and hTAS2R39 agonists with 1,10-Phenanthroline plus Thiamine in rat intestinal segments at doses close to their minimally effective concentration (Table 1). Figure 2a shows that ileal tGLP1 secretion was clearly stimulated by simultaneous stimulation with 1,10-Phenanthroline plus Thiamine. We then treated ileum segments with compounds that can bind both receptors but with a lower EC50 for hTAS2R5 than for hTAS2R39, i.e., B2gallate and (-)-epicatechin [19] (Table 1). B2 gallate tended to stimulate tGLP1 in ileum and CCK in the duodenum, while epicatechin significantly increased CCK secretion (Figure 2b).

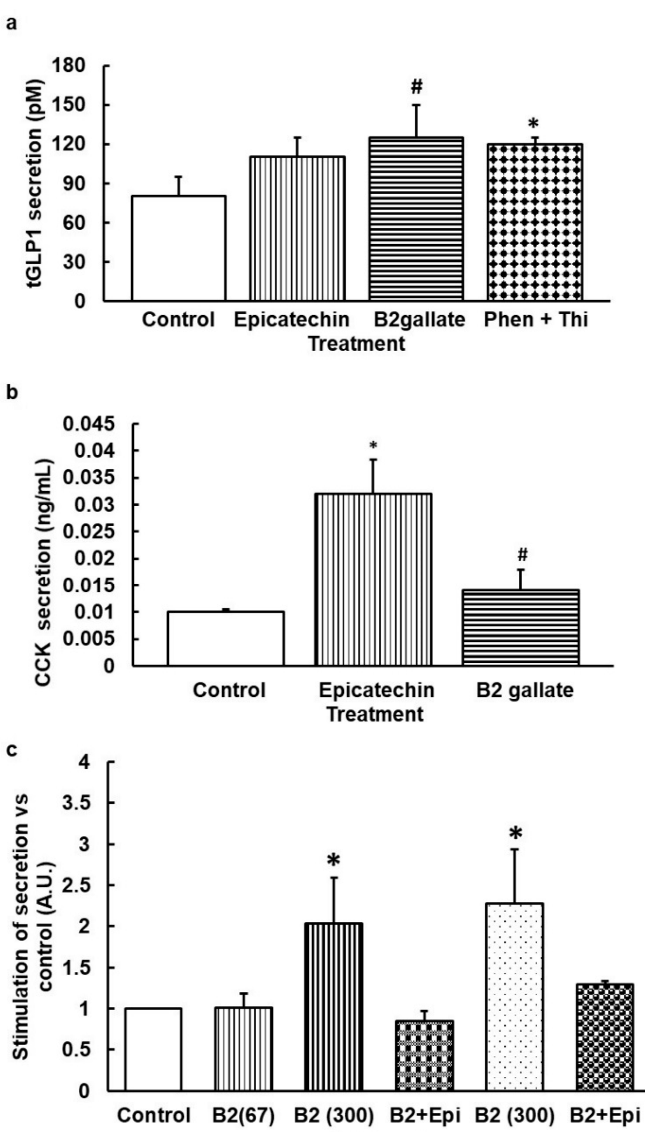


Figure 2. Ex vivo intestinal rat secretions induced by simultaneous stimulation by hTAS2R5 and hTAS2R39 agonist (**a**) and (**b**) or by Procyanidin B2 (**c**). (**a**) Segments of ileum were treated with epicatechin 1 mM, B2gallate 20 µM for 30 min or 1,10-Phenantroline 150 µM + Thiamine 1 mM for 45 min. (**b**) Segments of duodenum were treated with epicatechin 1 mM or B2gallate 20 µM for 30 min. (**c**) Segments of ileum (for tGLP1, vertical and squared lines) and duodenum (for CCK, dotted columns) were treated with B2 67 or 300 µM for 30 min, or B2 300 µM + epicatechin 1 mM for 45 min. Afterwards, medium was collected and respective enterohormones in the medium were quantified by ELISA (n = 6–10 segments). Mean ± SEM. (**c**) Results are calculated versus basal respective secretion in each hormone (Arbitrary Units, A.U.) * denotes $p < 0.05$ vs. control, # indicate $p < 0.1$ vs. control.

A different response was obtained with EGCG, which binds with lower EC50 to hTAS2R39 and also binds to TAS2R5 (Table 1) and hTAS2R43 [19]. EGCG 30 µM limits tGLP1 secretion in ileum segments (79.41 ±7.90 pM vs. control: 81.14 ± 13.41 pM, p = 0.04).

Finally, we tested Procyanidin B2, which has not yet been defined as a ligand for any TAS2R (though it has been identified as bitter with a threshold recognition of 0.485 mM [21]). Figure 2c shows that Procyanidin B2 increases tGLP1 and CCK at a dose of 300 µM. Its combination with epicatechin prevents these effects (Figure 2c).

3.3. Stimulation with Agonists of hTAS2R14 Increases GLP1 Secretion

One of the most promiscuous bitter taste receptors in humans is TAS2R14 [12]. Flufenamic acid is a selective agonist for this receptor, with a minimally effective concentration of 10 μM [11]. Rat intestine segments treated with Flufenamic (FFA) acid increased tGLP1 secretion (Figure 3), reduced CCK (0.60 ± 0.17 vs. control (1.00 ± 0.26); $p = 0.004$) and did not significantly modify PYY (1.15 ± 0.47 vs. control (1.00 ± 0.35); $p = 0.84$).

Vanillic acid (VAN), another selective ligand for TAS2R14 with an EC50 of 151.17 μM [17] also increased tGLP1 secretion in ileum segments, as did protocatechuic acid (PCA), another ligand of TAS2R14 (and TAS2R30) [17] (Figure 3).

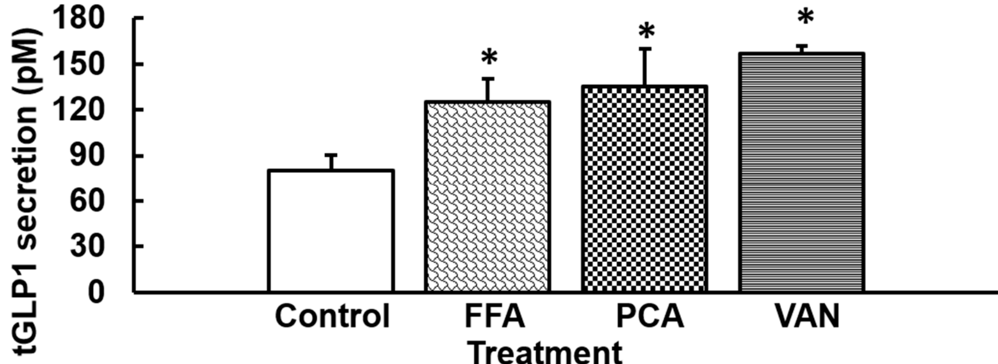


Figure 3. Ex vivo ileum rat total GLP1 secretion induced by hTAS2R14 agonists. Rat segments of ileum were treated with Flufenamic acid(FFA) 50 μM, protocatechuic acid(PCA) 300 μM or Vanillic acid(VAN) 300 μM for 30 min. Afterwards, the medium was collected and total GLP1 was quantified by ELISA (n = 6–10 segments). Mean ± SEM. * denotes $p < 0.05$ vs. control.

We also tested the effects of simultaneous activation with 1,10-Phenantroline (agonist for hTAS2R5) and Flufenamic acid (agonist for hTAS2R14), these being two stimulators of GLP1 secretion. Simultaneously treating ileum segments of rat intestine with the combination of Flufenamic acid (50 mM) and 1,10-Phenantroline (150 mM) did not induce tGLP1 secretion (control: 70.77 ± 7.47 vs. Phenantroline + Flufenamic acid: 104.01 ± 11.89 pM).

3.4. Agonists That Increase GLP1 and CCK Are More Effective in Limiting Food Intake

Acute administration of 1,10-Phenantroline to rats led to a reduction in food intake (Figure 4a). The same treatment tended to induce higher levels of active GLP1 in the portal vein thirty minutes after administration (t0 = 1.00 ± 0.15; t30 = 1.63 ± 0.4, p-value = 0.09, arbitrary units relative to secretion at t0). There were no clear effects on PYY or CCK at this time point (CCK ng/mL: t0 = 0.42 ± 0.04; t30 = 0.7 ± 0.07 control; t30 = 0.63 ± 0.10 1,10-Phenantroline; PYY pg/mL: t30 = 40.1 ± 3.15 control; t30 = 47.7 ± 8.9 1,10-Phenantroline). Neither was there a clear effect on glucose (results not shown).

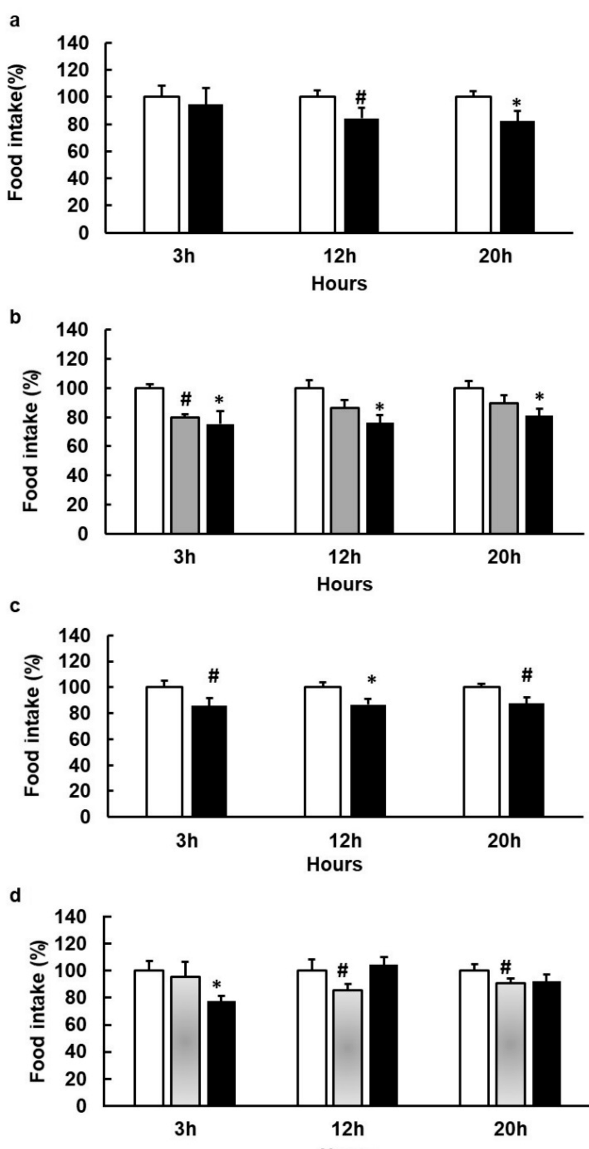


Figure 4. Food intake (FI) changes induced by acute doses of agonists of bitter taste receptors in female rats. Animals (n = 8–10/treatment) were treated one hour before dark period with an acute dose of 1,10-Phenantroline 200 mg/kg~290 µM (black columns) (**a**); 244 mg/kg~0.84 mM epicatechin (grey columns) or 300 mg/kg~1 mM epicatechin (black columns) (**b**); epicatechin + B2 + ECg (200 + 62 + 18 mg/kg) (black columns) (**c**); vanillic acid (252 mg/kg), grey columns) or Vanillic acid (252 mg/kg) + epicatechin (244 mg/kg) (black columns) (**d**); tap water as vehicle (white columns). Food intake was measured at the times indicated after the start of the dark period starts (Mean ± SEM). * denotes $p < 0.05$ vs. control; # indicate $p < 0.1$ vs. control.

An acute dose of around 0.84 mM of epicatechin tended to inhibit food intake, whereas an acute dose of around 1 mM clearly limited food intake (Figure 4b). This dose, increased levels of active GLP1 in the portal vein forty minutes after treatment (t0 = 1.00 ± 0.19; t20 = 1.56 ± 0.5; t40 = 4.69 ± 1.8, p-value = 0.03, arbitrary units relative to secretion at t0) and CCK secretion (CCK ng/mL: t0 = 0.68 ± 0.02; t40 = 0.78 ± 0.13 control; t40 = 0.96 ± 0.12 epicatechin). However, it did not change glycemia (results not shown).

Procyanidin B2 at a dose of 0.11 mM did not affect food intake (Figure S1). However, the same dose of B2 plus epicatechin (0.74 mM) and epicatechin gallate (ECg) at a total dose of 0.84 mM did reduce food intake (Figure 4c).

Vanillic acid tended to reduce food intake (Figure 4d). The combination of epicatechin with vanillic acid, both of them at doses that reduce food intake, was only effective three hours after treatment and was not effective thereafter (Figure 4d).

3.5. Stronger Agonism of hTAS2R39 Than hTAS2R5 Can Stimulate Food Intake

Rats treated with Thiamine (a selective hTAS2R39 ligand) at a dose of 7.5 mM, which is much higher than the effective concentration (Table 1), did not modify food intake (Figure S2). Neither did epicatechin gallate, which also binds hTAS2R39, with an EC50 of 88.2 µM [22] (Figure S3).

Stimulation with epicatechin 0.3 mM + 21.8 µM of EGCG (equivalent to simultaneous stimulation of hTAS2R39 and hTAS2R5) did not change food intake. We also found no effects when we doubled the epicatechin dose (0.78 mM) (Figure S4). Interestingly, when we added a selective hTAS2R39 agonist such as epicatechin gallate (at a dose that has no effect on food intake) to epicatechin to reach a total dose of 0.84 mM (at which epicatechin alone had no effect) we observed a stimulation of food intake (Figure 5a). This effect was also found with a simultaneous treatment of epicatechin plus procyanidin B2 (Figure 5b).

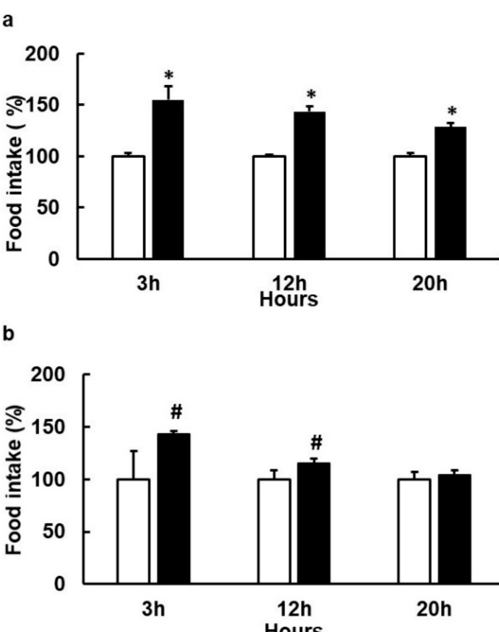


Figure 5. Changes in food intake (FI) induced by acute doses of agonists of bitter taste receptors in female rats. Animals (n = 8–10/treatment) were treated one hour before the dark period with an acute dose of epicatechin (234 mg/kg) + epicatechin gallate (14 mg/kg), a whole dose ~0.84 mM (black columns) (**a**); epicatechin (213 mg/kg) + B2 (62 mg/kg), a whole dose ~0.84 mM (**b**). White columns indicate the control group treated with tap water as vehicle. Food intake was measured at the times indicated after the start of the dark period starts (Mean ± SEM). * denotes $p < 0.05$ vs. control, # denotes $p < 0.1$ vs. control.

4. Discussion

We hypothesized that the specific stimulation of bitter taste receptors located in the gastrointestinal tract can produce a secretome that modulates food intake in rats. Knowledge of the role of these receptors in that location is scarce, though their ability to increase ghrelin secretion has been proven in two situations [15,16]. Here, we show that stimulation by some agonists for human TAS2R may be used as an on/off mechanism to elicit enterohormone secretions that modulate food intake in the organisms.

Our study is mainly based on the definitions by Meyerhof et al. [11] regarding the compounds that bind and activate human bitter taste receptors. To select the ligands, we worked with three

receptors with different selectivities: a highly selective hTAS2R5, a moderately selective receptor such as hTAS2R39 and a highly promiscuous hTAS2R14. We stimulated intestinal segments with the agonists of the human TAS2Rs and measured their ability to induce gastrointestinal secretions that participate in the control of food intake. The most consolidated changes due to metabolic surgery (which modulates food intake) on the secretome in humans are increased GLP1 and PYY [4]. Here, we have found that in rats, the most effective changes in secretome for reducing food intake are increases in GLP1 and CCK, which do not affect PYY. The common denominator in these approaches is the increase in GLP1. In fact, the only approved drug for managing body weight via enterohormone mechanisms is based on GLP1 analogues such as liraglutide [23].

Increased GLP-1 is obtained with a specific agonist (1,10-Phenanthroline) or with agonists that preferentially bind to hTAS2R5 (epicatechin or B2 gallate). However, we also found an increase in GLP-1 via the stimulation of hTAS2R14—in this case together with a reduction in CCK, which, with vanillic acid, also tends to reduce food intake. There is no additive effect between epicatechin and vanillic acid and this cotreatment antagonizes their ability to stimulate tGLP1 secretion, which, as expected, limits their respective ability to inhibit food intake. Since we are working with theoretically defined receptors, we can postulate different interactions between these ligands to interfere with tGLP1 secretion. They could interfere intracellularly producing crosstalk between intracellular signaling [24], or there could be a desensitizing phenomenon, as has been defined for hTAS2R14 [25]. When the combination of epicatechin and vanillic acid was tested in vivo, at three hours we observed a reduction in food intake, probably due to epicatechin. Afterwards, when vanillic acids become effective (12 h onwards), the effects of the combination are lost, which could be due to heterologous desensitization. We obtained the same secretome profile (higher GLP1 and CCK) with procyanidin B2 at 300 μM. This procyanidin has not been shown to bind to any hTAS2R at the concentrations assayed (i.e., below 150 μM) [17]. When it was assayed at higher concentrations in intestinal segments, we found a similar secretome profile to that of epicatechin (a hTAS2R5 + hTAS2R39 agonist). Contrastingly, with the combination of procyanidin B2 with epicatechin assayed on intestinal segments, any differences on the secretion of neither GLP1 nor CCK were observed. This finding suggests that procyanidin B2 may act as a partial agonist of the effects of hTAS2R5. The effects of procyanidin B2 alone seem to correspond to those of agonists of hTAS2R5 and hTAS2R39 but in combination with another agonist, it cancels these effects [26]. When we used B2 alone with rats, we observed no effect. This was because we were working with a dose of 110 μM, which is closer to the dose of 67 μM (which was shown to be ineffective in the studies on secretome) than to the effective dose of 300 μM.

Stimulating only PYY secretion, as produced by specific hTAS2R39 stimulation, appears to be ineffective in reducing food intake in the rat. On the other hand, some combinations that preferentially target hTAS2R39 signaling (such as epicatechin plus epicatechin gallate) at a dose of over 0.84 mM do stimulate food intake. Previous studies with epicatechin showed a trend towards the stimulation of octanoyl ghrelin secretion in murine cells, while epicatechin gallate, a specific ligand for hTAS2R39, clearly stimulated octanoyl ghrelin—an effect that was abrogated by a specific antagonist for hTAS2R39 [15]. From these in vitro studies, it could be suggested that hTAS2R39 stimulates PYY and octanoyl ghrelin secretions. It was not possible to measure octanoyl ghrelin in our study because its presence in rat intestine is too low to be accurately measured. However, we cannot rule out the stimulation of ghrelin secretion in vivo that contributes to the orexigenic effect of this combination. We observed a similar stimulation of food intake when epicatechin plus procyanidin B2 was administered. We also treated ghrelin-producing cells with B2 or B2 gallate but octanoyl ghrelin secretion remained unchanged [15]. Therefore, if our hypothesis is that hTAS2R39 stimulation is related to the stimulation of food intake, we may suggest that B2 counteracts the effect of epicatechin in hTAS2R5 and allows only the stimulation of epicatechin in hTAS2R39.

Surprisingly, the combination of epicatechin plus procyanidin B2 and epicatechin gallate (all at non-effective doses for inhibiting food intake) leads to a reduction in food intake in the rat. To explain this finding, we postulate that all the ligands of hTAS2R39 in the mix compete amongst themselves at

the level of the receptor or at other stages between the initial stimulation and the final effect on food intake, while the effects linked to hTAS2R5 remain unaltered. Nevertheless, we are unable to prove this with our data.

In addition to the different number of bitter taste receptors between species (25 in humans and 35 in rodents), there are different agonisms for the different sets of TAS2Rs possessed by each species [13]. 1,10-Phenantroline binds five bitter taste receptors in mice (mTas2r). Neither Thiamine nor Flufenamic acid has been tested in mice. Epicatechin binds two mTas2r while EGCG binds only one (mTas2144). In this study, we have been working on rats, about whom there is little information regarding TAS2R and their orthologues in humans, or their respective specificities against different ligands. Their proximity to mice can be used as a reference but extreme caution must be taken when extrapolating these results to humans. As an example, Avau et al. proved that intra-gastric stimulation induced a TAS2R-dependent delay in gastric emptying in mice that, when assayed in human volunteers, increased satiation [27]. Another aspect to address in the future should be gender effects since recently gender differences have been reported in humans [28,29]. Beyond these considerations, the importance of our study is the evidence that the stimulation with specific bitters produces enterohormone secretions linked to food intake modulation. However, specific attention must be paid to the possible differences between rat and human isoforms.

Finally, we used our hypothesis to explain the satiating effect of some doses of grape-seed derived procyanidins (GSPE), which we did not obtain when we used a very similar but slightly different (cocoa) extract [30]. Epicatechin, procyanidin B2, epicatechin gallate, vanillic acid and other ligands of hTAS2R5, hTAS2R14 and hTAS2R39 [17] are constituents of grape seeds. We showed that a grape-seed proanthocyanidin extract can increase GLP1 secretion, GIP and PYY [31,32] and limit food intake at doses above 350 mg/kg Bw [33]. Table S2 summarizes the abundance of ligands for hTAS2R5, hTAS2R14 and hTAS2R39 in GSPE, Cocoanox and the satiating combination (epicatechin + B2 + ECg). GSPE contains selective ligands for hTAS2R5 and very few amounts of ligands for hTAS2R14. The highest amounts of ligands are for hTAS2R5 and hTA2r39, together with selective ligands for hTAS2R39, which suggests competition by hTAS2R39 effects and enables the stimulation of enterohormones induced by hTAS2R5, which produces satiety. The case of Cocoanox resembles stimulation by epicatechin plus B2: either there is no effect or, depending on the ratio between both, food intake increases [30].

5. Conclusions

Food intake can be adjusted by gastrointestinal stimulation with compounds that bind to specific bitter taste receptors. This mechanism produces enterohormone secretions that can explain these effects on food intake. Specifically, the ligands of hTAS2R5 stimulation produce an anorexigenic effects in rats, whereas ligands of hTAS2R39 acts as an orexigenic. Further studies in humans are required to prove this strategy as means of controlling food intake.

6. Patents

There is a patent submitted on this manuscript P202030846.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/12/3784/s1, Figure S1: Food intake after an acute dose of procyanidin B2 in female rats, Figure S2: Food intake after an acute dose of Thiamine in female rats, Figure S3: Food intake after an acute dose of epicatechin gallate in female rats, Figure S4: Food intake after an acute dose of epicatechin + EGCG in female rats. Table S1: Compounds tested for secretion in intestinal segments and food intake studies and their concentration, Table S2: Molarity of respective ligands of hTAS2R in GSPE and Cocoanox (mM).

Author Contributions: C.G.-B.: conceptualization, methodology, investigation, visualization, writing; A.M.-G. and C.G.-Q.: investigation and data acquisition; J.-A.F.-L. and X.R.: investigation, resources and review; C.T.-F. and J.Á.-R.: resources and critical review; E.R.-G. and R.B.-D.: methodology, critical review and funding; M.T.B.: conception of design and funding acquisition; X.T.: conception of design, formal analysis, funding acquisition; A.A. and M.P.: conceptualization, writing, funding acquisition and project administration. All authors have read and agreed to the published version of the manuscript.

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

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Functional and genomic comparative study of the bitter taste receptor family TAS2R: insight into the role of human TAS2R5

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Nonstandard abbreviations:

hTAS2R: human bitter taste receptors

hTAS2R5: human bitter taste receptor 5

hTAS2R39: human bitter taste receptor 39

hTAS2Ri: Human bitter taster receptor “i”

mTas2r105: mouse bitter taste receptor 105

mTas2r144: mouse bitter taste receptor 144

TAS1R: family of taste receptor 1

TAS2R: family of taste receptor 2

GI: gastrointestinal

GLP-1: Glucagon-like peptide-1

SNPs: single nucleotide polymorphisms

PGG: pentagalloylglucose, a hydrolyzable tannin

EGCG: Epigallocatechin gallate

EC_{50}: half-maximun effective concentration

Abstract

Bitterness is perceived in humans by 25 subtypes of bitter taste receptors (hTAS2R) that range from broadly tuned to more narrowly tuned receptors. hTAS2R5 is one of the most narrowly tuned bitter taste receptors in humans and there is no consensus about its role. In this genomic study, we compare the possible role of hTAS2R5 with that of the proteins of the TAS2R family in rat, mouse and pig. A phylogenetic tree of all the proteins from human, pig, mouse and rat with a TAS2R domain showed that hTAS2R5 has no ortholog or close paralog. By comparing the ligands that are common to hTAS2R5 and other members of the family, we observed that hTAS2R39 is the receptor which shares most ligands with hTAS2R5. In mouse, some of these ligands bind to mTas2r105 and mTas2r144. mTas2r144 seems be the receptor that is most similar to hTAS2R5 because they are both activated by the same ligands and have affinities in the same range of values. Then, we can conclude that hTAS2R5 does not have a similar functional gene in humans, it is activated by exclusive ligands and its closest functional ortholog in mouse is mTas2r144.

1. Background

The first step in taste sensing takes place in the mouth. The predominant consensus holds that there are five basic tastes—sweet, bitter, salty, sour, and "umami" [1]. It remains a matter of debate whether fatty acids elicit an independent taste perception [2]. At the molecular level, taste perception depends on several G-protein related receptors and ionic channels located in the gustatory papillae [3]. Sour and salty tastes are sensed via ion channels and signal that dietary acid is present in spoiled foods or salts essential for maintaining the water balance of the body, respectively. Sweet or umami tastants that help to identify energy-rich nutrients are sensed via heterodimers of subtypes of the taste 1 receptor family (TAS1R). Bitter tastants that may indicate potentially harmful compounds are identified by different subtypes of the taste 2 receptor family (TAS2R). As well as being located in the mouth, these taste receptors have recently been reported in other areas of the human body (these are known as ecnomotopic receptors [4]). They are found in the gastrointestinal (GI) tract, adipose tissue, the airways and the pancreas [5]. Their role in these locations is far from clear.

Bitterness protects individuals from unhealthy natural products [6]. However, not all toxins are bitter and, likewise, not all bitter compounds are toxic [7]. Many bitter compounds have health benefits and it has been suggested that healthier diets have a higher component of bitter-tasting ingredients, including bitter vegetables [8]. It is believed that the receptive ranges of bitter taste receptor repertoires match the profiles of bitter chemicals that species encounter in their diets. Human and mouse genomes contain pairs of orthologous bitter receptor genes that have been conserved throughout evolution. In humans, bitterness is sensed by 25 subtypes encoded by 25 functional TAS2R loci, which reside on chromosomes 5, 7 and 12 [3, 4, 6]. In addition to these genes, humans also carry 11 TAS2R pseudogenes [7]. Mice have 35 genes and 5 pseudogenes on chromosomes 5, 6 and 15 [9]. Lossow and col have demonstrated that mouse taste 2 receptors, like their human counterparts, vary greatly in their breadth of tuning, ranging from very broadly to extremely narrowly tuned receptors. However, when compared with humans, mice have fewer broadly tuned receptors and a considerable number of narrowly tuned receptors, which supports the idea that a large receptor repertoire is the basis on which specialized receptors evolve. Lossow and col have also shown that sequence-orthologous bitter taste receptors have distinct agonist profiles. Species-specific gene expansions have enabled further diversification of bitter substance recognition spectra [10].

Human TAS2R5 is one of the most narrowly tuned bitter taste receptors in humans. According to Di Pizio *et al.*, TAS2R5 and TAS2R49 are the only specialized TAS2Rs activated by exclusive bitter compounds: 1,10-Phenantroline and cromolyn, respectively [11]. TAS2R49 seems to have a physiological role in the upper airways. Notably, the only compound that activates it is a common drug for treating asthma [12]. For TS2R5 there is no consensus about its role. In this paper, we review the possible role of TAS2R5 in the human body, its possible orthologs in other species similar to humans and commonly used as a model for human pre-clinical studies, and the structural similarity with other receptors based on common ligands.

2. Methods

2.1. Systematic review

The electronic databases Web of Science, Scopus and Pubmed were searched for relevant studies using the keywords TAS2R5 AND T2R5 (until March 2021). In total, 58 studies were selected to investigate the state of art on the function of human TAS2R5.

2.2. Transcriptomics from The Human Protein Atlas

Tissue microarray data was dowloaded from the publicly available The Human Protein Atlas (http://www.proteinatlas.org). The data consists of the consensus transcript expression levels (NX value) in 62 tissues based on transcriptomics data from three different studies: HPA, GTEx and FANTOM5. This data is based on The Human Protein Atlas version 20.1 and Ensembl version 92.38.

2.3. Genomic analyses of TAS2R

We downloaded the peptide sequences from all the genomes available in version 102 of the Ensembl database (http://www.ensembl.org/) of human (*Homo sapiens*: GRCh38.p13), pig (*Sus scrofa*: Sscrofa11.1), mouse (*Mus musculus*: GRCm38.p6), rat (*Rattus norvegicus*: Rnor_6.0), platypus (*Ornithorhynchus anatinus*: mOrnAna1.p.v1), opossum (*Monodelphis domestica*: ASM229v1), Tasmanian devil (*Sarcophilus harrisii*: mSarHar1.11), common wombat (*Vombatus ursinus*: bare-nosed_wombat_genome_assembly), hyrax (*Procavia capensis*: proCap1), hedgehog

(*Erinaceus europaeus*: eriEur1), American black bear (*Ursus americanus*: ASM334442v1), dog (*Canus lupus familiaris*: CanFam3.1), alpaca (*Vicugna pacos*: vicPac1), vaquita (*Phocoena sinus*: mPhoSin1.pri), sperm whale (*Physeter catodon*: ASM283717v2), goat (*Capra hircus*: ARS1), cow (*Bos taurus*: ARS-UCD1.2), American beaver (*Castor canadensis*: C.can_genome_v1.0), mouse lemur (*Microcebus murinus*: Mmur_3.0) and macaque (*Macaca mulatta:* Mmul_10).

From this dataset, we selected the TAS2R proteins in these species using the Hidden Markov Models (HMM)-search software HMMER 3.3 [13] and the Pfam model PF05296 for the TAS2R domain, obtained from version 31 of the Pfam database [14]. We used the gathering threshold (GA) as the search parameter.

The TAS2R domains of the retrieved sequences (n=300) were aligned using the multiple alignment program MAFFT 7 and the algorithm E-INS-I [15]. Poorly aligned positions were removed from the alignment using trimAL with automated1 algorithm [16]. From the resulting TAS2R protein alignment, we constructed a phylogenetic tree using the IQ-TREE 2.0.3 software, under the JTT substitution model with 4 Γ rate categories and empirical state frequencies [selected with the ModelFinder IQ-TREE module). Statistical supports were obtained from 1000 UFBS bootstrap iterations [17]. The resulting phylogenetic tree was mid-point rooted using the R phangorn 2.53 library [18], and visualised using the phytools 0.6–60 [19] and ape 5.3 libraries (plot.phylo) [20]. Groups of orthologous genes from our phylogenetic analysis were identified with Possvm software [21]. Finally, we compared the aminoacid identities and similarities (with BLOSSUM62 matrix) between all TAS2R sequences from the untrimmed alignments, using the dist.alignment function in the seqinr 4.2-5 R library [22].

3. Results and discussion

3.1. What we can learn from current literature

The human TAS2R5 gene is located on chromosome 7q31.3-q and encodes the bitter taste receptor. Its expression pattern has been summarized in “The human protein atlas” (Figure 1). The consensus dataset showed that the highest mRNA expression is in skin, epididymis, cerebellum and ovary. It is based on the quantification of this mRNA either by Taqman probes [23], [24] or specifically designed primers [25] (recruited in Supplementary Table 1). There is no data based on protein quantification.

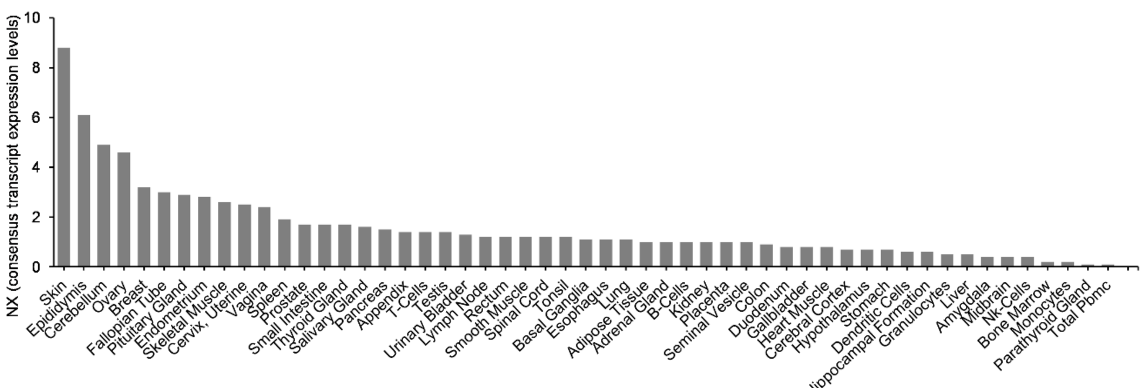

Figure 1. The Human Protein Atlas transcriptomic data for TAS2R5 https://www.proteinatlas.org/ENSG00000127366-hTAS2R5/tissue.

Although it is highly expressed in skin, there is little data on the role it plays. A positive correlation has been reported between increasing age and TAS2R5 gene expression in not-sun-exposed skin (suprapubic) (as well as human TAS2R4, TAS2R14 and TAS2R20) [25]. No correlation has been reported in sun-exposed skin. TAS2R5 is expressed in human myometrial cells. Specific stimulation with 1,10-phenanthroline dose-dependently suppressed the oxytocin-induced contraction of myometrium [26]. In the brain, TAS2R5 has been related to Parkinson's disease. TAS2R5 and TAS2R50 are downregulated in premotor and Parkinsonian stages in frontal cortex area 8 in the brains of patients with Parkinson's disease [24]. Taste function-related genes, including TAS2R5 and TAS2R3, might also be state-specific mania markers in bipolar disorder [27]. Recently, TAS2R5, together with TAS2R4, TAS2R14 and TAS2R39 expression, was confirmed by immunohistochemistry to be present in human choroid plexus epithelial cells, which suggests that TAS2Rs play an active role at the human blood-cerebrospinal fluid barrier as surveyors of the bloodstream and cerebrospinal fluid compositions [28].

Specific stimulation has shown that TAS2R5 is related to the relaxation of human bronchi [23]. The exclusive agonist of TAS2R5 (1,10-phenanthroline) induced the relaxation of human bronchi, whereas the TAS2R7 agonist cromoglycate and malvidin-3-glucoside were ineffective up to 10 mM and 30 µM, respectively. Phenanthroline induced relaxation at concentrations as low as 10 µM, 10-fold lower than the threshold concentration in a study performed with HEK cells, suggesting the involvement of TAS2R5 [5].

In the gastrointestinal tract, TAS2R5 has been colocalized in the human enteroendocrine L cell, where GLP-1 secretion is stimulated by 1,10-phenanthroline [29). Moreover, bitter agonists exclusive to TAS2Rs such as TAS2R5 and TAS2R10 have stimulated ghrelin secretion in fundic cells [30].

As observed for other bitter taste receptors, several single nucleotide polymorphisms (SNPs) affect the perception of bitterness in TAS2R5. The 4 SNPs across TAS2R3, TAS2R4 and TAS2R5 are located close to each other on chromosome 7, forming a haplotype in our sample. The TGAG homozygotes perceived twice as much bitterness as CCGT homozygotes, while the heterozygotes exhibited intermediate values. Within the CCGT/TGAG haploblock, 2 of the polymorphisms encode amino acid substitutions in TAS2R4 and TAS2R5. It is unclear from present data whether the variation in coffee bitterness seen for this haplotype results from the functional alteration of T2R5 or influences on transcription that alter the expression [31]. Additionally, recent studies in this allele have revealed that TGAG homozygotes show a significantly lower body mass index [32]. The bitterness of alcohol is known to be the major taste component that influences alcohol consumption [33]. Choi et al showed an association between the TAS2R5 rs2227264 variant and total alcohol intake [34). The TAS2R5 rs2227264 variant also influenced rice wine drinking status and the rs1859646 SNP is located in the intron of the TAS2R5 gene [35]. In the elderly, Mikołajczyk-Stecyna *et al.* [36] showed an association between the SNPs of the TAS2R5 gene and the frequency of grapefruit intake, and between the simultaneous effects of polymorphisms within TAS2R3 and TAS2R5 and the frequency of eating Brassica vegetables in general. Moreover, the SNPs of the selected TAS2R genes may be associated with the lipid profile, serum level of glucose and CRP, depending on the frequency of consumption of particular bitter-tasting items [36].

Understanding how similar human TAS2R5 is to the other members of the TAS2R protein family and how it is related to other bitter taste receptors in other animal models could provide important insight into its function and specificity. Moreover, since TAS2R5 is a membrane protein which needs external stimulation to be effective at modifying cell function, the information available from the study of its ligands and those in common with its closely related receptors would increase understanding of its specificity and its role in the organism. Thus, the similarity of TAS2R5 with other TAS2Rs and information about the ligands of these related receptors will be discussed below.

3.2. Phylogenetic study of human TAS2R5

To gain insight into TAS2R5 function by finding its closest related proteins, either a human paralog or an ortholog from another species, we constructed a phylogenetic tree with the proteins containing the TAS2R domain in humans and other mammals (Figure 2). Among the species selected were pig, mouse and rat because a considerable number of studies have been made on their perception of bitterness and they have often been used as animal models for human studies. The other mammalian species were selected to ensure a taxonomically balanced sampling.

We identified 24 human TAS2R genes and 276 homologs in 19 other mammalian genomes. Our phylogenetic analysis identified groups of orthologous genes (Figure 2) and ascertained the evolutionary relationships between homologs in human and other animal models (Table 1).

We found that, of the 24 subtypes of human TAS2R, 8 had orthologs in pig, mouse and rat, 13 had orthologs in rodents (mouse and rat) but not pig, and only TAS2R9 had a pig ortholog and no rodent orthologs. Only TAS2R5 and TAS2R8 have no orthologs in pig, mouse or rat. However, rather than being human-specific, we find that both of these genes belong to ancient orthologous groups that include homologs from multiple placental mammals and were secondarily lost in rodents (OG2 and OG3 in **Figure 2**).

An examination of our phylogenetic reconstruction also supports an early origin for TAS2R5 and TAS2R8, in spite of their restricted taxonomic distribution in extant genomes. According to our phylogenetic tree, the closest paralogs of TAS2R5 genes are in either the TAS2R4 or TAS2R38 orthologous groups. Both these groups contain marsupial and placental homologs, thus pointing to an equally early origin for TAS2R5. Similarly, TAS2R8 has a well-supported sister-group relationship to a large clade that contains many orthologous groups including marsupial and mammalian species. This topology indicates that it, too, emerged early in mammalian evolution in spite of its currently limited taxonomic distribution (only in primates, pig, and *Ursus americanus* in our dataset).

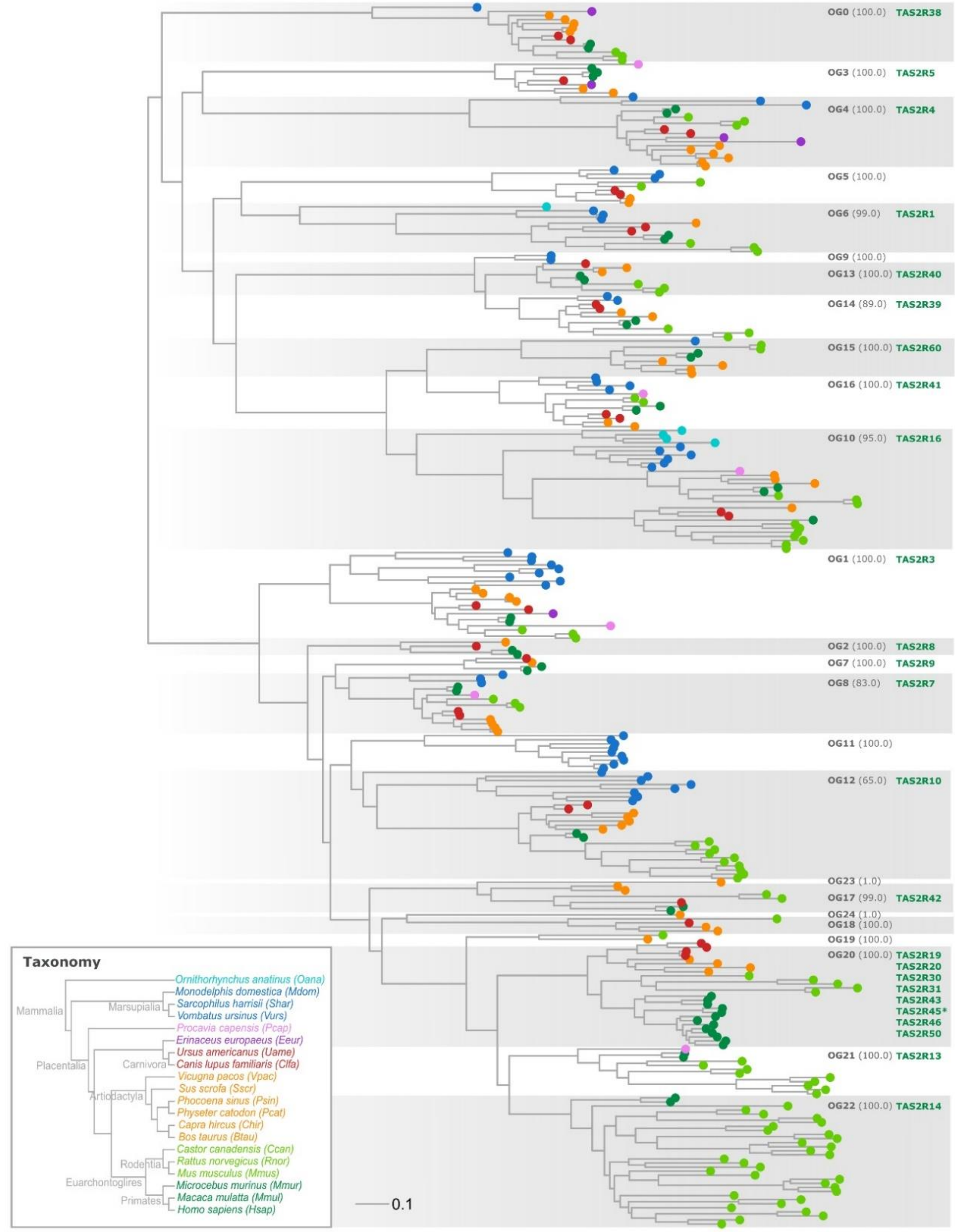

Figure 2. Phylogenetic tree of TAS2R. Species are coloured according to their taxonomic families. Orthologous groups (OG) are formed by a set of genes from the different studied species that descend from the same common ancestor. OG are listed in grey with their statistical support in parenthesis. For each OG, only the human TAS2Rs are highlighted in dark green (*Although TAS2R45 is listed as a pseudogene and therefore

not included in our dataset, we have added it to this figure because it is very similar to the human TAS2R in the OG20 orthologous groups).

Table 1. Orthologous groups and gene presence in selected model species

Orthologous groups (human and pig genes in UPPER CASE, rodent genes lower case)	Orthologous Group	Human	Pig	Mouse	Rat
TAS2R1, Tas2r119	OG6	●		●	●
TAS2R3, Tas2r147	OG1	●	●	●	●
TAS2R4, Tas2r108	OG4	●	●	●	●
TAS2R5	OG3	●			
TAS2R7, Tas2r130	OG8	●	●	●	●
TAS2R8	OG2	●			
TAS2R9	OG7	●	●		
TAS2R10, Tas2r104, Tas2r105, Tas2r106, Tas2r107, Tas2r114	OG12	●	●	●	●
TAS2R13, Tas2r13, Tas2r102, Tas2r121, Tas2r124	OG21	●		●	●
TAS2R14, Tas2r103, Tas2r109, Tas2r110, Tas2r113, Tas2r115, Tas2r116, Tas2r117, Tas2r123, Tas2r125, Tas2r129, Tas2r140	OG22	●		●	●
TAS2R16, Tas2r118, Tas2r134, Tas2r143	OG10	●	●	●	●
TAS2R19, **TAS2R20**, **TAS2R30**, **TAS2R31**, **TAS2R43**, **TAS2R45***, **TAS2R46**, **TAS2R50**, Tas2r120, Tas2r136	OG20	●		●	●
TAS2R38, Tas2r138	OG0	●	●	●	●
TAS2R39, Tas2r139	OG14	●	●	●	●
TAS2R40, Tas2r144	OG13	●		●	●
TAS2R41, Tas2r126	OG16	●	●	●	●
TAS2R42, Tas2r131 (Mmus), Tas2r145 (Rnor)	OG17	●		●	●
TAS2R60, Tas2r135	OG15	●	●	●	●
Tas2r7l	OG5				●
TAS2R? (Not annotated), Tas2r122	OG18		●	●	
TAS2R? (Not annotated)	OG24		●		

* Although TAS2R45 is listed as a pseudogene and therefore not included in our dataset, we have added it to this figure because it is very similar to the human TAS2R in the OG20 orthologous groups.

Our phylogenetic analysis also allows us to identify cases of species- or taxa-specific diversifications of the TAS2R gene complement. For example, seven human genes (TAS2R19, TAS2R20, TAS2R30, TAS2R31, TAS2R43, TAS2R46, TAS2R50) emerged via primate-specific paralogy events within OG20. Similarly, we find rodent-specific duplications within OG21, which contains 10/11 recent paralogs in rat and mouse genomes, respectively. By contrast, other orthologous groups have less dynamic evolutionary histories, and have only one homolog per sampled species (e.g. TAS2R5).

It is worth noting that previous studies [10, 37–41] have listed 25 TAS2R genes in humans, of which our survey recovers 24. The only missing gene is TAS2R45, which is currently annotated as an unprocessed pseudogene in the Ensembl database (http://www.ensembl.org/). TAS2R45 is located in the genome next to its close paralogs TAS2R30, TAS2R43 and TAS2R46, which all emerged during the recent primate-specific diversification of OG20 homologs (Figure 2). We have highlighted TAS2R45 with an asterisk (*) in the figures of the human TAS2R subtypes to reflect its pseudogene annotation.

We studied the percentage of identity between all human TAS2Rs to gain further insight into how they are related (**Supplementary Figure 1A**). TAS2R5 shows that the identity between the other human TAS2Rs was moderate, with values ranging from 25 to 30% of identity, which confirms the distant paralogy observed in the phylogenetic tree. We observe the maximum percentage of identity (30%) between TAS2R7 and TAS2R5. As well as studying the identity values for related proteins, we also studied their similarity, in terms of the amino acids between two proteins that confer structural and functional similarity on their specific protein domain (**Supplementary Figure 1B**). We observe that TAS2R7 is the closest homolog of TAS2R5 in terms of sequence similarity (the only homolog with >50% similarity), in spite of their distant phylogenetic relationship. This proximity is due to the fact that TAS2R7 is a relatively slow-evolving orthologous group, as attested by the short stem branch in the gene tree (which reflects amino-acid substitutions per alignment position; Figure 2). In contrast, TAS2R8, the only other TAS2R without orthologs in rodents or pig, is more similar to its phylogenetically closer paralogs TAS2R7, TAS2R9 and TAS2R10, with identity percentages ranging between 38 and 47% and similarity percentages ranging between 70% and 75%. We also observe that the cluster of human TAS2R paralogs that belong to orthology group 20 (OG20) share high identity and similarity percentages, which confirms their close paralogy. Moreover,

we observe close paralogy between TAS2R39 and TAS2R40 (57% identity and 79% similarity) and TAS2R13 and TAS2R14 (50% identity and 75% similarity).

Table 2 summarises the phylogenetic relationships between human TAS2R proteins and all their homologs in pig, mouse and rat with an identity percentage higher than 50%. We observe that human TAS2R5 and TAS2R8 do not have any homologs in these species. Moreover, although we previously showed that TAS2R29, TAS2R30, TAS2R42, TAS2R46 and TAS2R50 have rodent orthologs, the percentage of shared identity is low. We found that pig TAS2R9 is analogous to human TAS2R7, and that mouse Tas2R144 is analogous to human TAS2R39. We also found that both human TAS2R39 and TAS2R40 are orthologs to pig TAS2R39.

The phylogenetic study shows that, as well as their closer phylogenetic relationship, the TAS2R repertoires of rodents and humans (which share 15 out of 21 orthologous groups) are more similar than those of humans and pigs (which share 10 out of 21). Furthermore, although TAS2R5 has orthologs in mammals, it does not have a close human paralog or an ortholog in pig, mouse or rat. Although they are not close paralogs, TAS2R7 is the most similar human TAS2R to TAS2R5.

Table 2. Human bitter taste receptors (TAS2R) and homologous genes with >50% aminoacidic identity in *Rattus norvegicus*, *Mus musculus* and *Sus scrofa*. For each human gene, all listed homologous genes in other species are orthologs, unless otherwise indicated (#).

Human	Pig		Mouse		Rat	
Gene	**Gene**	**Identity (%)**	**Gene**	**Identity (%)**	**Gene**	**Identity (%)**
TAS2R1			Tas2r119	51.7	Tas2r119	51.5
TAS2R3	TAS2R3	71.5	Tas2r137	63.9	Tas2r137	64.2
TAS2R4	TAS2R4	70.6	Tas2r108	67.0	Tas2r108	64.6
TAS2R5						
TAS2R7	TAS2R7	73.1	Tas2r130	68.3	Tas2r130	69.2
	TAS2R9 (#)	50.8				
TAS2R8						
TAS2R9	TAS2R9	70.4				
TAS2R10	TAS2R10	69.1	Tas2r104	55.3	Tas2r104	54.3
			Tas2r105	54.4	Tas2r105	55.0
			Tas2r106	57.4	Tas2r106	54.4
			Tas2r107	56.9	Tas2r107	58.2
			Tas2r114	57.0	Tas2r114	55.0
TAS2R13			Tas2r102	51.0	Tas2r13	50.0
			Tas2r121	57.3	Tas2r102	50.7
					Tas2r121	56.3
TAS2R14			Tas2r116	51.2	Tas2r125	50.6
			Tas2r125	50.6		
TAS2R16	TAS2R16	61.5	Tas2r118	53.3	Tas2r118	52.2
TAS2R19						
TAS2R20			Tas2r120	50.8		
TAS2R30						
TAS2R31			Tas2r120	51.2		
TAS2R38	TAS2R38	67.5	Tas2r138	65.3	Tas2r138	65.3
TAS2R39	TAS2R39	70.7	Tas2r139	55.1	Tas2r139	53.2
			Tas2r144 (#)	51.3		
TAS2R40	TAS2R39	56.1	Tas2r144	65.8	Tas2r144	64.3
TAS2R41	TAS2R41	70.6	Tas2r126	68.7	Tas2r126	69.7
TAS2R42						
TAS2R43			Tas2r120	50.2		
TAS2R45*			Tas2r120	50.2		
TAS2R46						
TAS2R50						
TAS2R60	TAS2R60	66.1	Tas2r135	58.2	Tas2r135	57.9

* Although TAS2R45 is listed as a pseudogene and therefore not included in our dataset, we have added it to this figure because it is very similar to the human TAS2R in the OG20 orthologous groups.

3.3. Search for functional orthologs of human TAS2R5

In this section we will evaluate how TAS2R5 and the homologs identified interact with their ligands. By so doing, we will gain further insight into how TAS2R5 is similar to the other receptors in terms of functionality.

According to the initial classification of human bitter taste receptors proposed by Di Pizio *et al.* [11), TAS2R14 and TAS2R46 are among the most promiscuous receptors and TAS2R13 and TAS2R50 are two of the most selective. In contrast, in the section above we observed that both TAS2R13 and TAS2R14 share considerable similarity to the cluster of paralogs that include TAS2R46 and TAS2R50. Therefore, the information we obtain from the ligands seems to be more useful than the similarity for elucidating the function of the receptors.

Di Pizio *et al.* [11] showed that human TAS2R5 (hTAS2R5) is one of two specialized TAS2Rs activated by exclusive bitter compounds. Subsequently, Soares *et al.* [38) studied the response of human receptor isoforms to condensed tannins. They tested the ability of each ligand to increase cytosolic calcium levels in HEK cells expressing different TAS2R isoforms. They found that (-)-epicatechin, procyanidin trimer C2 and PGG (pentagalloylglucose, a hydrolyzable tannin) can bind to hTAS2R5. Although (-)-epicatechin and PGG also had the ability to bind TA2R4 (hTAS2R4) and TAS2R39 (hTAS2R39), C2 was specific for hTAS2R5 [38). They suggested that the catechol or galloyl group (which has only one more hydroxyl group than catechol) is a critical (but not essential) feature for the interaction of polyphenol compounds with hTAS2R5. In subsequent studies, they included the following in the list of ligands for hTAS2R5: Procyanidin B1, B4, B2g and EGCG as condensed tannins and punicalagin as hydrolysable tannin [39]. Previous studies in the same system showed that hTAS2R5 can also be stimulated by denatonium saccharide and sucralose [10], and specifically stimulated by 1,10-phenantroline.

Although it was first classified into the specific group [11], to date hTAS2R5 has shown that it can bind to twelve molecules (**Supplementary Table 2**). Most of these compounds can also activate other human and mouse receptors [10] (**Supplementary Table 2**). The other human receptors that have ligands in common with hTAS2R5 are human TAS2R7 (hTAS2R7) and hTAS2R39. hTAS2R7 shares three ligands with hTAS2R5, sucralose, punicalagin and procyanidin B1. Interestingly, hTAS2R39 shares six ligands with hTAS2R5. To compare the sensitivity of these two human isoforms to stimulation by a

common ligand, we used EC_{50} in **Table 3**, when available. Procyanidin B2g, PGG and EGCG showed no preference to binding to any of them. According to these data, only (-)-epicatechin showed a higher affinity for hTAS2R5 than for hTAS2R39, since the EC_{50} is lower for hTAS2R5. This data is not available for denatonium saccharide or for sucralose, but they need doses close to the epicatechin dose to stimulate both receptors (on the millimolar scale). On the other hand, punicalagin, procyanidin B1, 1,10-phenanthroline and procyanidin C2, B4 and B7 are hTAS2R5 ligands, assayed on hTAS2R39 and which do not activate it. So, they are exclusive agonists for human hTAS2R5. There are also five different hTAS2R39 ligands that did not activate hTAS2R5.

Table 3. EC50, or dose used to activate, for shared ligands of hTAS2R5 and hTAS2R39

EC50 (μM)	**hTAS2R5**	**hTAS2R39**
Procyanidin B2G [39]	6.29 ± 3.22	9.11 ± 6.05
PGG (pentagalloylglucose) [38]	≥8.5	≥6.6
EGCG [38]	12.30 ± 3.63	8.50 ± 2.84
Epicatechin [38]	3210.0 ± 42.0^{a}	3800.0 ± 200.0^{b}
Dose that activates (mM)		
Denatonium saccharide [10]	3	3
Sucralose [10]	30	30

[a]Values with a different letter are significantly different ($P < 0.05$). Values with ≥ are estimates because the dose-response curves did not saturate

In the search for close-to-human species, some bitter sensing studies have been performed in pig [42, 43], mouse and rat but the only data available on ligand binding to Tas2r is in mouse [10]. Two mouse receptors are candidates for being functional orthologs of hTAS2R5 (**Supplementary Table 2**): Tas2r105 (mTas2r105) and Tas2r144 (mTas2r144). These two mouse receptors share the same four agonists with hTAS2R5: sucralose, epicatechin, denatonium saccharide and 1,10-phenanthroline. In addition, mTas2r144 shares one more ligand with hTAS2R5: epigallocatechin gallate (EGCG). **Table 4** compares the sensibility of these two mouse receptors to the ligands they have in common with hTAS2R5. The data does not enable a good comparison to be made since EC_{50} is not available for all the cases. As suggested by Lossow *et al.* [10], mTas2r105 seems to be more sensitive than hTAS2R5 to most of the ligands, but mTas2r144 shares more ligands with hTAS2R5. Most of the shared ligands with hTAS2R5 are common to both hTAS2R39 and the mouse receptors mTas2r104 and

mTas2r144, while sucralose is the only ligand common to hTAS2R7 and these mouse receptors.

Table 4. EC50, or dose used to activate, for shared ligands of hTAS2R5, mTas2r105 and mTas2r144

Dose that activates (mM)	**hTAS2R5**	**mTas2r105**	**mTas2r144**
1,10-Phenanthroline [10]	0.1-1[1]	1	1
Epicatechin [38]	3210.0 ± 42.0$	1	1
EGCG [39]	12.30 ± 3.63	-	0.01
Denatonium saccharide [10]	3	0.59 ± 0.21$	0.25 ± 0.01$
Sucralose [10]	30	4.9 ± 1.7$	50
EC_{50} (μM)$			

*Dose used to induce increase in cytosolic calcium

However, 1,10-phenanthroline, which binds mTas2r105 and mTas2R144 is not a ligand for hTAS2R39. Whether procyanidin B2g and PGG, two of the ligands shared by hTAS2R5 and hTAS2R39, also bind the mouse receptors has yet to be determined. Interestingly, the few studies available on mouse Tas2r144 reveal that, like hTAS2R5, it is expressed in the choroid plexus epithelial cells and may play a role in detecting alterations of the cerebrospinal fluid [44, 45]. Moreover, its expression is regulated by female sex hormones, which corroborates the hypothesis of the importance of bitter taste receptors in the female reproductive system.

Finally, we studied the structural similarity and identity of the receptors that are functionally similar to hTASR5: hTAS2R7, hTAS2R39, mTas2r105 and mTas2r144 (**Table 5**). We observe that while hTAS2R39 shares the most ligands with hTAS2R5, it is less similar to it than hTAS2R7. Likewise, although we have previously seen that mTas2r144 seems to function more similarly to hTAS2R5, mTas2r105 is more structurally similar to it. In fact, considering the phylogenetic tree (**Figure 2**), mTas2r105 is much further than mTas2r144 from hTAS2R5 and hTAS2R7 is much further than hTAS2R39 from hTAS2R5, which indicates the evolutionary relationship is conserved by functionality rather than by structural similarity. Therefore, according to current data, structurally similar TAS2Rs are not the closest functional orthologs to hTAS2R5.

So, to date, the exclusive stimulators of hTAS2R5 in humans are procyanidin B4, B7 [39], procyanidin C2 and 1,10-phenanthroline, and the closest functional ortholog in mouse of hTAS2R5 is mTas2r144.

Table 5. Matrix of identity (up, normal case) and similarity (down, bold case) percentages in human and mouse TAS2R that have ligands in common with human hTAS2R5

	hTAS2R5	hTAS2R7	hTAS2R39	mTas2r105	mTas2r144
hTAS2R5		29	24	27	23
		50	**47**	**48**	**25**
hTAS2R7	29		30	41	27
	50		**50**	**58**	**47**
hTAS2R39	24	30		27	49
	47	**50**		**48**	**64**
mTas2r105	27	41	27		24
	48	**58**	**48**		**45**
mTas2r144	23	27	49	24	
	25	**47**	**64**	**45**	

4. Conclusion

Our bibliographic review has suggested that hTAS2R5 has several roles. Besides its role in specific taste perception, hTAS2R5 may play a role in the ageing of skin, unlinked to exposure to the sun. In the brain, it seems that it is required to protect against Parkinson's disease, and it is involved in bipolar pathology. It may also play a role in pregnancy and participate in controlling enteroendocrine secretions and bronchodilation processes. Our comparative genomics study has not shed light on its possible role since it has no close paralog or ortholog, which highlights its uniqueness. Our functional study showed that it is bound by several ligands and is less selective than initially described. It also showed that its closest functional ortholog is mTas2r144. Although it is currently known that both hTAS2R5 and mTas2r144 are involved in the female reproductive system and the monitoring of cerebrospinal fluid, more research needs to be done to fully elucidate their role in the body.

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Authors' contributions: CG-B participated in the design of the study, performed the systematic review and drafted the manuscript; XG-B carried out sequence alignments and helped draft the manuscript, XT participated in data analysis, SG-V participated in the genomic analysis; ER-G helped draft the manuscript; RB-D participated in the systematic review; MTB participated in data analysis; MP participated in the design of the study and critically revised the manuscript; AA conceived and coordinated the study.

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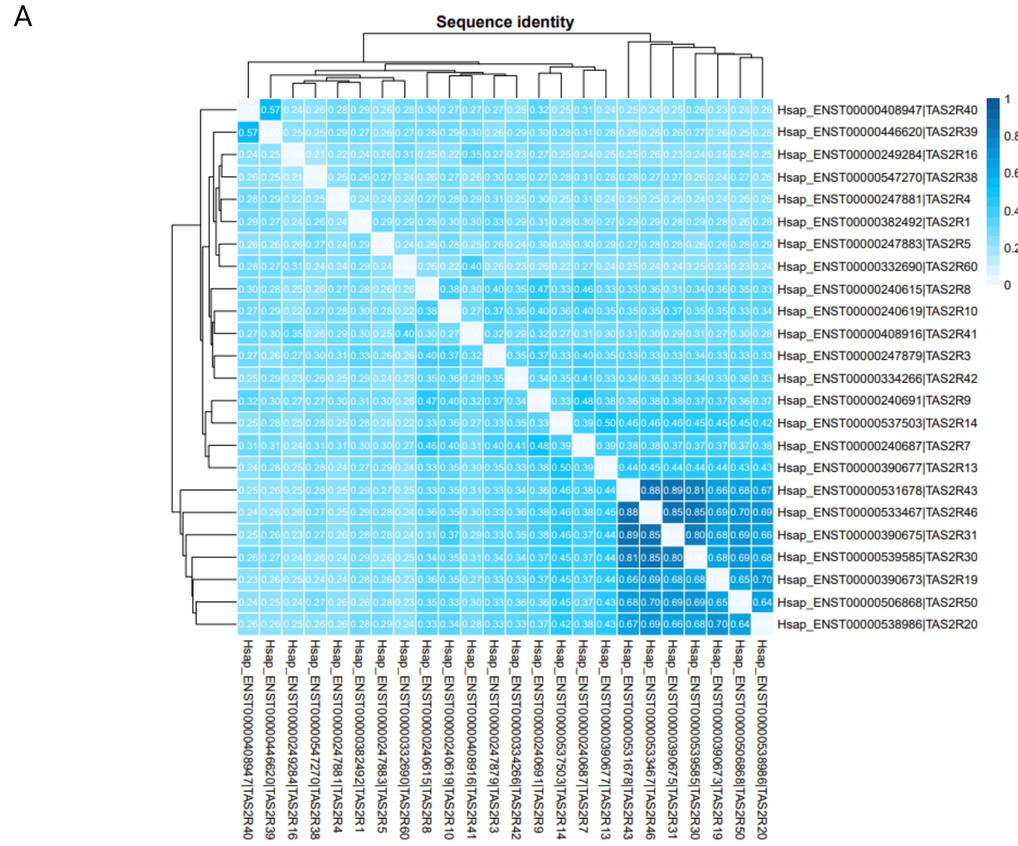
Supplementary materials

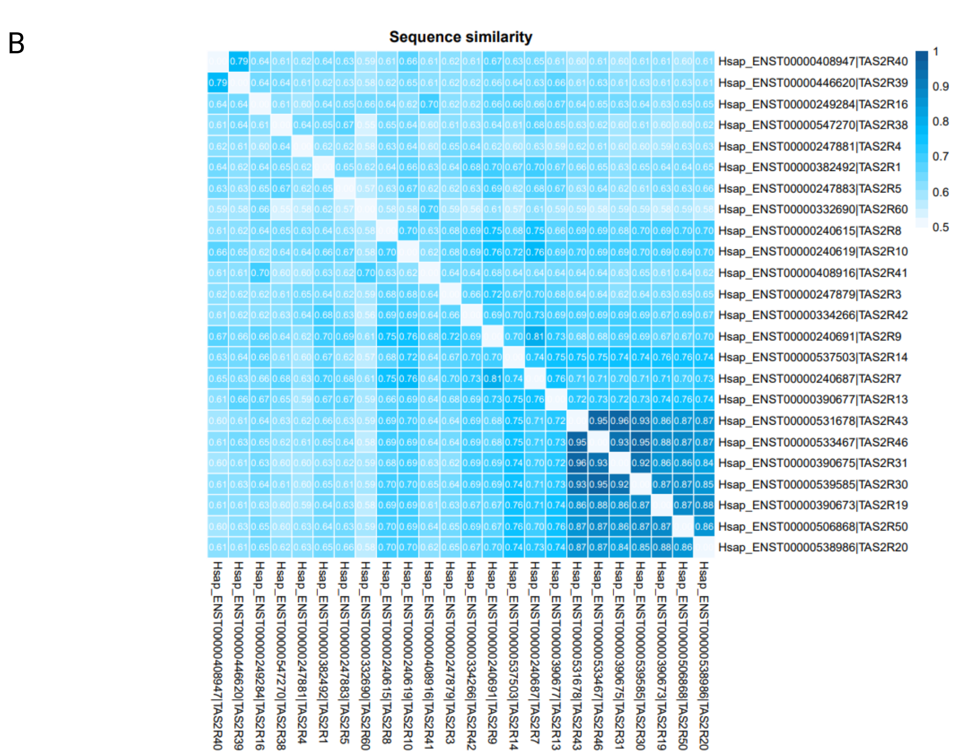
Supplementary table 1. Tools used to characterize TAS2R5

Methodology	Reference
PCR-RT for human (TaqMan® array based on predesigned reagents (Assay-on-Demand®, Life Technologies)	[1]
PCR-RT for human (primers -5’-3’ -667 bp)	[2]
PCR-RT for human (TaqMan-TAS2R5: TTTCTTGTTTCCTCTGGGATGCTGA	[3]
PCR-RT for human and mouse (primers Tas2R)	[4]
PCR-RT (primers Tas2r105); CCACCAGCCTAAGCATCTTC; CGTCCTTCATCACCTTCACA; 165 bp	[5]
PCR-RT for mouse (primers Tas2r)	[6]

A



B



Supplementary Figure 1: (A) Identity among human bitter taste receptors. (B) Similarity among human bitter taste receptors.

Supplementary table 2. Ligands of human TAS2R5 that bind to other human (hTAS2R) or mouse TA2R (mTas2r)

Bitter Taste Receptors	hTAS2R5	hTAS2R7	hTAS2R39	mTas2r105	mTas2r144
Ligands Shared with human TAS2R5	12	3	6	4	5
1,10-Phenanthroline	✓			✓	✓
Sucralose	✓	✓	✓	✓	✓
Epicatechin	✓		✓	✓	✓
Denatonium saccharide	✓		✓	✓	✓
Punicalagin	✓	✓			
PGG (Pentagalloylglucose)	✓		✓		
Procyanidin B2G	✓		✓		
EGCG	✓		✓		✓
Procyanidin B1	✓	✓			
Procyanidin C2	✓				
Procyanidin B4	✓				
Procyanidin B7	✓				
Azathioprine			✓		
Acetaminophen			✓		
Denatonium benzoate			✓		
Chloroquine			✓		
Chlorpheniramine			✓		
Pyrocatechin			✓		
Diphenidol			✓		

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GENERAL DISCUSSION

GENERAL DISCUSSION

The enteroendocrine system integrates the exogenous signals from the diet and orchestrates metabolism through humoral and neuronal signalling. There are two main physiological functions regulated by the enteroendocrine system: the control of food intake and the control of the endocrine function of the pancreas. The diseases comprised within metabolic syndrome are characterized by insulin resistance and a decline on pancreatic insulin production. The fact that most of the enteroendocrine secretions remain unaltered in the metabolic syndrome makes the enteroendocrine system an adequate target to treat these diseases. GLP1-based drugs, which are GLP1R-agonists or DPP4-inhibitors, are currently used to reduce body weight and improve insulin resistance and more research is focused on the study of GLP1 secretagogues that stimulate the endogenous production of GLP1 and their anti-diabetic potential [2]. Moreover, bariatric surgery is also performed to treat obesity and it does not only reduce food intake because the reduction of the stomach volume, but it also enhances a healthier enteroendocrine secretion that promotes a decreased food intake [3]. Besides, the enteroendocrine system can be naturally modulated by bioactive compounds present in the diet, but the exact mechanism underlying this modulation remain vastly unclear [4], [5]. Moreover, the knowledge on the field of the enteroendocrine system is quickly evolving and increasing, since new enterohormones with unknown functions and new functions for those already known are being identified [6], [7]. Thus, understanding the mechanisms by which bioactive compounds are modulating enterohormones and all the implications of enterohormone modulation is of highly importance for the field of metabolic diseases.

Polyphenol-rich diets have many beneficial health effects such as reduced risk of cardiovascular disease [8], low-grade inflammation associated with the metabolic syndrome and colorectal cancer [9] and some other such as improved insulin sensitivity and reduced food intake and body weight [10], [11] that are directly connected to the bioactivity of polyphenols on the enteroendocrine system. Previous work from our research group revealed that a supplementation with a grape seed proanthocyanidins extract (GSPE) is able to improve insulin resistance in rats by potentiating GLP1 production [12]. Moreover, GSPE supplementation in rats is reported to modulate pancreatic beta-cell function [13]. At lower dietary doses, the positive effect of GSPE in the signalling pathways of the endocrine pancreas in insulin-resistant conditions is well characterized [14], [15]. However, the effect of GSPE at the dose necessary for

enteroendocrine stimulation in the regulation of the endocrine pancreas is not fully known since its effects in glucagon production in an insulin resistance-situation are not defined. Hence, we assessed in an *in vivo* study in rats the effect of different GSPE treatments in cafeteria diet-induced insulin resistant rats **(Manuscript 1)**. Animals fed with cafeteria diet 12 weeks showed peripheral insulin resistance, as evidenced by the increased indicators HOMA-IR and HOMA-beta, in comparison to the control group. A group of rats was given a pre-treatment of GSPE that consisted of a daily dose of 500 mg/kg for 10 days before the cafeteria diet. While a group of rats with the pre-treatment were fed with cafeteria diet for 12-weeks (PRE-12), another group with the same pre-treatment were fed with cafeteria diet for 17 weeks (PRE-17). We found that, while PRE-12 rats exhibited significantly higher glucagon plasma levels in fasting conditions, PRE-17 rats showed lower glucagon levels in milder fasting conditions than the cafeteria group. Thus, GSPE has long-lasting effects on the pancreatic glucagon production, as revealed by the increased sensitivity to the fasting state of the rats with a GSPE pre-treatment. This long-lasting effect of GSPE goes in agreement with that observed in a previous study in which the same treatment was able to induce epigenetic changes in the ileal expression of GLP1R [16]. Although GLP1 plasma levels were not altered by the treatment at week 17, interesting results were obtained in an oral glucose load at week 14. Cafeteria-fed rats with the GSPE pre-treatment showed a higher GLP1 secretion in response to the glucose oral load than the cafeteria group. Accordingly, previously reported data show that PRE-17 rats have increased genetic expression of GLP1 in the ileum [16]. There was a tendency to increased GLP1R in the pancreas in GSPE pre-treated rats. These findings suggest that, although basal GLP1 is not higher in GPSE pre-treated rats, these animals can generate a higher response of GLP1 upon stimulation. As evidenced in studies where GLP1 GLP1R-KO mice, GLP1 signalling is essential for glucose-induced glucagon inhibition, while insulin secretion remained unaltered by the lack of GLP1R [17]. Thus, the enhanced GLP1 response by the GSPE pre-treatment could be related to the more sensitive regulation of pancreatic glucagon production in these rats. This finding correlates with the fact that a higher GLP-1 secretion has been related to a better pancreatic functioning, together with a higher sensitivity to GLP-1 [22]. In contrast to the preventive treatment, a simultaneous treatment of GSPE to the cafeteria (SIT) produces different results. These animals showed an inability to produce enough insulin to maintain normoglycaemia in fasting conditions in the 14-week of treatment. In non-fasting conditions at 17-weeks, glucagon levels were lower in SIT rats than in cafeteria rats. Moreover, GLP1 plasma levels tended to be lower in this group, while

genetic expression of GLP1 in the pancreas remained unchanged, indicating a that GLP1 signalling more similar to the cafeteria group than when GSPE was administered preventively. Thus, GSPE can produce beneficial effects on the endocrine pancreas through enteroendocrine modulation when administered preventively rather than in combination with a cafeteria diet, at the assayed doses.

As mentioned above, it would be very beneficial in the advance of the knowledge about the enteroendocrine system and its based therapies to decipher which are the exact compounds that exert the bioactive activities from GSPE. With this purpose, we performed an *ex vivo* study with human colon and pig intestinal samples **(Manuscript 2)**. *Ex vivo* experiments are widely used for the study of the enteroendocrine system, since they allow the maintenance of the morphology of EECs and the intestinal architecture surrounding them, as opposed to *in vitro* experimentation with intestinal secretory cell lines. However, the limited access to fresh human intestinal samples leads to the use of animal experimental models instead. The easy availability to fresh pig intestinal samples from the upper part of the GI tract where several enterohormones are produced and the similarity between these two species, make the pig a good experimental model for the study of enteroendocrine secretions in humans. Nevertheless, we could also access and study human colon biopsies, were human PYY and GLP1 are produced. In addition, the *ex vivo* studies in pig were performed in Ap-to-Bas, a setup where the tissue is placed creating an apical compartment, which resembles the intestinal lumen, and a basolateral compartment, offering a higher control on the administration of the treatments and measurements on the enteroendocrine response and a major similarity to *in vivo* conditions [18]. Thus, we compared the effects of GSPE in the stimulation of pig and human colon and dissected which polyphenols within the extract, alone or in combination, are able to stimulate enteroendocrine secretions in pig intestinal segments. We showed that, GSPE induced a higher PYY secretion in human descending colon than in ascending colon. Accordingly, basal secretion of PYY, was also higher in descending than in descending colon. However, GSPE was unable to stimulate GLP1 secretion in either location. Results in pig differed slightly from those obtained in human. We studied PYY secretion in pig duodenum and descending colon, where it is known to be expressed [19]. In contrast to that observed in human, GSPE-induced PYY secretion is higher where the basal expression was lower, namely the descending colon, although it also induced PYY secretion from the duodenum. Moreover, GPSE induced GLP1 secretion from pig ascending colon, with no GLP1 effect in descending colon. In addition, we studied the effect of GSPE on duodenal CCK production, but no significant effect was

found. The fact that PYY and GLP1 are not equally stimulated by GSPE depending on the location of the GI tract suggests that the chemosensory machinery in the secretory cells is different along the GI tract. In fact, although not shown in this thesis, we have recently identified that a chemosensor that may be involved in the detection of GSPE in the GI tract, TAS2R39 (discussed below), is differently expressed along the GI tract, being highly expressed in the duodenum, followed by the descending colon. In this direction L cells have been reported to be different depending on their location on the GI tract and species. While proximal L-cell contain CCK and GIP in addition to GLP1, distal L-cells contain preferably GLP1 and PYY. In addition, glucose uptake in distal L-cells seems to act independently to SGLT1, the mechanism used by proximal L-cells [20]. Moreover, although PYY and GLP1 are produced by the same EECs, we have seen that they are not stimulated by the same stimulus. This different stimulation is supported by the fact that PYY and GLP1, in human L-cells, are stored in different vesicles [21]. Similar results are obtained in *ex vivo* pig ileum, where a sucralose treatment stimulates GLP1 but not PYY secretion [22]. Next, we evaluated the responses of pig intestine to pure compounds abundant on GSPE, alone and in combination. We found that in pig duodenum procyanidin B2 is able to induce PYY and not CCK secretion and, conversely, catechin and a mixture of catechin and epicatechin did not stimulate PYY secretion but increased CCK secretion. However, we did not identify any pure compound that reproduced the GSPE-induced GLP1 secretion in pig colon. Although procyanidin B2 has previously been shown to have an effect in reducing food intake [23], ours is the first evidence on its ability to modulate enterohormone secretion. In addition, the fact that procyanidin B2 alone and GSPE stimulate PYY but not CCK secretion but catechin alone or in combination with epicatechin stimulates duodenal CCK provides evidence that EECs respond differently and selectively to stimulus. Specifically, this finding suggests that catechin can stimulate CCK secretion, but other compounds found in GSPE may be able to inhibit the catechin-induced secretion from duodenal I-cells while being able to stimulate PYY secretion through specific receptors in L-cells.

We therefore focused on the specific machinery involved in intestinal sensing of polyphenols that allows enteroendocrine regulation. A previous work by our research group evidenced that epicatechin gallate stimulated ghrelin secretion from a murine ghrelinoma cell-line through the bitter taste receptor (TAS2R) mTas2r39, as a specific antagonist prevented this stimulation [24]. This ghrelin stimulation in a TAS2R-independent way was further confirmed in a recent study on human fundic cells [25]. Although the evidence on polyphenol-induced enterohormone secretion through TAS2R

activation is scarce, most polyphenols have been identified in the last decade as TAS2R agonists [1]. Thus, we hypothesized that that specific stimulation of bitter receptors located in the gastrointestinal tract by polyphenols and other agonists could generate an enteroendocrine response that regulates food intake. To do so, we used rats as experimental model to perform *in vivo* studies of food intake that we would relate to the results obtained in *ex vivo* studies **(Manuscript 4)**. Out of the 25 subtypes of TAS2R family, we decided to focus on a selection based on our previous evidence. Since, in the previous study, we identified some pure compounds that are able to stimulate enteroendocrine secretions, we focused on the known TAS2R for those identified molecules. Specifically, hTAS2R14 and hTAS2R39, which are bound by most monomeric polyphenols, and hTAS2R5, which in addition to hTAS2R39, is bound by epicatechin at different affinities, among other polyphenols such as ECG, EGCG and B2 gallate [26]. Since most polyphenols bind more than one of these selected receptors, we also studied the enterohormone response to non-polyphenols that are identified as specific ligands for these receptors. In the *ex vivo* studies in rat intestinal segments we found that GLP1 secretion is increased by the hTAS2R5 specific agonist 1,10-Phenantroline and by polyphenols that preferentially bind to hTAS2R5 over the other receptors, such as epicatechin and procyanidin B2 gallate. Similar results were obtained by Park et al [27], which reported increased GLP1 secretion in response to 1,10-Phenantroline from a human enteroendocrine cell line. Accordingly, *in vivo* food intake studies revealed that hTAS2R5 agonists successfully reduce food intake. Moreover, the hTAS2R14 specific agonist flufenamic acid, and the phenols procatechuic acid and vanillic acid that are also ligands of hTAS2R14 are also able to induce GLP1 secretion, while reducing CCK secretion. Besides, hTAS2R5 and hTAS2R14 ligands had no additive effect in GLP1 stimulation *ex vivo*. Similarly, the combination of epicatechin with vanillic acid was not able to reduce food intake in a sustained way, since an initial food intake reduction, probably due to the food intake-limiting action of epicatechin, was then lost most likely due to the action of vanillic acid, unable to limit food intake. Although it does not have an identified TAS2R, we assayed procyanidin B2, which, was unable to modulate food intake alone but induced GLP1 and CCK secretion, similarly to the results obtained with epicatechin. However, when procyanidin B2 and epicatechin were assayed together, their effects were cancelled, revealing a potential antagonist role of procyanidin B2 on the receptors bind by epicatechin. PYY stimulation was not achieved by any of the assayed hTAS2R specific agonists. Although the hTAS2R39 specific agonist thiamine showed a tendency to increase PYY secretion, it had no effect in food intake. In fact, the

combinations of polyphenols that preferentially bind to hTAS2R39, such as epicatechin with ECG, induced food intake. This finding goes in agreement with the previous results on ghrelin stimulation achieved through TAS2R39. Contrastingly, the addition of procyanidin B2 to epicatechin and ECG, reversed the outcome and reduced food intake, supporting the antagonist role of procyanidin B2 on the receptors involved in the sensing of epicatechin. Knowing that the relative amount of each TAS2R ligand is relevant to predict the outcome of extract rich in polyphenols, we used this information to assess the differences between GSPE and a cocoa extract, Cocoanox. While GSPE has been shown to reduce food intake, Cocoanox does not have a clear satiating effect [28], which correlates with the hTAS2R5/hTAS2R39 agonist amounts ratio. In sum, we confirmed for the first time that the stimulation of intestinal TAS2R modulates food intake, in an orexigenic or anorexigenic way, through the activation of specific secretomes from the enteroendocrine system. Specifically, we have identified that, while ligands of hTAS2R5 produce an anorexigenic outcome, hTAS2R39 ligands acts as orexigenic in rats. Moreover, we confirmed, as observed in the *ex vivo* study in pigs, that, although structurally similar, bioactive molecules can compete by means of having higher affinities of being able to antagonise chemosensors expressed in EECs so they are able to produce different enteroendocrine responses depending on their relative abundance in the intestinal lumen.

This new knowledge about enteroendocrine modulation by bitter molecules is of high interest in translational applications in humans, since it would allow not only the reduction of intake and body weight as attempted with drugs based on the enteroendocrine system to treat the metabolic syndrome, but it could also be used to develop orexigenic outcomes. Specifically, it could be of great interest to increase appetite in people who has lost it due to illnesses or severe treatments such as chemotherapy, a recurrent health problem which is currently not addressed by a specific treatment [29]–[32]. However, the extrapolation of our results in rats to human is very complicated. On the one hand, ligands bind receptors with different affinities in one species or another. On the other hand, the repertoire of receptors is very different between species. Humans have 25 subtypes of TAS2R family while rodents have 35 [33]. In particular, our results on modulation of rat intake by hTAS2R5 and hTAS2R39 specific agonists are difficult to extrapolate because, while we know orthologs of hTAS2R39 in other species, such as Tas2r139 in rodents, currently there is not any ortholog known for hTAS2R5. For this reason, we have carried out a bibliographic and bioinformatics study with the aim of addressing the uniqueness of hTAS2R5 and try to find its closest relative

in the animal model species with available information regarding TAS2R ligands **(Manuscript 5)**. Besides the before mentioned function of hTAS2R5 in the enteroendocrine system [27], hTAS2R5 is expressed in other areas of the body, where its function remains vastly unknown. However, specific stimulation with 1,10-Phenanthroline has revealed that in can regulate bronchodilation in the lungs [34]. Similarly, 1,10-Phenanthroline is able to supress the oxytocin-induced contraction of the myometrium in the uterus, indicating a role in the female reproductive system [35]. Besides the highest expression of hTAS2R5 is in the skin, there is not any evidence on its function [36]. In contrast, several studies have linked its expression in the brain with the evolution of neurodegenerative disorders [37], [38].. In addition, polymorphisms of hTAS2R5 condition the perception of bitterness, which has implications in physiology such as consumption of alcohol and vegetables and exhibiting a certain lipidic and glycaemic profile [39], [40]. Without a consensus on the role of hTAS2R5, we moved on to the construction of a phylogenetic tree of all the TAS2R variants in human and in the model species rat, mouse and pig. With this study, we aimed to identify the closest homolog to hTAS2R5 and therefore, gain knowledge on their activity. Among, the 25 subtypes from the human TAS2R family, only hTAS2R5 and hTAS2R8 had no ortholog among the model species. The phylogenetic tree revealed that these two receptors belong to ancient orthologous with placental mammals that were lost in rodents. Moreover, we identified that some human TAS2R belong to orthologous groups with other species rich on duplications which results on receptors with high structural similarity among them originated from genomic diversifications. In contrast, hTAS2R5 belong to a less dynamic orthologous group which resulted in it being the only human homolog in it. Although the closes paralogs to hTAS2R5 appear to be hTAS2R4 and hTAS2R38, hTAS2R5 shares the highest identity and similarity values with hTAS2R7 (30% of identity and 50% of similarity). Nevertheless, these values are low in comparison to other paralogs, such as hTAS2R8, the only other paralog without orthologs, which shares similarity percentages over 70%. Thus, the results obtained with the phylogenetic tree highlighted the uniqueness of hTAS2R5 among the TAS2R from human, pig and rodents. Finally, we compared the TAS2R homologs from the selected species with known agonists in common to hTAS2R5 in an attempt to identify which receptor, although not phylogenetically or structurally related, shared the most ligands and therefore, behave similarly to hTAS2R5. Out of the twelve agonists identified so far for hTAS2R5, hTAS2R7 and hTAS2R39 were the human homologs that shared the highest number: three and six, respectively. By comparing the half maximal effective

concentration (EC50) of these receptors for the shared ligands we found that the affinity of epicatechin for hTAS2R5 is higher than for hTAS239. Regarding the homologs in other species, there is only information about ligands affinities for mice isoforms. We found that mTas2r105 and mTas2r144 share the same four ligands with hTAS2R5 and that mTas2r144 shares one additional ligand. Out of the identified hTAS2R5 ligands, some were specific for it, such as 1,10-Phenanthroline, since they do not bind similar receptors like hTAS2R39, and they were also found to be ligands for mTas2r105 and mTas2r144. Besides being the mice receptor that shares the most ligands with hTAS2R5, mTas2r144 also shows more similar affinities for the ligands to those of hTAS2R5 than the other mouse isoform, mTas2r105. Therefore, we postulated that mTas2r144 may act as a functional ortholog to hTAS2R5. Moreover, mTas2r144 has been identified in some of the locations where hTAS2R5 is expressed, suggesting a similar role. mTas2r144 expression in the brain is found to be regulated by female sex hormones, which also indicates a function on the reproductive system, as seen for hTAS2R5 [41], [42].

Finally, to fully comprehend the enteroendocrine system is not only necessary to understand its chemosensory machinery, but also its secretory mechanisms. As mentioned before, the use of a good experimental model is crucial obtaining results close to the reality in physiology but, the models available to study the enteroendocrine system have several limitations. The use of Ap-to-Bas set-up in pig *ex vivo* studies allowed us to characterize the relevance of apical stimulation of the receptors for procyanidin B2 and the mixture of catechin and epicatechin in the secretion of PYY and CCK, respectively **(Manuscript 2)**. However, to study human enteroendocrine secretions, we most frequently use the *ex vivo* model of intestinal explants, as we did in the previously mentioned work about GSPE-induced secretions. In *ex vivo* intestinal explants, the tissue is placed in a culture media with the treatment mimicking the luminal content on the intestine and the secretions are collected over time and analysed from the same media. Although this model allows a high throughput from difficult-to access intestinal human samples, it offers a limited resem e to physiology because it does not preserve the intestinal vectoriality of the established mechanism of luminal nutrient-sensing and basolateral secretions. Recent findings by Stevens *et al.* [43] showed that human colon stimulation with rebaudioside or casein in an *ex vivo* set-up that maintains intestinal vectoriality, InTESTine, resulted in increased apical secretion of GLP1, without providing an explanation for this unknown new target of the enteroendocrine secretions. PYY secretions, although not modified by the treatments, were also found in the apical compartment. This finding, combined with the difficulties we have experimented in

translating the results obtained in model species to the results from human experiments, lead us to compare the results obtained by stimulating human colon enteroendocrine secretions in a non-vectorial model, i.e., intestinal explants, and in a vectorial model, Ussing chambers. Moreover, we aimed to study the relevance of potential apical enteroendocrine secretions in the intestinal lumen **(Manuscript 3)**. For this reason, we studied the enteroendocrine response of human colon in these two set-ups to meat peptone, a known common secretagogue for PYY and GLP1, the enterohormones produced in human colon [44]. We found that the stimulation by the treatment observed of PYY and GLP1 in explants, when looking at the apical and basolateral compartments of Ussing chambers separately, corresponded only to the apical compartment, where the secretions were increased. Thus, we first show that meat peptone is able to induce luminal, and not basolateral, PYY and GLP1 secretion. Besides, in non-stimulated conditions, basal secretions of both enterohormones were higher in the apical compartment than into the basolateral. Since activity of enterohormones is controlled by the cleavage of luminally expressed DDP4, we wondered what the implications of cleaved PYY and GLP1 in the lumen may be. While it is known that cleaved PYY is active through Y2 receptors that are expressed in the colon mucosa and responsible for delaying gastric emptying [45], [46], little is known about the bioactivity of cleaved GLP1 and the presence of its receptors within the GI tract. Although full GLP1 is active through GLP1R, cleaved GLP1 has been shown to be active in a GLP1R-independent way, not in the regulation of glucose metabolism but in newly identified functions such as the proliferation and differentiation of preadipocytes [47]. More recent work has proposed glucagon receptor (GCGR) to be the receptor for cleaved GLP1 in the pancreas [48]. Thus, to understand the relevance of luminal, and potentially cleaved GLP1, we evaluated the presence of its classic receptor, GLP1R, and GCGR. We first showed that, not only GLP1R and GCGR are expressed in the luminal cells of human colon mucosa, but also, they had different expression patterns in other cell types of the mucosa. While GLP1R and not GCGR is expressed in the lamina propria, where the nerve endings, endothelial cells and immune cells are located, GCGR but not GLP1R is expressed in the crypts of Lieberkühn, were the intestinal stem cells and mucus-producing goblet cells are. Thus, this finding suggests possible paracrine roles of GLP1 in the human colon mucosa through different receptors. The presence of GLP1R and GCGR in the cells facing the lumen could indicate a direct L-cell autocrine stimulation or paracrine regulation of GLP1 secretion from the neighbouring cells, as it has previously been suggested in upper parts of the GI tract with the GLP1-somatostatin regulatory loop [49], [50]. Besides, the presence of one ore either

receptor in other cell types of the mucosa indicates a possible role of GLP1 in the modulation of the immune cells in the lamina propria, accordingly to the previously reported anti-inflammatory activity of GLP1-based therapies in reducing immune infiltration [51], and a potential novel function of the mucus secretion. Therefore, the knowledge about the enteroendocrine system is still expanding. Although we have found interesting new ways to modulate it in favour of human health, much more research needs to be done in order to fully comprehend how the enteroendocrine system impacts in many physiological roles in the organism.

Considering all the results obtained in this thesis, we can affirm that glucagon is more sensitive to the fasting state due to the action of GSPE in the modulation of enterohormone secretion. Particularly, GSPE enhances GLP1 secretion and GLP1R signalling in the pancreas which can be associated to the improved glucagon sensitivity. Moreover, we have identified some of the molecules responsible for the enteroendocrine action of GSPE and that the relative abundance of one molecule or the other is determinant for the enteroendocrine effect they produce. Specifically, while a single molecule has an effect, it can be counteracted by other molecules within the extract. Another important factor to take into consideration is the possible different arrange of chemosensors along the GI tract, since GSPE unequally stimulates GLP1 secretion when administered to one or other position of the GI tract where GLP1 is normally produced. The fact that molecules alone or in combination have different effects goes in agreement with what we have observed when studying polyphenols with known affinities for TAS2R. We have identified that depending on the relative abundance of hTAS2R5 and hTAS2R39 agonists, a different enteroendocrine response is produced. Therefore, the affinity of polyphenols for TAS2R and their expression in specific localities of the GI tract define what the enteroendocrine effect of a combination of polyphenols will be. Moreover, we have described for the first time that flavanols activate TAS2R which results in certain enteroendocrine secretions that ultimately modulate intake. These results are of great interest for the development of anti-diabetic drugs, among other health applications to modulate food intake, but have the main limitation of difficult extrapolation to humans. In order to better understand the effects described in rats, we conducted a comparative genomic study to find orthologs to the human TAS2R5, which had no previously defined homologs. Although we found that the mTas2r144 mouse receptor could be a functional ortholog of hTAS2R5, more studies are needed to make the most of this food intake-modulation technology. Finally, not only stimulation mechanisms are important for finding applications of enterohormone secretagogues in human health, but we also need

to fully understand the functions of the enterohormones we stimulate. Knowledge about the functions of enterohormones is constantly evolving. In this direction, we have found that GLP1 in the colon, which is the location of the GI tract that produces the most, is secreted predominantly into the lumen than in the basolateral part, as previously thought. In addition, we found for the first time that GLP1R and GCGR, which in some cases also acts as a GLP1 receptor, are expressed in various cell types of the colonic mucosa. Therefore, more research is needed to fully understand the functions of this enterohormone in the colonic lumen in order to advance in the field of the use of bioactive molecules as antidiabetic therapies.

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CONCLUSIONS

CONCLUSIONS

The main conclusions obtained from this thesis are:

1. **GSPE modulates glucagon in rats fed with a cafeteria diet.**
    - The moment at which the GSPE is administered seems to be a key point in modulating the endocrine pancreas, since preventive and simultaneous treatments versus cafeteria diet induce different endocrine regulations.
    - The glucagon modulation is related to the effects of GSPE on GLP-1 ileal production
    - Glucagon is more sensitive than insulin to the modulation by GSPE.

2. **Polyphenols stimulate secretion of enterohormones differentially depending on different factors:**
    - The enteroendocrine cells location along the GI tract.
    - The administration of polyphenols alone or in combination with others.
    - The species; specifically, we have observed clear differences among human, pig and rat.

3. **In human colon, GLP1 and PYY are secreted and act into the lumen of human colon.**
    - Basal GLP1 and PYY secretion is higher in the apical than in the basolateral compartment.
    - Apical PYY and GLP1 secretion can be enhanced by meat peptone stimulation.
    - GLP1R together with GCGR, which has been related to GLP1 activities in some cases, are expressed in several cell types of the human colon mucosa, indicating that GLP1 is active in the lumen.

4. **Food intake can be modulated by flavanols-induced enteroendocrine secretions through bitter taste receptors (TAS2R) stimulation.**
    - The ligands of hTAS2R5, such as epicatechin, produce an anorexigenic effects in rats, whereas a stronger agonism of hTAS2R39, such as with a combination of epicatechin and epicatechin gallate, acts as orexigenic.

- In contrast to other human TAS2R, hTAS2R5 seems to be unique to humans, without close orthologs in the model species, and has no close paralogs among the other human subtypes. However, the mouse mTas2r144 subtype is revealed as a functional ortholog of hTAS2R5 because responds to the same stimulus and is expressed similarly.

CONCLUSIONS

Les principals conclusions obtingudes d'aquesta tesi són:

1. **El GSPE modula el glucagó en rates alimentades amb una dieta de cafeteria.**
    - El moment en què s'administra el GSPE sembla ser un punt clau per a la modulació del pàncrees endocrí, ja que els tractaments preventius i simultanis versus la dieta de la cafeteria indueixen diferents regulacions endocrines.
    - La modulació del glucagó està relacionada amb els efectes de GSPE sobre la producció ileal de GLP-1
    - El glucagó és més sensible que la insulina a la modulació per GSPE.

2. **Els polifenols estimulen la secreció d'enterohormones diferencialment en funció de diferents factors:**
    - La localització de les cèl·lules enteroendocrines al llarg del tracte gastrointestinal.
    - L'administració de polifenols sols o en combinació amb altres.
    - L'espècie; específicament, hem observat diferències clares entre humans, porcs i rates.

3. **En el còlon humà, GLP1 i PYY es secreten i actuen en la llum del còlon humà.**
    - La secreció basal de GLP1 i PYY és més alta al compartiment apical que al basolateral.
    - La secreció apical de PYY i GLP1 es pot millorar mitjançant l'estimulació de la peptona de la carn.
    - GLP1R juntament amb GCGR, que s'ha relacionat amb activitats de GLP1 en alguns casos, s'expressen en diversos tipus cel·lulars de la mucosa del còlon humà, fet que indica que GLP1 és actiu a la llum.

4. **La ingesta d'aliments es pot modular mitjançant secrecions enteroendocrines induïdes per flavanols mitjançant l'estimulació dels receptors del gust amarg (TAS2R).**
    - Els lligands de hTAS2R5, com l'epicatequina, produeixen efectes anorexigènics en rates, mentre que un agonisme més fort de hTAS2R39, com ara amb una combinació d'epicatequina i epicatequina gal·lada, actua com orexigenica.
    - En contrast amb altres TAS2R humans, el hTAS2R5 sembla ser únic per als humans, sense ortòlegs propers espècies model, ni paràlogs propers entre els altres subtipus humans. No obstant això, el subtipus del ratolí mTas2r144 es revela com un ortòleg funcional de hTAS2R5 perquè respon als mateixos estímuls i s'expressa de manera similar.

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ANNEX

ANNEX

Published papers

Grau-Bové C, González-Quilen C, Terra X, Blay MT, Beltrán-Debón R, Jorba-Martín R, Espina B, Pinent M, Ardévol A. (2020) Effects of Flavanols on Enteroendocrine Secretion. *Biomolecules*. 1;10(6):844. doi: 10.3390/biom10060844

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González-Quilen C, **Grau-Bové C**, Jorba-Martín R, Caro-Tarragó A, Pinent M, Ardévol A, Beltrán-Debón R, Terra X, Blay MT (2020). Protective properties of grape-seed proanthocyanidins in human ex vivo acute colonic dysfunction induced by dextran sodium sulfate. *European Journal of Nutrition*. 60(1):79. doi: 10.1007/s00394-020-02222-3

Submitted papers

Grau-Bové C, Grau-Bové X, Terra X, Garcia-Vallve S, Rodríguez-Gallego E, Beltran-Debón R, Blay MT, Ardévol A, Pinent M. Functional and genomic comparative study of the bitter taste receptor family TAS2R: insight into the role of human TAS2R5. Submitted to *FASEB Journal* (01/08/2021)

Grau-Bové C, González-Quilen C, Cantini G, Nardini P, Espina B, Bani D, Terra X, Blay MT, Rodríguez-Gallego E, Luconi M, Ardévol A, Pinent M. GLP1 exerts paracrine activity in the intestinal lumen of human colon. Submitted to *European Journal of Endocrinology* (14/08/2021)

Patent

Ardévol A, Pinent M, **Grau-Bové C**, Blay MT, Terra X, Beltrán-Debón R, Rodrñiguez-Gallego E, González-Quilen C, Sierra-Cruz M, Miguéns-Gómez A. Composición y método para modular la ingesta de alimentos. (núm. Sol·licitud: 2020PAT-04) (August 2020).

Communication posters and oral presentations in congresses

Grau-Bové et al. Modulation of food intake by differential TAS2R stimulation in rat. The 1st International Electronic Conference on Nutrients - Nutritional and Microbiota Effects on Chronic Disease. Nov 2020. Online (Poster).

Grau-Bové et al. The study of enterohormone secretion in human colon is conditioned by the ex vivo experimental model. NuGo Week, From foodomics to nutrigenomics. Sep2019, Bern, Switzerland (Poster)-

M. Pinent et al. Differential expression of bitter taste receptors in animal species used as human models. Alimentòmica, XI Seminar on Food and healthy lifestyles. Jul2019, Barcelona, Spain (Poster)-

M. Pinent et al. Differential expression of bitter taste receptors in animal species used as human models.42nd Congress of the Spanish Society of Biochemistry and Molecular Biology. Jul2019, Madrid, Spain (Poster)-

Grau-Bové et al. Grape seed proanthocyanidins extract stimulates enterohormone secretion in different location of porcinegastrointestinal tract. Invinotec - 2nd National Congress on Wine Biotechnology. Oct2018, Tarragona, Spain (Oral presentation).

The enteroendocrine system is located in the gastrointestinal tract and controls appetite and endocrine pancreatic activity, among other functions. Thus, bioactive compounds that stimulate the enteroendocrine system are therapeutic candidates for treating pathologies related to these functions. Previous research has identified a grape-seed proanthocyanidin extract (GSPE) as antidiabetic for its β-cell function enhancement abilities and its appetite-suppressing activity at least partly through activating the enteroendocrine system. Moreover, our group has linked the polyphenol-induced enteroendocrine secretions to the stimulation of some bitter taste receptors (TAS2R) in vitro, but whether it results in an altered food intake has not been studied yet. Since little is known of the mechanisms used by polyphenols to stimulate secretory mechanisms of the enteroendocrine system, there is a need to fully comprehend this system to specifically target it with a therapeutic strategy.

For this reason, this thesis addressed whether GSPE-induced enterohormone secretions modulate pancreatic glucagon production, and whether these secretions are regulated through the specific stimulation of TAS2R leading to a differential control of food intake. This hypothesis was assessed with in vivo studies in rats and ex vivo studies in intestinal samples.

We found that glucagon was higher sensitive than insulin to GSPE which correlates with enhanced ileal GLP1 secretion. Moreover, we identified that while some polyphenols stimulate basolateral secretion of enterohormones differentially depending on the location in the gastrointestinal tract; but also that GLP1 and PYY are secreted and act into the lumen of human colon. Finally, we showed that flavanols act through TAS2R, the selective stimulation of which can be used to increased or reduced food intake.

To sum up, this thesis shows that polyphenols influence pancreatic glucagon production through a stimulation of the enteroendocrine system, which occurs through the activation of TAS2R. Polyphenols' activation of TAS2R in turn also modulates the food intake.