TASTE PERCEPTION BEYOND FLAVOUR: BITTER TASTE RECEPTORS IN THE GASTROINTESTINAL TRACT AND THEIR ROLE IN AGEING

Florijan Jalševac

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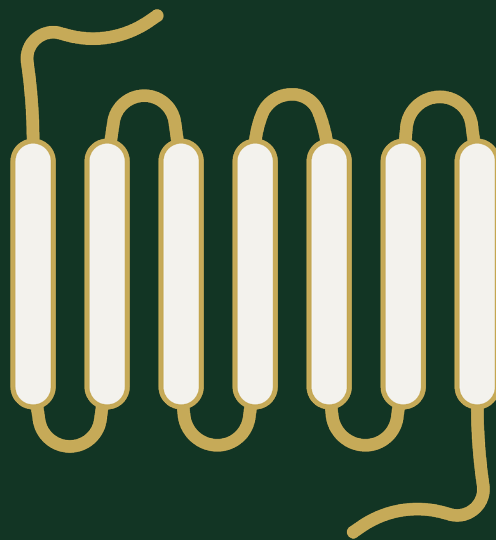
Doctoral Thesis

Taste Perception Beyond Flavour: Bitter Taste Receptors in the Gastrointestinal Tract and Their Role in Ageing

Florijan Jalševac

2024



UNIVERSITAT ROVIRA i VIRGILI

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Taste Perception Beyond Flavour: Bitter Taste Receptors in the Gastrointestinal Tract and Their Role in Ageing

Doctoral Thesis

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Tarragona, 2024

FAIG CONSTAR que aquest treball, titulat "**Taste Perception Beyond Flavour: Bitter Taste Receptors in the Gastrointestinal Tract and Their Role in Ageing**", que presenta **Florijan Jalševac** 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 "**Taste Perception Beyond Flavour: Bitter Taste Receptors in the Gastrointestinal Tract and Their Role in Ageing**", que presenta **Florijan Jalševac** para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Bioquímica y Biotecnología de esta universidad.

I STATE that the present study, entitled "**Taste Perception Beyond Flavour: Bitter Taste Receptors in the Gastrointestinal Tract and Their Role in Ageing**", presented by **Florijan Jalševac** 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.

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An expert is a person who has made all the mistakes that can be made in a very narrow field.

Neils Bohr

Everything is theoretically impossible until it is done.

Robert A. Heinlein

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SUMMARIES

Summary

The population of the world is growing ever older, and with it, the incidence of age-associated disorders is on the rise. The constant internal and external stressors are balanced by homeostatic systems, which minimise their negative effects. Dysregulation of metabolic homeostasis is one of the main drivers of many age-accompanying disorders. Chemosensory receptors of the gastrointestinal tract (GIT) can be influenced to elicit several beneficial effects. One group of these receptors are the bitter taste receptors (TAS2R). TAS2R and their ligands, such as grape seed proanthocyanidin extract (GSPE) have been proposed as novel ways to achieve healthier ageing.

In this dissertation, we discuss the diversity in the TAS2R throughout GIT, their evolution with ageing, and focus on establishing which out of 26 human receptors are more likely to have a role in the progress of ageing. Additionally, we focus on how GSPE and its constituents influence the changes in the presence of TAS2R in the GIT, and how they influence the secretory potential of the GIT.

We discovered that some receptors are more present in specific segments of the GIT, and interestingly, the ageing upregulated the expression of a handful of TAS2Rs in the colon mucosa. TAS2R5 was labelled as the most probable receptor to have important roles in the progress of ageing, due to its correlations with several parameters that are known to impact overall health. Finally, GSPE has proven to be a potent regulator of TAS2R expression, and its constituent epicatechin has been shown to impact the secretory role of the GIT cells.

In summary, this thesis presents TAS2R as interesting novel targets for potentially impacting the progress of ageing, due to their involvement in the centre of metabolic regulation and possible activation with bioactive compounds, which could elicit multiple beneficial effects.

Resum

La població del món està envellint, i amb això, la incidència de trastorns associats a l'edat està augmentant. Els estressors interns i externs es compensen amb sistemes homeostàtics, que minimitzen els seus efectes negatius. La desregulació de la homeòstasi metabòlica és un dels principals factors de trastorns associats a l'edat. Els receptors quimiosensorials del tracte gastrointestinal (GIT) poden ser influïts per aconseguir efectes beneficiosos. Un grup d'aquests receptors són els receptors del gust amarg (TAS2R). Els TAS2R i els seus lligands, com l'extracte de proantocianidines de llavor de raïm (GSPE), s'han proposat com a noves maneres d'aconseguir un envelliment més saludable.

En aquesta tesi, discutim la diversitat dels TAS2R al llarg del GIT, la seva evolució amb l'edat i ens centrem en establir quins receptors són més propensos a tenir una funció en el progrés de l'envelliment. A més, estudiem com el GSPE i els seus components influeixen en els canvis en la presència de TAS2R al GIT i com afecten el potencial secretor del GIT.

Hem definit que alguns receptors estan més presents en segments específics del GIT i que, l'envelliment augmenta l'expressió de diversosTAS2R a la mucosa del còlon. A través de l'anàlisi de la correlació de TAS2R5 amb diversos paràmetres que es coneixen per afectar la salut general, se l'ha identificat com el receptor que més probablement té una funció important en el progrés de l'envelliment. Finalment, el GSPE ha demostrat ser un regulador potent de l'expressió de TAS2R, i el seu constituent epicatequina ha demostrat afectar la funció secretora de les cèl·lules del GIT.

En resum, aquesta tesi presenta els TAS2R com dianes noves interessants per modular potencialment el progrés de l'envelliment; gràcies a la seva implicació en la regulació metabòlica i la possible activació mitjançant compostos bioactius.

Resumen

La población mundial está envejeciendo y, con ella, la incidencia de los trastornos asociados a la edad aumenta. Los constantes estresores internos y externos se equilibran mediante sistemas homeostáticos, que minimizan sus efectos negativos. La desregulación de la homeostasis metabólica es una de las principales causas de trastornos que lo acompañan. Los receptores quimiosensoriales del trato gastrointestinal (GIT) pueden ser influenciados para provocar efectos beneficiosos. Un grupo de estos receptores son los receptores del gusto amargo (TAS2R). Los TAS2Rs y sus ligandos, como el extracto de proantocianidinina de semilla de uva (GSPE), se han propuesto como nuevas maneras de conseguir un envejecimiento más saludable.

En esta tesis, discutimos la diversidad de los TAS2Rs en el GIT, su evolución con el envejecimiento y nos centramos en establecer qué receptores son más propensos a tener una función en el progreso del envejecimiento. Además, nos enfocamos en cómo el GSPE y sus componentes influyen en los cambios en la presencia de TAS2R en el GIT y cómo afectan su potencial como estimuladores de secreciones.

Hemos definido que algunos receptores están más presentes en segmentos específicos del GIT y, curiosamente, el envejecimiento aumenta la expresión de unos determinados TAS2R en la mucosa del colon. El TAS2R5 se etiquetó como el receptor más probable para tener una función importante en el progreso del envejecimiento, debido a sus correlaciones con parámetros que se conocen por afectar la salud general. Finalmente, el GSPE ha demostrado ser un regulador potente de la expresión de TAS2R, y su constituyente epicatequina ha demostrado afectar la función secretora de las células del GIT.

En resumen, esta tesis presenta a los TAS2R como interesantes objetivos para afectar potencialmente el progreso del envejecimiento, gracias a su implicación en la regulación metabólica y la posible activación con compuestos bioactivos, que podrían provocar efectos beneficiosos.

LIST OF ABBREVIATIONS

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5-HT	Serotonin
AMPK	AMP-Activated Protein Kinase
CALHM1/3	Calcium Homeostasis Modulator 1/3
cAMP	Cyclic Adenosine Monophosphate
CCK	Cholecystokinin
ChgA	Chromogranin A
DAG	Diacylglycerol
DPP4	Dipeptidyl-Peptidase 4
EECs	Enteroendocrine Cells
ENaC	Epithelial Na+ Channel
ER	Endoplasmic Reticulum
G protein	Guanine Nucleotide-Binding Protein
GDP	Guanosine 5′-Diphosphate
GIP	Gastric Inhibitory Polypeptide
GIT	Gastrointestinal Tract
GLP-1	Glucagon-Like Peptide-1
GLUT4	Glucose Transporter Type 4
GPCRs	G Protein-Coupled Receptors
GSPE	Grape Seed Proanthocyanidin Extract
GTP	Guanosine 5′-Triphosphate
$G_{\alpha gust}$	A-Gustducin
IL-25	Interleukin 25
INSL5	Insulin-Like Peptide 5
IP_3	1,4,5-Triphosphate
IP_3R3	IP3 Receptor 3
mGluR	Metabotropic Glutamate Receptor
mTORC1	Mechanistic Target of Rapamycin Complex 1
NPY	Neuropeptide Y
NSAID	Nonsteroidal Anti-Inflammatory Drugs
OTOP1	Otopetrin 1
PDE1A	Phosphodiesterase 1A

PIP_2	4,5-Biphosphate
PLCβ2	Phospholipase Cβ2
PTC	Phenylthiocarbamide
PYY	Peptide YY
SGLT1	Sodium/Glucose Cotransporter 1
SREBP-2	Sterol Regulatory Element-Binding Protein 2
TAG	Triacylglycerol
TAS	Taste Receptors
TAS1R	Taste Receptor Type 1
TAS2R	Taste Receptor Type 2
TMD	Transmembrane Domain
TRP	Transient Receptor Potential
$VGNa^+C$	Voltage-Gated Na+ Channels
VSCCs	Voltage-Sensitive Ca2+ Channels
WHO	World Health Organization

INTRODUCTION

INTRODUCTION

1. LOSS OF HOMEOSTASIS THROUGH LIFE WITH THE FOCUS ON THE INTESTINE

The world is experiencing a great demographic shift. By 2030, over 1 billion people will exceed the age of 65. This unprecedented surge in the elderly population presents a unique set of challenges for societies worldwide. While initially observed in high-income countries, the World Health Organization (WHO) estimates that by 2050, two-thirds of the global population over 60 will reside in low- and middle-income countries [1,2]. This demographic shift necessitates proactive measures to address the needs of the ageing population.

1.1. Ageing definition and its hallmarks

Though a universal biological process, ageing itself is such a complex interplay of changes that defining it simply is anything but simple. In essence, ageing can be defined as a natural process where a gradual decline in physiological functions occurs. It's experienced by most animals and manifests as a gradual increase in both physical and cognitive impairments, often accompanied by age-associated disorders and diseases [3].

The complexity of ageing makes it impossible to single out a specific reason for why it happens, as changes on the molecular levels are caused by a multitude of reasons. Nonetheless, in recent years, there has been a focused effort to establish the hallmarks with the greatest impact on how we age, and thus, pillars of ageing were proposed [4,5]. Overall, they are divided into 3 major categories: primary hallmarks represent damage accumulated with age that affects the genome, proteomes, and organelles; the antagonistic hallmarks demonstrate the mechanisms of response to damage; and finally, integrative hallmarks manifest when the damage that occurs due to dysregulation of the first two groups cannot be compensated anymore. These hallmarks and their complex web of interactions are the subjects of study of gerontology, whose definitions have been established on the basis of 3 rules: a) they manifest as we age; b) experiments modulating these systems change the rate of ageing; and c) with correct interventions, there is a possibility to decelerate, stop, or even reverse the process [6].

1.2. The homeostasis of metabolism

While we often associate the consequences of ageing with later life, scientific understanding reveals that the underlying molecular mechanisms begin to change much earlier. This period,

marked by the gradual loss of cellular homeostasis, presents a crucial window of opportunity. By intervening at this early stage, we might be able to prevent or ameliorate future age-related dysfunctions and extend the period of health and well-being.

Most researchers consider that the ageing process itself starts with sexual maturation [7]. It is worth noting that chronological and biological ages are not the same, and there are some diseases that cause accelerated ageing, while the hunt for ways to slow down ageing has been a matter of investigation for millennia [8].

1.2.1. Role of Metabolism in the Homeostasis

Life itself, as we understand it now, is a complex set of biological systems where all must function in unison to maintain conditions that allow an organism to survive. One of the qualities that separate life from matter is the capacity to maintain homeostasis. Homeostasis itself can be explained as a balanced state of the internal environment, indicating that physical conditions in the system are such that enable the optimal conditions to survive [9,10]. The constant environmental pressures that are exerted on an organism continuously influence these physiological conditions. But to support life, tight regulation of internal factors, such as pH, body temperature, concentrations of ions (calcium, sodium, potassium, iron), glucose levels, blood pressure, and many more, is required [11–13]. Thus, a buffer system that can negate these potentially threatening factors was developed, known as homeostasis. One key component that contributes to achieving homeostasis is metabolism [14]. This set of anabolic and catabolic reactions that release energy from molecules and create building blocks for cellular structures gives rise to the functions that create and sustain life. However, metabolism is not flawless. The very processes that maintain homeostasis can generate imperfections and waste products, such as misfolded proteins, DNA and RNA damage, and malfunctioning organelles [15]. Cells respond to this damage with repair and clearance mechanisms, but this process is not perfect either and over time, the damage accumulates, impeding cellular functioning [4–6]. Gladyshev labelled this damage as “deleteriome”, a deleterious consequence of metabolism-associated accumulation of cellular damage [16]. This gradual accumulation of damage to the cell ultimately results in lower adaptability and lower metabolic flexibility, one of the ageing hallmarks [17,18]. The decrease in the capability of balancing anabolic and catabolic reactions and the accumulation of cellular damage could also explain the lower metabolic rate in aged individuals. Damaged components may overwhelm the body's cellular waste disposal and repair mechanisms. As a potential consequence, or perhaps simply due to the damage itself, the overall cellular metabolism slows down.

1.2.2. Nutritional Impact on Homeostasis

While regular medical checks, higher quality of medical care and medicine, and early detection of age-associated disorders for timely intervention are all effective ways of promoting longer health, they all require medical intervention. Somewhat paradoxically, the lack of resources and nutrients is one of the most effective ways to improve cellular maintenance and mitigate damage caused by cellular metabolism [19–21]. In recent years, nutritional interventions like caloric restriction and intermittent fasting have emerged as promising, non-invasive, and more widely implementable approaches to promoting healthier ageing [22,23]. Nutrition is a key factor in ageing, and unlike medical interventions, nutritional strategies offer a non-invasive and potentially more scalable approach to public health. Conversely, the overnutrition prevalent especially in the Western world significantly undermines health and accelerates the onset of various disorders [24,25]. Considering this, it becomes evident that by regulating our diet we can have meaningful and long-lasting effects on our health. Caloric restriction, a well-established intervention, reduces calorie intake while maintaining essential micronutrient and macronutrient uptake over a given period [26,27]. Intermittent fasting is another popular approach where in place of reducing the size of the meal, a meal is skipped entirely. Apart from these two methods, many more have been established in the literature [28]. Upon nutrient restriction, a change in the metabolic profile can be observed, as the energy production, which in normal conditions is driven by glucose, is switched to lipid metabolism and the production of ketone bodies. Between meals, the liver uses glycogenolysis and gluconeogenesis to maintain blood sugar levels. However, glycogen stores are limited, and during extended fasting periods, the body activates alternative pathways like lipolysis and ketogenesis. Tissues such as the brain and muscles have high energy requirements. In fasting conditions, the ketone bodies produced by the liver transform into acetyl-CoA, which can enter the Krebs cycle and provide ATP for cellular functions [29,30].

On a cellular level, different mechanisms are triggered that in principle, enable the cell to utilize the available nutrients and recycle the metabolic byproducts more efficiently. This can be observed in variate ways. One important signalling system that is activated upon starvation of nutrients is the AMP-activated protein kinase (AMPK) pathway [31]. AMPK is sensitive to AMP levels, which rise when cellular processes deplete ATP and nutrient intake is low [32,33]. Increased AMP triggers AMPK activation, which promotes cellular energy balance in two ways: the inhibition of anabolic reactions and turning on the catabolic mechanisms. The former is achieved through inhibition of lipid synthesis and glycogenesis. Moreover, AMPK is also responsible for reprogramming the metabolism (inhibition of gluconeogenesis and metabolism

of lipids) [34–37]. AMPK-stimulated catabolic reactions encompass the metabolism of macromolecules for energy requirements and overall activation of autophagy. Studies in animal models such as Drosophila and C. Elegans have shown the importance of AMPK complex activation for better longevity. Overexpression of AMPK subunits extended the lifespan of animals, while the mutations resulting in inactivation of this complex noticeably lowered the length of their lives [38–40].

While AMPK regulates and establishes the balance when nutrients are scarce, the abundance of nutrients activates the regulator of growth, the mechanistic target of rapamycin complex 1 (mTORC1) [41,42]. González et. al. have likened AMPK and mTORC1 to yin and yang, representing the opposing yet interconnected forces that regulate cellular metabolism [43]. While AMPK promotes catabolism, the effect of mTORC1 is the opposite: it promotes cell growth, gene transcription and protein synthesis, and an overall increase in anabolism [44]. Interestingly, inhibiting mTORC1, which AMPK can do, has proven beneficial effects on the mechanisms of maintenance, such as autophagy, and leads to extension of life in animal models [45–47]. In essence, the balance of these mechanisms and others are the invisible leaders of homeostasis and are key to achieving homeostasis, while any deficiency eventually leads to dysfunction.

1.3. Homeostasis Dysfunction of the Gastrointestinal Tract

Interacting with the environment, sensing our surroundings, incorporating nutrients, and disposing of byproducts are all important contributing factors for achieving homeostasis, and are factors where the gastrointestinal tract (GIT) has a substantial role. The understanding of this system has evolved greatly in the last decades, from its important, but obvious role of nutrient absorption, to what is now known as a key component in the defence of the organism, and the biggest endocrine organ in humans.

Normally functioning intestinal tissue is implicated in protection against the environment, but is also responsible for detecting, observing, and relaying information on a variety of signals [48–51]. A balance between the renewal of the epithelial cells and cellular shedding and apoptosis is needed for the continuation of intestinal homeostasis. This balance is under pressure from both external and internal factors and depends on the progress of stem cell regeneration of the epithelia, the influence of microorganisms and the ingested nutrients, as well as on inflammatory stress. Due to this overwhelming exposure to the environment, the cells of the GIT are characterised by a high renewal rate; most of the cells of the GIT are renewed in less than a week [48]. However, such a rapid regeneration and constant presence of external stressors exert a great challenge on the present stem cells, and eventually, their capacity to proliferate and differentiate

diminishes, which leads to eventual stem cell exhaustion and the diminishment of the barrier function of the intestine [4]. Just like any other tissue, the ageing GIT is associated with numerous changes (**Figure 1**), which all contribute to diminished capacity and the development of chronic inflammation [52,53].

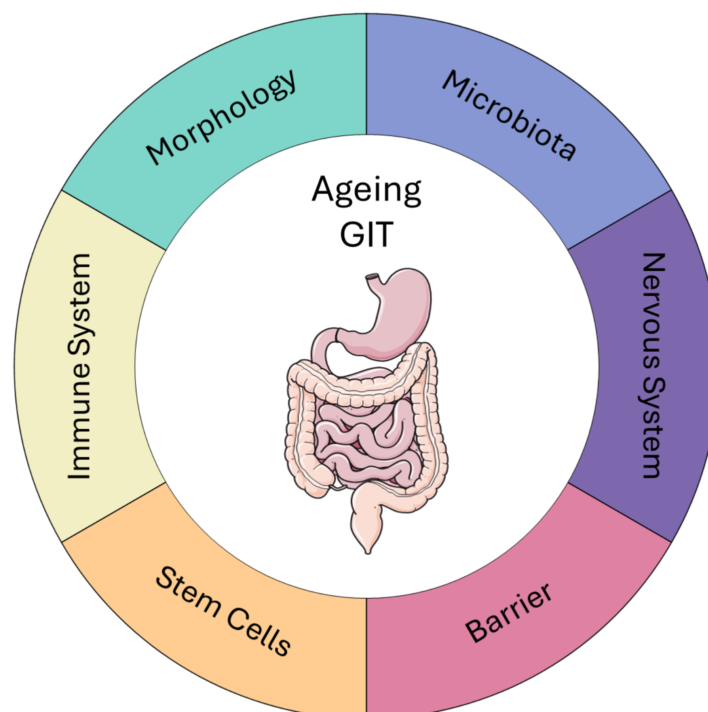

Figure 1: ELEMENTS OF THE GIT THAT ARE AFFECTED BY AGEING: During ageing, all the constituents that compose the functionality of the GIT are affected. Adapted from [54].

1.3.1. Dysfunction and consequences in the stomach

Ageing affects the functionality of each segment of the GIT. For example, one of the characteristics of the stomach that is affected by ageing is the rate of gastric emptying [55,56]. The complex movement that is involved in gastric emptying encompasses a coordinated move from the smooth muscles around the stomach, pylorus and duodenum, all controlled by the autonomic nervous system. The contractability of these muscles diminishes with ageing [57]. The slower emptying of the stomach content has multiple effects. First, the slower emptying of the stomach triggers the sensation of satiety, lowering the overall food intake, a possible explanation of age-associated anorexia [58]. Additionally, gastric emptying has also been associated with blood pressure, as gastrointestinal have an impact on it. The secretion of these hormones depends on the presence of food in the intestine; as the gastric emptying is slowed down, the release of hormones is delayed, which finally manifests as postprandial hypotension [59].

When considering the secretion of gastric acid, a consensus has not been reached. While historically it was reported that lower secretion of gastric acid was associated with ageing, recently this has come into question, mostly due to the recent inclusion of Helicobacter pylori as a factor [60,61]. However, H. pylori has been associated with inflammation, a condition prevalent in homeostasis dysfunction [62]. In any case, less acidic conditions in the stomach and subsequently the duodenum cause the overgrowth of the intestinal and foreign bacteria, leading to a higher chance of infections and inflammation[63]. Finally, due to the increased incidence of medications that affect the gastric mucosa, like nonsteroidal anti-inflammatory drugs (NSAID), and the overall lower protective capacity of the barrier against gastric acid, there is a higher occurrence of gastritis and ulcers [64,65].

1.3.2. Dysfunction and consequences in the intestine

One important aspect of the intestine that has a significant impact on its absorptive capacity is the morphology of the intestine. The main cells of the intestinal mucosa are enterocytes, and these are the cells that carry the typical structure of microvilli, which greatly increase the overall surface area of the intestine [66]. Overall, researchers have found that with increasing age, morphological changes include the lower density of the villi [67]; however, the precise effect of these changes on the overall absorptive function of the intestine is not clear, as Valentini et. al. showed no observable differences between young and aged groups [68]. Something that has been observed however is the age-induced change in the permeability of the intestine [69]. Permeability depends on the paracellular transport, incidence of apoptosis of the mucosal layer of the intestine, and the transcellular permeability [70]. There are various causes that influence the parameters of intestinal permeability, but all of them have a common end, that is, the disorder known as leaky gut [71]. Low-grade inflammation and changes in intestinal microbiota, two characteristics observed in ageing, are associated with the incidence of leaky gut syndrome [72]. The higher permeability of this membrane greatly reduces its defensive capability, and with it, there is an increased potential for toxic compounds and infiltration of pathogens into the systemic circulation, which can have grave consequences.

Homeostasis dysfunction also appears in the colon. While it is more common in the latter stages of ageing, the occurrence of motility disorders has a noticeable effect on quality of life [73]. While the complexity of the disorder makes it difficult to distil to a singular cause, animal models have shown that disruptions in neural functioning, control and muscle weakening play a major role in the incidence of motility disorders [74]. A major factor that has a major impact on the overall functioning of the organisms is located in the lower intestinal tract: the microbiota. The

microorganisms that inhabit our intestines play important roles in occurrences of cancer, metabolic disorders, cardiovascular diseases, and even psychological disorders [75]. This is a highly dynamic environment, one that is influenced by factors such as our diet and lifestyle. Another important role that microbiota plays is the process of ageing itself, most notably through its influence on the inflammatory regulation in the intestine and systemically, and the manifestation of inflammation [76–78].

2. SENSING THE ENVIRONMENT: FOCUS ON TASTE AND BITTER RECEPTORS

The way we perceive our surroundings, how we recognise the stimuli, and how we respond to the cues from our environment, are of utmost importance for our survival. The immense pressure of our surroundings and the importance of recognising danger and safety influenced the evolutionary development of mechanisms for detection, interaction, and response to stimuli: senses [79–81]. These biological systems are fundamental for nearly all the aspects of cognition, behaviour and thought, and are what allow us to gather information through the detection of stimuli. This occurs in the specialised family of cells known as sensory cells. These cells exist in many forms (such as chemoreceptors, mechanoreceptors, and thermoreceptors) and are the building blocks of the sensory organs [82–85]. Upon their stimulation with external cues, the signal from the sensory organ is transduced towards the central nervous system, where the processing of the stimuli in the sensory cortices takes place, with the final result being the interpretation of the signal [86–88]. Due to the importance and need for speciality, we can find numerous senses in living beings. One of the most fundamental ways to separate the senses is based on whether they detect the stimuli from our external environment, called exteroception, or if they are responsible for detecting the cues from the inside of the organism itself, known as interoception [89]. Exteroception is detected by our external sensory organisms: skin, nose, mouth, ears, and eyes, which colloquially detect the 5 basic senses of touch, taste, smell, sound and sight [90]. While many generations have been taught that these are the only senses we have, it has been well-documented that complex organisms can detect a myriad of internal signals as well. The vestibular system of the inner ear is responsible for the awareness of balance, coordination, and movement; proprioception detects the position of our body in space through proprioceptors located in the muscles and joints; and noxious stimuli of pain is detected by nociceptors found in the skin, as well as internally [91–94]. Moreover, hunger, lack of oxygen in the blood, and thirst are just some of the sensations we experience through our chemoreceptors

and osmoreceptors, while some animals have evolved capabilities for the detection of the Earth's magnetic field, moisture, wider ranges of the electromagnetic field, and can even perceive the world through echolocation [95–97]. And sensory receptors can detect not just the presence of a specific stimulus, they can transfer information about the quality of the cue itself.

While interoceptive perception is just as important for overall well-being, we are much more aware of the sensation we perceive from the clues from the external environment, like for example, the sensation of taste.

2.1. Taste Sensing

Gustation, or sense of taste, is one of the colloquial 5 senses and is responsible for perceiving the chemical composition of the food that we are ingesting. The complexity and importance of taste sensation can be seen in its lengthy prenatal development and rapid maturation: first taste buds are observed in around the 9th week of gestation, and they are rapidly developed in the first year [98–101]. It is important to distinguish the perception of taste from the perception of flavour: while taste is the sensing of the non-volatile chemical makeup of the nutrients through specific taste receptors, flavour is a complex interplay of taste, odour, temperature, and other chemical sensations which our brain interprets in union [102]. Due to the nature of the diversity of taste, the role taste receptors serve is vast, but we can briefly characterize them as nutrient sensors that upon their activation with the food components send signals to the brain about the composition and safety of the food [103].

And while taste is a ubiquitous sensation we experience on a constant basis, the area itself is still very much unknown. For example, we have yet to reach a consensus on just how many tastes can we detect: canonically, humans can detect 5 basic tastes: salty, sour, sweet, umami, and bitter [104]. However, there is an ever-increasing opinion that there is a 6th taste we can detect, the one of taste of fat [105,106]. For a sensation to be labelled as a specific taste, a few specific criteria have to be met: there has to exist an effective stimulus, which has to be detected by a specific family of taste cells, located in the taste organ, and this signal has to be transduced by afferent nerves from the taste buds to the taste-processing centres of the brain. Finally, the perceived taste must be specific, that is, should elicit specific signals independent of other taste qualities and with no other physiological responses [107]. CD36 and GPR120 are 2 proteins that have been associated with the detection of fatty acids, and these proteins do send signals through afferent nerves to the brain; however, is it still unclear whether the sensation is independent of other tastes, and some research has suggested that activating fatty acids receptors causes an increase

of triacylglycerol (TAG) levels in the blood; thus, the sensation of fatty taste is still a matter of debate [107,108].

2.2. Mechanism of Taste Reception

2.2.1. Taste bud – the Organ of Taste

This peripheral part of the gustatory system is a complex comprised of a circuit of cells, called taste cells, which are primarily located on the tongue, but also on the palate, epiglottis, and pharynx [109]. Traditionally, the tongue has been recognised as an important part of taste perception, as it houses the sensory endings that are papillae [110]. In these papillae, we can find the taste buds, a cluster of 50-100 morphologically elongated epithelial cells. Canonically, in the taste bud, we can find 4 different types of cells, aptly named type I or dark NTPdase2+ cells, type II or light phospholipase Cβ2 (PLCβ2+) type, type III or SNAP25+ cells, and basal cells (sometimes classified as Type IV) [111]. The function of the basal cells is still not well known, and it has been postulated that these cells are in fact immature precursors, however, this is yet to be established.

2.2.2. Salty and Sour Sensations

The most abundant cell type found in the taste bud is Type I, and this is where the perception of salty taste takes place (**Figure 2**) [112,113]. Detection of salt is of vital importance due to the importance of both sodium and chloride levels for the normal functioning of the cellular system and signalling transduction. Preventing hypernatremia and dehydration is of vital importance, however, so is the necessity to uptake sufficient quantities of salt. This dichotomy drove the evolution of salt perception in 2 different and opposite ways, depending on the concentration of the salt itself [114–116]. At low salt concentrations, the sensation elicits a positive sensation in the brain, but in higher quantities, the signal is interpreted as aversive. The appetitive response to salt is exclusive for detecting sodium through the Amiloride-sensitive epithelial Na+ channel (ENaC), which allows the entry of Na+ into the cell [114,117]. The aversive taste of salt is triggered by higher quantities and how it occurs remains not completely known. It has been suggested that uptake of Cl- into the cells may be required, and the most prevalent theory is that this taste is mediated through the unique simultaneous activation of both bitter and sour receptors [115,118,119].

Like the perception of salt taste, the perception of sour sensation is detected by ion channels. Sour taste is detected by the type III cells in the taste buds, and the sensation can be characterized as a taste of hydrogen protons derived from organic and inorganic acids. Type III cells are the only

cells in the taste buds that contain conventional synaptic connections with the afferent nerves. Sour taste is detected by the proton channel otopetrin 1 (OTOP1). Its gene, Otop1 was detected exclusively in type III taste cells and later it was established as the protein with a direct role in the sour taste signal transduction [120–122].

2.2.3. Sweet, Umami, and Bitter Sensations

Detection of the other three tastes, sweet, umami and bitter, is distinct from the salt and sour sensations (**Figure 2**). The main difference is in the type of their receptors: while salty and sour tastes are detected through the influx of ions through specific ion channels, the sensation of sweet, umami and bitter taste are detected by a family of receptors belonging to G protein-coupled receptors (GPCRs) [123]. Taste receptors, known as TAS, are located in type II cells of the taste bud, and like the other ones, are elongated in nature, but unlike other cells of the taste bud, display a single villus on the apical side of the cell. Another morphologically distinct trait these cells possess is their atypically large mitochondria located close to the innervating afferent nerves [124,125]. The glycoproteins responsible for detecting sweet, umami and bitter are structurally similar to other members of the GPCR family: these are transmembrane receptors, comprised of 7 transmembrane α-helix domains (TMD), with the starting NH2-terminus located extracellularly and a COOH-intracellular segment. TAS family can be separated into 2 subfamilies: Taste receptor type 1 (TAS1R) and Taste receptor type 2 (TAS2R) [126,127]. These 2 subfamilies can be further divided into individual members. The former one is comprised of 3 different members, aptly named TAS1R1, TAS1R2 and TAS1R3, and these are the receptors responsible for the detection of sweet and umami tastes. Both of these stimuli are appetitive, as sweet receptors detect high-energy sugars, while umami can be best described as savoury taste, reminiscent of broths and amino acids, like L-glutamate [128,129]. Interestingly, heterodimerization is crucial for their functionality: for detecting sweet taste, TAS1R2 and TAS1R3 are joined; and TAS1R1 + TAS1R3 heterodimer detects the umami taste [130,131]. One distinct characteristic of these receptors is the long NH2-terminus formed in the shape of a Venus flytrap module, which is likely the location of the binding site of sugars, although multiple binding sites in these receptors have been observed. Additionally, animal Tas1r3 knock-out models displayed a variable response to stimuli, indicating a Tas1r-independent signal transduction: sweet taste is postulated to be detected through glucose transporter type 4 (GLUT4) and sodium/glucose cotransporter 1 (SGLT1). At the same time, it is theorised that taste-specific variants of metabotropic glutamate receptors (mGluR1 and mGluR4) stimulate the umami signalling [132–135]. The other TAS family, TAS2R, is a much more diverse set of receptors,

with great discrepancies also observed between the species: in humans, 26 functional TAS2R have been observed, while in rodents (mice and rats), approximately 35 transcripts were discovered. Some animals have basically lost all functional Tas2r, while others possess a set of 80 different receptors [136–140]. Somewhat paradoxical to the diversity in the number of TAS2R is the fact that they are responsible for the detection of solely one taste: the bitter one. These receptors have represented a challenge for their classification among the GPCRs: there have been suggestions that TAS2R are representative of class A or rhodopsin-like GPCRs; now others classify them as members of frizzled receptors, while others consider them as a separate class of receptors [141,142]. Unlike sweet and umami, bitter taste stimuli are aversive. It becomes evident why when we consider the fact that many compounds we interact with and potentially see as food sources, especially those of plant origin, can be toxic; thus, bitter receptors have evolved as primarily a protective mechanism to prevent ingestion of potentially toxic compounds. Additionally, this also helps us understand the presence of various TAS2Rs: due to the overwhelming diversity of possible toxins, a wide range of receptors are required to establish sufficient protection against intoxication. However, not all bitter compounds are toxic, as, for example, many medications do activate bitter receptors at their pharmacological concentrations [143,144].

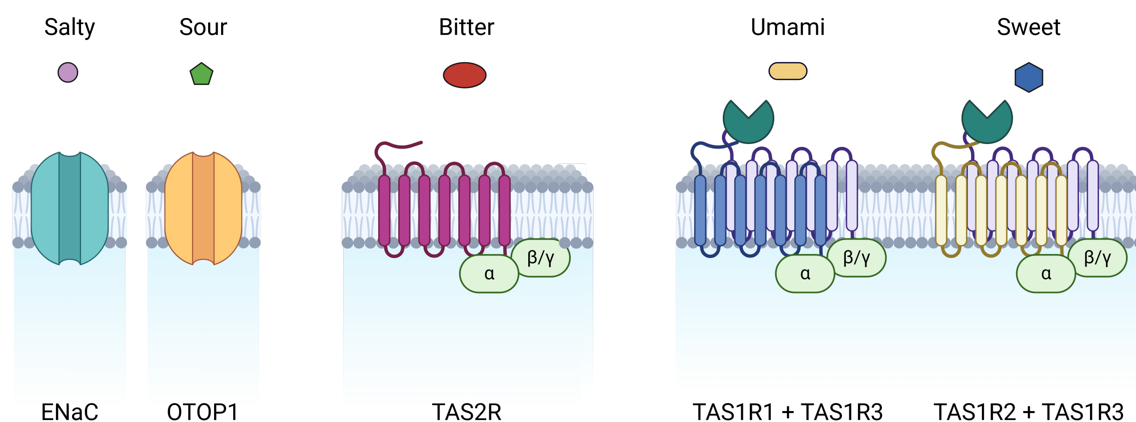

Figure 2: RECEPTORS FOR 5 BASIC TASTES. For detecting salty and sour tastes, specialised ion channels are required, while bitter, umami and sweet tastes are detected by members of the GPCR family. Created with BioRender.com.

2.3. Genetics of Bitter Taste Receptors

In the human genome, databases such as NCBI and HUGO Gene Nomenclature Committee report 39 transcripts of receptors that pertain to the TAS2R family, located on chromosomes 5, 7 and 12 [145,146]. However, up to date, only 26 of them have been identified as being expressed as functional proteins, with the latest one being characterized as functional in 2023, implying the

likelihood other TAS2R genes could also be functional [136,147]. Interestingly, bitter receptor genes belong to a small group of intronless genes. Of the 26, most have been de-orphanized, thus we know at least 1 ligand for the majority of human TAS2R, with some having multiple known activators [147–149]. Polymorphism of bitter receptors has been observed long before the establishment of bitter receptors: in the 1930s, phenylthiocarbamide (PTC) was used to describe discrepancies in taste sensation. It was shown that some people tasted PTC as extremely bitter, some described it as mildly bitter, while others described it as not bitter at all [150]. However, it took almost 70 years to establish why these discrepancies occur: it was shown that allelic differences in the gene for TAS2R38 cause some people to be supertasters, while others were non-tasters [151]. Since then, more polymorphisms of other receptors have been established, however, clear implications and great effects on tasting or health have yet to be described [152].

Genetic diversity between different species was originally explained to be the consequence of the diet, as herbivores are much more likely to be exposed to the toxic compounds from their diet than carnivores that usually consume freshly caught prey. Somewhat oxymoronically, herbivores seemed to be also less sensitive to bitter stimuli, but the assumption is valid from an evolutionary standpoint, as high sensitivity would result in higher aversion to food. But the story is much more complex than originally proposed, and it has been shown that the response to bitter stimuli is species-specific, and not agonist-specific [153–157]. Finally, the impact of the diet on taste perception can be seen in species such as penguins, dolphins, and others, which contain solely pseudogenes in their genomes [140,158,159].

2.4. Structure and Functionality of Bitter Receptors

Most TAS2R receptors expressed in humans are around 330 amino acids long [160]. The vast majority of TAS2R structures have been predicted by simulations, as only TAS2R14 and -46 have been successfully crystallised and have known structures [161,162]. From simulations of TAS2R39, the binding pocket was predicted to be located deep inside the transmembrane helices III, V, VI and VII [163]. The exact interactions between specific TAS2R and their ligands are poorly understood. However, recent studies on TAS2R14 have uncovered a brand-new interaction profile of bitter receptors: in the study, one binding site was found extracellularly, where cholesterol was bound to the receptor, while another allosteric binding site was discovered intracellularly, where a bitter ligand was bound [161]. Further investigations on the amino acid sequence of TAS2R1 revealed that the replacement of asparagine-66 and asparagine-24 made the receptor unresponsive, as they are key for creating hydrogen bonds across different TMDs, and with the

agonist itself. Unlike TAS1R, TAS2R do not possess the Venus flytrap module extracellularly but have a short terminus, and unlike TAS1R, they also do not require dimerization for their functionality, although some research does suggest that they can create both homodimers and heterodimers [164,165].

TAS1R and TAS2R proteins do not colocalise in the type II cells of the taste buds, thus the appetitive and aversive sensing mechanisms appear to require separation. On the other hand, at first, it was theorised that all isoforms of bitter receptors are expressed in each cell, however, it is now believed that in the same cell, just some of the bitter receptors are produced, which could indicate that tasting cells are specifically tuned and it is possible to distinguish between different bitter sensations [166–168].

2.5. Bitter signalling

2.5.1. Receptor Promiscuity, Species Homology, and Ligand Specificity

As mentioned before, in humans we can encounter 26 active isoforms of TAS2R, as well as a handful of pseudogenes. While they are similar in their structure, the nominal changes still have major effects on their functionality. The accepted way of categorising TAS2R is based on the number of ligands they interact with. There are some discrepancies, but most researchers agree to distribute the bitter receptors in 3 to 4 groups: the most promiscuous receptors bind tens of different ligands; highly selective receptors with just a handful or even no ligands known; the receptors that interact with an average number of ligands; and the final group which is somewhat distinct, as here are the receptors which recognise specific motifs [147]. While 26 human receptors have active isoforms, a handful of them are still orphans, meaning there are no known ligands that bind to them. Currently, these receptors are TAS2R19, -42, -45, and -60 (**Figure 3**) [147]. One of the main difficulties in the search for the ligands has been their genetic variability, and the fact that some receptors express various haplotypes, some of them not responding to stimulus. This was the case with TAS2R9 and -41, which were for the longest labelled as orphan receptors, but their ligands were eventually discovered [169,170]. The latest TAS2R which has been confirmed as active and de-orphanized was TAS2R2 [136]. While most TAS2R have been shown to be activated by natural or some synthetic compounds, the majority of these tests have been done in cellular assays, measuring the rise in intracellular Ca2+ levels upon stimulation, leaving uncertain their activity in organisms at non-toxic concentrations. In humans, the most promiscuous receptor is TAS2R14 [171,172]. On the other side, TAS2R3, -5, -8, -9, -13, .41, -49, and -50 are the most specific receptors, with only a couple of known ligands. The rest can be labelled as moderately promiscuous, except for 2 receptors: TAS2R16 and -38, which seem to be

specialised to detect specific structural motifs [147]. Due to their evolutionary development as a protective mechanism, it is not surprising that TAS2R have far fewer antagonists compared to agonists. The main role bitter antagonists have been proposed for is their ability to block bitter sensations triggered by drugs, improving patient compliance. Antagonists for only some TAS2R have been found, and additionally, agonists for some receptors can inhibit the signalling of different TAS2R [173–178].

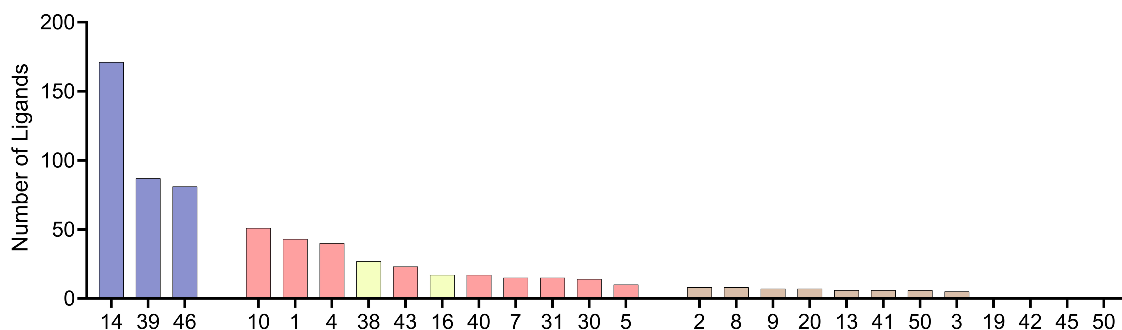

Figure 3: THE PROMISCUITY OF HUMAN TAS2R. The most promiscuous bitter receptor is TAS2R14, which binds to 171 different ligands. On the other hand, 4 TAS2R have for now no known ligands. The colours distinguish between the aforementioned groups depending on the promiscuity of TAS2R. Adapted from [179].

The use of animal models, although not perfect, has been instrumental in expanding our knowledge about bitter receptors. It is important to familiarize ourselves with the nomenclature of bitter receptors in different species. When these receptors were first discovered, the naming was done according to the following convention: T2RX, with X marking a number of the receptors. But soon it was replaced by the now prevalent method: TAS2RX (taste receptor, type 2, member X). A few different nomenclature rules have been introduced to distinguish between the species: first, before the usual “TAS2RX” label, a letter indicating species can be written (h – human, r – rat, m – mouse, gg – chicken, mg – turkey, xt – frog, tg – zebra finch). Additionally, the nomenclature follows the accepted norms of writing, where for humans, all letters are written in upper case (TAS2RX), while for non-human species, only the first letter is written in upper case (Tas2rX). The most prevalent study models for bitter receptor exploration are rodents, thus, to easier distinguish between humans and mice and rats, a special numbering was devised: for humans, bitter receptors have one- or two-digit numbers (1-99), while for rodent bitter receptors are labelled by three-digit numbers (100-199). The mouse genome contains 40 Tas2r transcripts, while in rats, we can find 38 different transcripts [180,181]. Studies investigating the evolutionary pathways of bitter receptors in multiple species identified 19 one-to-one gene orthologs between

mammals and rodents, with more specific research on connections between humans and mice establishing 11 pairs of human and murine bitter receptors [182,183]. The amino acid sequence analysis of human, mouse and rat bitter receptors revealed that most human TAS2R do share at least 50% of their sequence with at least one rodent Tas2r (**Table 1**) [184].

Table 1: LIST OF RODENT ORTHOLOGS AND PROTEIN SEQUENCE IDENTITY. Most human TAS2R have at least one rodent bitter receptor gene ortholog. Some have more than one, and likewise, some human receptors share ortholog genes with multiple other human TAS2R. The aminoacidic identity displayed here is presented only for the identity > 50%. # Although from an evolutionary standpoint, TAS2R39 and mouse Tas2r144 are not orthologs, they do share significant identities in their amino acid sequence. Adapted from [184].

	RAT		MOUSE	
Human	Gene Ortholog	aminoacidic identity %	Gene Ortholog	aminoacidic identity %
TAS2R1	Tas2r119	51.5	Tas2r119	51.7
TAS2R2	*	*	*	*
TAS2R3	Tas2r137	64.2	Tas2r137	63.9
TAS2R4	Tas2r108	64.6	Tas2r108	67.0
TAS2R5	/		/	
TAS2R7	Tas2r130	69.2	Tas2r130	68.3
TAS2R8	/		/	
TAS2R9	/		/	
TAS2R10	Tas2r104	54.3	Tas2r104	55.3
	Tas2r105	55.0	Tas2r105	54.4
	Tas2r106	54.4	Tas2r106	57.4
	Tas2r107	58.2	Tas2r107	56.9
	Tas2r114	55.0	Tas2r114	57.0
TAS2R13	Tas2r13	50.0	Tas2r13	
	Tas2r102	50.7	Tas2r102	51.0
	Tas2r121	56.3	Tas2r121	57.3
	Tas2r124		Tas2r124	
TAS2R14	Tas2r103		Tas2r103	
	Tas2r109		Tas2r109	
	Tas2r110		Tas2r110	
	Tas2r113		Tas2r113	
	Tas2r115		Tas2r115	
	Tas2r116		Tas2r116	51.2
	Tas2r117		Tas2r117	
	Tas2r123		Tas2r123	
	Tas2r125	50.6	Tas2r125	50.6
	Tas2r129		Tas2r129	
	Tas2r140		Tas2r140	
TAS2R16	Tas2r118	52.2	Tas2r118	53.3
	Tas2r134		Tas2r134	
	Tas2r143		Tas2r143	
TAS2R19	Tas2r120		Tas2r120	

	Tas2rl36		Tas2rl36	
TAS2R20	Tas2r120 Tas2rl36		Tas2r120 Tas2rl36	
TAS2R30	Tas2r120 Tas2rl36		Tas2r120 Tas2rl36	
TAS2R31	Tas2r120 Tas2rl36		Tas2r120 Tas2rl36	51.2
TAS2R38	Tas2rl38	65.3	Tas2rl38	65.3
TAS2R39	Tas2rl39	53.2	Tas2rl39 Tas2r144 (#)	55.1 51.3
TAS2R40	Tas2rl44	64.3	Tas2rl44	65.8
TAS2R41	Tas2rl26	69.7	Tas2rl26	68.7
TAS2R42	Tas2rl45		Tas2rl31	
TAS2R43	Tas2r120 Tas2rl36		Tas2r120 Tas2rl36	50.2
TAS2R45	Tas2r120 Tas2rl36		Tas2r120 Tas2rl36	50.2
TAS2R46	Tas2r120 Tas2rl36		Tas2r120 Tas2rl36	
TAS2R50	Tas2r120 Tas2rl36		Tas2r120 Tas2rl36	
TAS2R60	Tas2rl35	57.9	Tas2rl35	58.2

2.5.2. How Bitter Taste Signalling is Detected

The signalling cascade for bitter receptors is highly similar to the signalling pathways of sweet and umami tastes (**Figure 4**). The signalling cascade of TAS2R is that of a typical GPCR: when the ligand successfully establishes the interactions with the amino acid residues, a conformational change in the structure of the protein is induced [185]. The protein that gives its name to this family of receptors, guanine nucleotide-binding protein o G protein, is a heterotrimeric messenger protein which plays a significant role in the signalling cascade. The aforementioned conformational change elicits a response from the accompanying G protein: the dissociation of its subunits, which are known as Gα, Gβ and Gγ [186,187]. The α-subunit contains the all-important guanine sight, where in a non-stimulated state a guanosine 5′-diphosphate (GDP) is located. Upon the ligand binding to the TAS2R, GDP quickly dissociates from its site and is replaced by guanosine 5′-triphosphate (GTP). This exchange is the signal for the following dissociation of the subunits, which separate into 2 parts, the Gα subunit, and Gβγ [188,189]. Subsequently, the signal is transmitted through the cell through these 2 subunits. In the case of bitter signalling, the Gβγ heterodimer finds and activates PLCβ2, which in turn hydrolyses the inner membrane component phosphatidylinositol 4,5-biphosphate (PIP2) into inositol 1,4,5-

triphosphate (IP3) and diacylglycerol (DAG) [190,191]. IP3 travels to the endoplasmic reticulum (ER), where it binds and opens the IP3 receptor, which for bitter translation was discovered to be IP3 receptor3 (IP3R3). The opening of IP3R3 causes the release of Ca2+ from the ER storage, which is also stimulated by DAG. The influx of Ca2+ is a gating signal for transient receptor potential (TRP) melastatin family members 4 and 5 (TRPM4 and TRPM5) [191–193]. Opening of these 2 channels causes an influx of sodium, depolarizing the cellular membrane, creating an action potential through voltage-gated Na+ channels (VGNa+C), resulting in the opening of the large-pore ion channel called calcium homeostasis modulator 1/3 (CALHM1/3) [194,195]. The last step in the signalling cascade that takes place is the secretion of ATP through the opened CALHM1/3 channels.

The second G protein subunit, Gα, is the unit which contains the binding site for GTP, It has been postulated that it also plays a signalling role, however, the consensus has not been reached up to which point it is involved in the signalling cascades. One theory suggests that this subunit acts through cyclic adenosine monophosphate (cAMP) and phosphodiesterase 1A (PDE1A), possibly regulating TRPM channels, which we have seen above [196,197].

It is also worth noting that there is evidence showing activation of bitter taste receptors without the involvement of PLCβ2. For example, the bitter compound denatonium directly modulates ion channels. Nicotine, by binding to its nicotinic acetylcholine receptor also elicits a bitter response, while caffeine modulates the IP3R3 receptor, terminating in the excitation of the cell [198–200].

The number of GPCRs found in humans is in hundredths, thus there is a necessary distinguishing factor to differentiate between the observed stimuli. Apart from different structures and classes of GPCRs, one key distinguishing factor between the receptors is the structure of the associated G protein. In case of bitter receptors, Gαi-2, Gαi-3, Gα14, Gα15, Gαq, Gαs, Gβ1, Gβ3 and Gγ13 have all been reported [197,201]. However, the most significant discovery of taste receptor-associated G proteins was the discovery of α-subunit exclusive for taste receptor-containing cells called α-gustducin (Gαgust) [202–204]. First described in 1992, researchers noticed that its amino acid sequence was highly similar to α-transducin, a G protein that is present in the retinal rods and cones and plays an important role in sight perception [205]. Gαgust presence was observed on the apical membrane, as well as in the cytoplasm, and its importance for taste sensing was confirmed with animal knock-out models, where the lack of this protein resulted in a significant reduction of response to bitter stimulants [206]. However, the knock-out model did not produce total inhibition to bitter stimulants, and it was later discovered that other Gα proteins are present, albeit in lower quantity, in the taste cells, including the homologous α-transducin. It was due to

these similarities and colocalization of these two α-subunits that researchers postulated that α-Gαgust interacts with phosphodiesterase, which was later shown to be PDE1A [197,207].

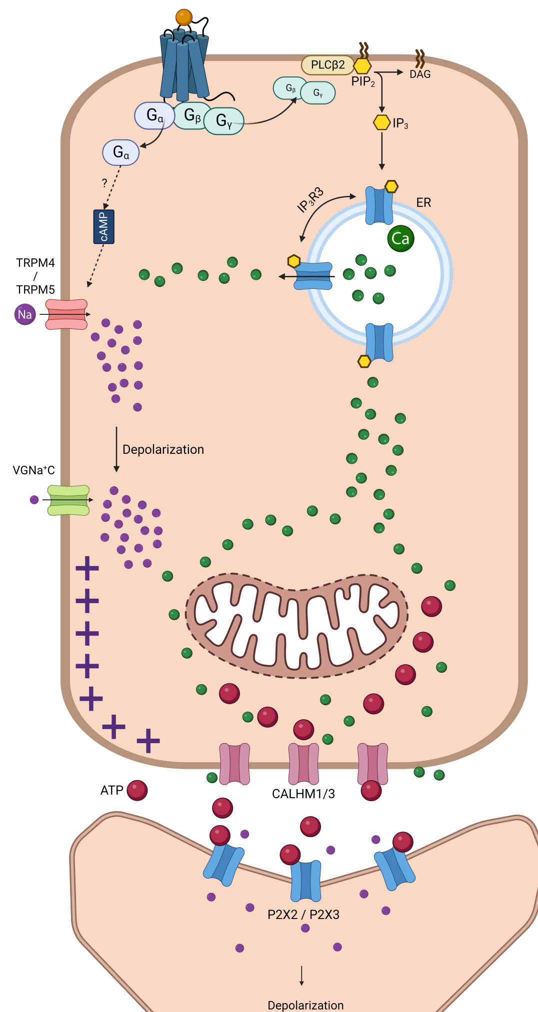

Figure 4: BITTER SIGNALLING. Upon establishing the interactions between the TAS2R and its agonist, the conformational change in the receptor's structure is induced. This in turn allows the dissociation of the G-protein complex, and the separation of Gα and the Gβγ subunits. The latter stimulates the creation of IP3, which activates the secretion of Ca2+ from the endoplasmic reticulum (ER). This triggers the influx of Na+ from the intercellular space, which triggers the creation of action potential. Upon this signal, ATP is secreted into the space between the taste cell and the neuron through CALHM1/3 channels, and ATP finally binds to P2X2/P2X3 channels, which allows the entry of Na+ into the neuron, propagating the signal further down to the gustatory nucleus in the brain. The Gα is presumed to be involved in the signalling as well, through the cAMP-led activation of TRPM channels. Adapted from [208]. Created with BioRender.com

2.6. Afferent Innervation of the Bitter Receptors

Only type III cells of the taste bud display neuron-like characteristics, meaning that those are the only cells that contain direct connections with afferent nerves and the ubiquitous signalling machinery, transducing the signal with the release of synaptic vesicles containing serotonin (5-HT) [209]. Bitter-sensing tastes use a different transmitter: the purinergic ATP. This occurs not through the release of the neurotransmitter vesicles, but through the direct secretion of ATP

through the CALHM1/3. The use of ATP as the signalling molecule might also explain the unusual mitochondria found in type II cells, as their location close to the basal membrane, where afferent nerves can be found, is an ideal location for the production of signalling ATP. The branches of cranial nerves close to gustatory cells present P2X receptors, specifically P2X2 and P2X3, which in their extracellular domain contain binding sights for ATP [210]. The efflux of ATP into the extracellular space and consequent binding to the P2X receptors opens these cation channels, allowing the influx of cations, which then propagates the signal downstream.

2.7. Bitter Taste Receptors in Other Tissues

While their name reveals where they were first discovered, and what is their role, it soon became apparent that the name might hide their true significance. The first inclinations that these receptors might appear in the rest of the tissues came with the discovery of taste-specific signalling proteins in other tissues. Firstly, the signalling molecules that participate in taste sensation, namely Gαgust and structurally similar α-transducin, were found in rat intestinal and pancreatic cells [211,212]. This finding spurred investigations into the presence of GPCR taste receptors themselves, and subsequent studies confirmed their expression in these tissues. Notably, soon it became evident that bitter receptors are found in essentially all major tissue groups: from the brain, the respiratory system, and the GIT to the skin and the reproductive systems [213–217]. It goes without saying that taste perception in these tissues does not occur, thus the reasoning that these receptors are implicated in other pathways was the logical conclusion. And while in some tissues their presence has been partially explained, in others, their roles remain a mystery. For example, TAS2R were found in the blood-cerebrospinal fluid barrier, and while their role has not been characterized, it has been postulated that they are involved in homeostasis and potentially have neuroprotective and anti-inflammatory capacity [218]. Next, there are theories that different members of bitter receptors function as nutrient sensors in the heart tissue [219], with many other tissues investigated as well.

While TAS2R have been detected in a wide range of tissues, the most extensive body of knowledge regarding TAS2R function currently exists in two distinct tissues. These are the respiratory system and the GIT, which is not surprising when we consider that these tissues are the ones most exposed to the environment, where their function as sensory mechanisms is most important and discernible.

The first is the respiratory system, where TAS2R have been discovered in the epithelium of airways, the nasal cavity and the bronchi, as well as in the lung tissue, the smooth muscle of the airways, the pulmonary artery and the lung macrophages. The observed diversity of cell types

expressing TAS2Rs within the respiratory system aligns with their proposed functional repertoire. These receptors have been implicated in processes such as bronchodilation, respiratory innate defence, modulation of susceptibility to respiratory infections, and inhibition of cytokine secretion. Furthermore, emerging evidence suggests their roles in regulating additional signalling pathways [214,220–224].

The other system where the knowledge about the taste receptors has been greatly expanded is the GIT.

3. THE BITTER TASTE RECEPTORS OF THE GUT AND THE REGULATION OF ENTEROHORMONES

The gut represents the biggest area to be in contact with the external environment. It is a complex network of cells, organs, and systems, which all together play important roles in the absorption of nutrients, defence of the organism, and hormonal secretion. TAS2R are one of many chemosensory receptors located in the GIT wall that interact with the ingested nutrients and are important regulators of the body's largest endocrine system.

3.1. Bitter receptors as regulators of enterohormones

Enteroendocrine cells (EECs) scattered throughout the GIT function as the body's endocrine system. These cells produce a diverse array of approximately 30 distinct enterohormones, collectively constituting the enteroendocrine system [225,226]. This complex hormonal network plays a vital role in regulating digestion, nutrient absorption, appetite, and numerous other metabolic processes. Their central role in the regulation of satiety and hunger could potentially make them a valuable tool in the regulation of caloric intake for the purpose of caloric restriction. Unlike traditional endocrine organs with distinct glandular structures, the intestine, despite being the largest endocrine organ in the body, does not resemble the rest of the hormone-secreting organs: it does not follow the typical glandular morphology, but the secreting EECs cells are located all throughout the intestine, where they represent around 1% of epithelial cells of the GIT [227]. Considering their effect on food intake, enterohormones can be broadly classified into two functional categories: orexigenic hormones, which stimulate appetite, and anorexigenic hormones, which promote satiety.

The intestinal bitter taste receptors came to prominence in the late 1990s and early 2000s. This period marked the initial discovery of Gαgust, a key taste signalling molecule, in the intestinal

mucosa of rats [211]. This finding provided the first evidence for a potential taste signalling apparatus in the GIT. A significant advancement in our understanding of TAS2R function in the intestine came when researchers established colocalization of TAS2R transcripts and chromogranin A (ChgA), a specific marker of EECs throughout the gastric and intestinal tissues [228,229]. Soon after, TAS2R were established to appear and have important functions throughout the GIT (**Table 2**).

Table 2: BITTER STIMULATION OF THE GIT: Bitter receptors can be found through the GIT, in a myriad of different cells. Stimulations of these cells with different bitter agonists has been shown to elicit specific responses. Only a couple of specific TAS2R have been confirmed to be involved in the signalling cascades, as most studies use non-specific bitter agonists to elicit a result.

TAS2R whose involvement is known	TAS2R that are activated by bitter agonists which elicit a response	Cells	Role
-5, -10 [230];	-4, -8, -10, -13, -30, -38, -39, -43, -46 [230]	P/D1 cells	Regulation of Ghrelin Secretion
-10, -43 [231];	-1, -4, -8, -10, -13, -30, -38, -39, -43, -46 [232]	Parietal Cells	Secretion of Gastric Acid
/	-3, -4, -7, -8, -10, -13, -30, -39, -43, -46 [233]	Gastric Smooth Muscle cell	Regulation of Gastric Emptying
/	-7, -14 [234]; -4, -8, -10, -13, -30, -38, -39, -43, -46 [235]	Enteroendocrine I Cells	Regulation of CCK secretion
-5 [236];	-4, -7, -10, -14, -31, -39, -40, -43, -46 [237]	Enteroendocrine L Cells	Regulation of PYY and GLP-1 Secretion
/	-4, -7, -10, -14, -16, -31, -39, -40, -43, -46 [238]	Tuft Cells	Regulation of IL-25 Secretion
-10 [239]		Paneth Cells	Regulation of defensins Secretion
/	4, -8, -10, -13, -30, -31, -39, -43, -46 [239]	Goblet Cells	Regulation of mucus protein Secretion
/	-4, -8, -10, -13, -30, -38, -39, -43, -46 [240]	Smooth muscle cells	Regulation of Contractability

3.1.1. Ghrelin and other orexigenic hormones

Ghrelin, the most well-known orexigenic hormone, is secreted by P/D1 cells in the stomach as the prohormone pro-ghrelin. Within these cells, pro-ghrelin undergoes post-translational modifications before its release into the bloodstream. Ghrelin acts on the central nervous system to stimulate the secretion of orexigenic peptides, ultimately increasing hunger and food intake. Additionally, it suppresses insulin secretion and promotes gastric emptying [241]. The ghrelin receptor belongs to the GPCR family, similar to TAS2Rs [242]. In addition to ghrelin, insulin-like peptide 5 (INSL5) and motilin are also considered orexigenic hormones.

The stomach is one of the first organs documented to express TAS2Rs and their associated signalling molecules were documented. A pioneering study by Wu et al. provided early evidence for the presence and functionality of TAS2Rs in gastric tissue. They identified key bitter signalling components, such as Gαgust, in both antral and fundic rat mucosa [243]. Given the specificity of these proteins to taste-sensing cells, the researchers investigated the presence of taste receptors in these tissues. Their analysis revealed transcripts for multiple known rat Tas2rs within the gastric mucosa. These findings were further supported by Janssen et al., who demonstrated colocalization of ghrelin with Gαgust in mice. Moreover, oral administration of a bitter compound mixture to mice resulted in a noticeable increase in plasma ghrelin levels, most likely with Gαgust involvement [244]. Interestingly, the animal model also showed a biphasic effect on food intake. While bitter treatment initially increased food intake within the first 30 minutes, it was followed by a decrease in intake over the next 4 hours. Something similar was observed by our group as well. In the study of Serrano et. al., an interesting dichotomy was seen, as depending on the agonist used, the secretion was either induced or inhibited, while the mix of the compounds had no effect. Likewise, in their animal model, an acute dose of the bitter mixture increased ghrelin levels, whereas a sub-chronic 8-day treatment resulted in decreased ghrelin secretion [245].

Human studies also support the functional role of TAS2Rs in ghrelin regulation. A study by Wang et al. demonstrated the colocalization of at least one TAS2R (TAS2R5) with ghrelin in human primary fundic cultures. Additionally, stimulation with specific bitter compounds (emetine, 1,10-phenanthroline, and chloroquine) increased ghrelin secretion, confirming TAS2R functionality in P/D1 cells of the gastric mucosa [230]. However, the precise mechanisms by which different bitter compounds regulate ghrelin secretion remain a topic of debate. While some studies, like those mentioned above, suggest TAS2Rs stimulate ghrelin release, others report contrasting effects. For instance, studies investigating the treatment of patients with bitter-tasting quinine hydrochloride have shown a decrease in plasma ghrelin levels [246,247].

Regarding the rest of the orexigenic hormones, little is known. While motilin does seem to be lowered by bitter agonists [247], the detailed implication remains to be elucidated.

3.1.2. Anorexigenic hormones

Anorexigenic hormones are released in response to food intake and modulate tissue responses to available nutrients. Glucagon-like peptide-1 (GLP-1), secreted by L-cells in the distal part of the GIT, and gastric inhibitory polypeptide (GIP), produced by K-cells in the duodenum and jejunum, are both prominent anorexigenic hormones [248,249]. Notably, these two

enterohormones are classified as incretins due to their ability to stimulate glucose-dependent insulin secretion [250]. While GIP mainly affects insulin secretion and does not have a direct influence on controlling satiety, GLP-1 does relate signals to the brain, stimulating the satiating effect, acts on the liver, lowers gluconeogenesis, slows down gastric motility and emptying, affects metabolism in muscle and bone tissues, and regulates the process of metabolism [251,252]. Apart from these 2 incretins, the intestinal endocrine system produces a vast array of enterohormones with diverse functions. Peptide YY (PYY), co-secreted with GLP-1 from L-cells, targets pancreatic β-islets, modulates neuropeptide Y (NPY) signalling, and inhibits gastric motility, ultimately contributing to satiety [253]. Cholecystokinin (CCK), released from I-cells primarily located in the duodenum and ileum, regulates gallbladder contraction and enzyme secretion from the pancreas and stomach. These actions contribute to feelings of fullness, although CCK may not directly influence the perception of satiety [254]. Each enterohormone has its specific receptor, through which it exerts its signalling, and these receptors are members of the GPCR family [255–257].

Built on the previously mentioned study of Höfer and Drenckhahn on the presence of Gαgust in the EECs, several studies have emerged exploring the role of TAS2Rs in the small and large intestine, suggesting a more extensive role for these receptors in gut chemosensory functions beyond bitterness [211]. Wu et al. identified Tas2r transcripts not only in the gastric mucosa but also in murine STC-1 EECs and the duodenal mucosa of mice and rats [243]. Notably, their study demonstrated that stimulating STC-1 cells with bitter compounds triggered an increase in intracellular $Ca2+$ levels. While Wu et al. found Tas2r transcripts solely in the duodenum and not in the rest of the segments [147], Rozengurt et al. provided evidence for a broader TAS2R presence. They identified Gαgust, a key bitter taste signalling molecule, localized within colonic L-cells [258]. These EECs are responsible for secreting gut hormones, suggesting a potential role for TAS2Rs in regulating hormone release. Furthermore, Rozengurt et al. identified bitter receptors in two human intestinal cell lines: HuTu-80, derived from the duodenum, and NCI-H716, isolated from the cecum. Functional TAS2R activity was confirmed in these cells by the observed increase in intracellular $Ca2+$ levels upon stimulation with the bitter compound phenylthiocarbamide, highlighting their functionality beyond the stomach [258].

Building on the evidence for TAS2R presence throughout the gut, Chen et al. demonstrated a functional link between bitter taste receptors and gut hormone secretion [235]. Their study showed that stimulating murine STC-1 cells, derived from the small intestine, with the bitter compound denatonium benzoate increased intracellular $Ca2+$ and triggered the release of CCK, a key gut hormone involved in satiety and digestion.

This link between bitter taste and CCK secretion was further confirmed in ex vivo experiments. One study used a purified steroid glycoside from the H. gordonii plant as a bitter stimulant on rat intestines and observed increased CCK release [234]. The same study by Le Neve et al. also demonstrated this secretory effect of the same compound in the human intestinal cell line HuTu-80 [234]. Interestingly, some research suggests a deeper connection between bitter taste and CCK. Studies have shown that Sterol regulatory element-binding protein 2 (SREBP-2) regulates bitter gene expression, and in STC-1 cells, SREBP-2 activation induced CCK secretion [228]. Limited human studies have yielded mixed results. Quinine hydrochloride administration in women showed increased CCK levels, whereas another study using quinine in men found no significant effect on plasma CCK levels [259,260].

Beyond CCK, TAS2Rs appear to influence the secretion of another key gut hormone GLP-1. Studies using human NCI-H716 cells, derived from the cecum, have shown that bitter compounds like quinine and denatonium benzoate stimulate GLP-1 release through a pathway involving Gαgust [261]. This link between TAS2Rs and GLP-1 secretion was further supported by Park et al., who observed a similar response in NCI-H716 cells using a specific TAS2R5 agonist 1,10-phenanthroline [236]. In a murine line, STC-1, a similar profile was observed. Studies using these cells demonstrated that berberine and phenylthiocarbamide, well-known bitter compounds, also triggered GLP-1 secretion via Gαgust [262].

The link between TAS2Rs and GLP-1 secretion extends beyond cell line studies. In human HuTu-80 cells, a specific TAS2R38 agonist, propylthiouracil, stimulated GLP-1 release [263]. This study also identified three additional candidate TAS2R38 activators that triggered GLP-1 secretion in mice. Animal models further support these findings. Kim et al. demonstrated that treating diabetic mice with the bitter compound Cucurbitacin B increased GLP-1 secretion, improved blood sugar control, and enhanced insulin release. This effect was mediated by AMPK, a cellular energy sensor, and involved Gαgust signalling [264]. Another study by Kok et al. investigated KDT501, a specific isohumulone from hops with anti-diabetic properties. In obese mice, KDT501 stimulated GLP-1 secretion through the mouse receptor mTas2r108. Chronic treatment also improved various diabetes and obesity-related parameters [265]. While a human study using KDT501 in prediabetic individuals showed positive effects on triglycerides, the involvement of bitter receptors was not assessed [266]. Adding further weight to this connection, Pham et al. demonstrated that GLP-1 co-localizes with TAS2R38 in intestinal L-cells, suggesting a physical association between the two [263]. Recent research further strengthens the evidence that TAS2R agonists significantly impact GLP-1 secretion, highlighting the potential role of bitter taste receptors in regulating GLP-1 release [237,267].

While the primary focus has been on GLP-1, some studies suggest TAS2Rs might also influence the secretion of PYY. It was observed that in human HuTu-80 cells, PYY co-localized with both GLP-1 and Gαgust, suggesting a potential link between bitter taste signalling and PYY secretion [258]. Further supporting this connection, the same study showed that stimulating NCI-H716 cells with phorbol 12-myristate 13-acetate and denatonium benzoate, a bitter compound, increased PYY secretion [261].

3.2. Bitter receptors in other GIT cells

But EECs represent just a small number of overall cells that can be found in the GIT and are not the only ones where bitter receptors can be observed. For example, they can be found in the chemosensory cells of the intestinal epithelium called tuft cells. These cells form an important part of the immune system in the GIT, as these are the chemosensory machinery that detects parasites, such as helminths and protozoa. Blocking of the TAS2R present in the tuft cells with a bitter taste inhibitor blocks the release of the cytokine interleukin 25 (IL-25) [268]. Bitter receptors have also been observed in the large intestine, more specifically, in the goblet cells, which are mucus-producing and thus have a protective role for the intestinal epithelium [269]. Finally, TAS2R transcripts were also found in the Paneth cells, which are another set of secretory cells located in the GIT, but they do not secrete enterohormones but antimicrobial peptides [270]. While the exact physiological roles of bitter receptors in all these cells have not been determined, their sheer presence is a testament that they have a regulatory role to play and gives credibility to the theory that TAS2Rs play important protective and immune-regulatory roles throughout the GIT.

While it has been known that TAS2Rs are present in different types of cells of the GIT, and the accompanying bitter sensing-related signalling proteins such as Gαgust have been well documented, the exact signalling pathways that activate the TAS2Rs in the GIT remain unconfirmed. However, recent studies showcasing the presence of taste transducers, such as Gαgust, and the effectors (PLCβ2, TRPM4) in the cells of the GIT do shed some light upon the mechanisms involved in extraoral bitter signalling. Most of what we know about bitter signalling in the intestinal cells comes from studies performed on cellular lines, especially on intestinal secretin tumour or STC-1 line. After establishing their presence in these cells [243], it was then discovered that upon bitter stimulation, there was a noticeable increase in the intracellular Ca2+ levels, which is something that was also observed in the type II taste bud cells upon bitter activation. However, in contrast to type II cells, in STC-1 cells this increase of Ca2+ seems to be caused by an influx from the extracellular environment and not ER [271]. It has been proposed

that this influx is mediated through L-type voltage-sensitive Ca2+ channels (VSCCs) [235]. This influx of Ca2+ is believed to be the signal for the EECs to secrete enterohormones. However, the signalling cascade remains poorly known.

Apart from hormonal secretion, TAS2Rs and associated bitter signalling components were identified in human gastric smooth muscle cells. Notably, Avau et al. demonstrated the presence of TAS2Rs in human gastric smooth muscle cells, revealing a direct influence of bitter stimuli on their contractile activity [233]. This finding suggests a potential role for TAS2Rs in gastric motility regulation. Furthermore, TAS2R transcripts were identified in gastric epithelial cells of the corpus/fundus region, as well as in the HGT-1 cell line, a recognized model for studying gastric acid secretion. In vitro studies using HGT-1 cells showed that stimulation with bitter compounds triggered the secretion of gastric acid [232,272]. These findings suggest a broader functional role for TAS2Rs within the stomach, potentially influencing both gastric motility and acid secretion.

4. TAS2R AS TARGETS FOR IMPROVING HOMEOSTASIS AND HEALTHIER AGEING

Just like any other system, ageing affects the bitter perception. Older adults are more likely to suffer malnutrition, due to a multitude of reasons, including loss of appetite and sensory desensitisation [273]. A meta-analysis of different studies found that overall detection thresholds of taste sensations, including bitter perception, are noticeably higher in the aged groups [274,275]. However, what kind of changes occur in detecting bitter perception is still unknown. Takeuchi et al. investigated the electrophysiological properties of taste cells in two different age groups of mice. They found no significant changes in these properties for type II taste cells [276]. While the mechanisms of age-induced change of bitter perception remain unknown, ultimately, there is a likelihood it is caused by multiple reasons, including changes in the taste bud number, the decline of nerves transducing the signals, and changes in the gustatory nucleus.

Despite observing these changes in the oral cavity, changes in the GIT have yet to be assessed. The impact of nutrition and dietary interventions on promoting healthier ageing, the role of the intestine in the regulatory mechanisms of metabolism and nutrient utilization, and the presence of TAS2R in different cells of the GIT imply a potential role of the bitter receptors on the processes that regulate metabolism, and thus potentially have important influence on the homeostatic maintenance [277]. Nutritional interventions are potent and useful ways to promote healthier ageing, however, there is an inherent flaw in their utilization: patient compliance. Application of these interventions requires strict compliance for long periods of time, usually for several weeks,

to have the desired effect. The abundance of food available to us, and the hedonic hyperphagia experienced by many significantly lowers the effectiveness of these interventions [278,279]. Thus, ways of activating the previously described pathways without the strict dietary requirements are in development and are known as caloric restriction mimetics [280].

The majority of these mimetics elicit their effect through the activation of autophagy. One reason why these mimetics have this effect is the regulation of protein acetylation. The process of acetylation is a posttranslational modification that is facilitated by lysine acetyltransferases and regulates the function of multiple metabolic enzymes [281]. Caloric restriction mimetics function mostly through deacetylation of protein, inducing changes in the cellular metabolism that favour the pathways of autophagy [282]. The exact mechanisms are still relatively unknown, but both inhibition of the mTORC1 complex and activation of AMPK pathways are involved, however, non-AMPK induction of autophagy has also been observed [283–285].

At the moment, there are a few compounds that have confirmed caloric restriction mimetic mechanism [286]. Interestingly, many of these compounds that mimic the caloric restriction effect are known bitter agonists. Aspirin, resveratrol, and spermidine have all been investigated for their ability to improve age-associated disorders, while also being known agonists of multiple bitter receptors [287–291].

In recent years, a new way of fighting health issues has emerged, the concept of nutritional interventions using the compounds found in our daily diet.

4.1. Bioactive polyphenols as activators of intestinal TAS2R

Polyphenols are a class of naturally occurring phytochemical compounds found in many plants. This is a vast group of compounds, with thousands of different members of these secondary plant metabolites identified until now [292]. One common characteristic, which gave its name to this family, is the presence of multiple phenol motifs in their structure. This diverse group of compounds can be divided into phenolic acids, flavonoids, stilbenes, and lignans. Flavonoids can be further divided into flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols [293,294]. These compounds share one common structural motif, which is two aromatic rings connected by three-carbon atoms that form an oxygenated heterocycle. This diverse class of compounds can be found in nuts, fruits, vegetables, tea, cocoa, and their products, like wine. Flavonoids can be found in glycoside forms, except for flavanols which can be esterified with gallic acid or in their aglycone form [295].

One polyphenol-rich source is grapes, especially the extract from its seeds called grape seed proanthocyanidin extract (GSPE) [296]. Mostly comprised of flavanols, it has been shown that

these compounds have anti-oxidative, anti-cancerogenic, anti-obesogenic, and anti-inflammatory effects [297–301]. One area where our group has made strides in is the role of GSPE and its regulatory effect on enterohormonal secretion, which we have explored in cellular models, ex-vivo experiments and animal studies. We have seen that the diverse mixture that is GSPE has complex interactions with the enterohormonal secretion. For example, we have seen that ex-vivo rat segments stimulated by GSPE induced secretion of GIP in the duodenum and GLP-1 and PYY in the ileum, while simultaneously decreasing the secretion of CCK in the duodenum [302]. A sub-chronic treatment with GSPE elicited an increased level of GLP-1 in rats, however, this was accompanied by an increase in the ghrelin levels [303]. This multifaceted effect could be partially down to its nature: this extract is a complex mix of polyphenols, primarily of catechin, epicatechin, epigallocatechin and their esters with gallic acid [304]. This mixture could explain the variability in its effect: many of these compounds that are found in GSPE are known bitter ligands [305], however, each individual compound interacts with its own TAS2R [306], meaning that one compound could elicit one reaction upon interacting with a bitter receptor, while another compound could elicit a different or even an opposite reaction through a different receptor.

As mentioned, enterohormones have an important regulatory role in food intake. With the multiple results indicating that GSPE can regulate how these hormones are secreted, it is highly likely that this effect will have a noticeable influence on overall eating habits. This was studied previously by our group, and we have observed a significant reduction in food intake after acute treatment with GSPE [307]. With further experiments reinforcing this observation, it soon became apparent that this effect could be potentially useful as a regulator of food intake and body weight. An 8-day sub-chronic treatment with GSPE investigated the effect of the sub-chronic effect of GSPE, and it was observed that this treatment lowered body weight, reduced food intake, as well as increased energy expenditure [308].

The aforementioned studies observed the food intake limiting effect of GSPE in healthy animals. However, where this GSPE effect could be utilized the most is in pathological conditions where metabolic dysfunction contributes negatively to overall health, as is the case in ageing. To establish if GSPE retains its effect on food intake, and to establish its positive effects on overall health, our group employed a model of cafeteria diet, as this is a well-established model for inducing metabolic dysfunction. Overall, we have demonstrated that GSPE can have beneficial effects on numerous dysfunctions. In animals fed with a cafeteria diet, GSPE was able to reverse the negative effect that this diet had on the secretion of GLP-1 [309]. Moreover, GSPE was shown to have an effective inhibitory effect on dipeptidyl-peptidase 4 (DPP4), which is a mechanism

employed by some type 2 diabetes medications [310]. The association with diabetes, which is a prevalent metabolic disorder and is associated with ageing, could indicate that GSPE possibly can have a promoting effect for healthier ageing. Indeed, our group has observed some of these effects. In an aged rat model (21 months old), we performed a sub-chronic GSPE treatment for 10 days, and after 11 weeks, the animals were sacrificed. During the treatment, the food intake was significantly lower, which was reflected by somewhat lower body weight on the 10th day, and the effect persisted for 8 weeks, most likely due to the observed lower visceral adiposity [311]. In addition, a lower incidence of tumours has been observed as well [311]. And when inspecting the GSPE protective role on the enteroendocrine system, the segment where the effect was most noticeable was the colon [312]. Specifically, GSPE reverted the age-induced downregulation of GLP-1, PYY and ChgA expressions in the colon, while the effect in other intestinal segments was not as significant.

Put together, the positive effect of GSPE on metabolic dysfunctions and regulation of food intake represented an interesting potential utility for improving age-associated disorders. This assumption is further expanded upon when we take into account that many polyphenols are currently being investigated as caloric restriction mimetics. Green tea-extracted epigallocatechin gallate has been shown to have multiple positive effects on age-related disorders, such as lowering inflammation, lowering cholesterol, producing antioxidative effects and having a positive effect on cardiovascular diseases being just some [313]. The same was observed for other polyphenols, such as epicatechin, epigallocatechin, catechin, gallic acid, caffeic acid, curcumin, and many others [286,314,315].

The exact mechanism of most of the bitter compounds remains unknown. However, for a select few there is some information available. Bitter berberine, for example, has been shown to increase the activity of SIRT3, which in turn activates the AMPK pathway, improving the biogenesis of mitochondria and reducing the reactive oxygen species damage [316,317]. Another bitter compound, quercetin, was shown to upregulate the expression of SIRT1, which is another activator of the AMPK pathways [318].

On the whole, polyphenols found in GSPE and other natural extract, have well-documented positive effects on multiple different physiological systems. Their potential role as promotors of healthier ageing is a compelling one, however, the novelty of the area brings with it many uncertainties. For example, how polyphenols elicit their effects, especially as caloric restriction mimetics, remains unknown. Due to their origin and characteristics, strong interaction with TAS2R could provide some insights into the functionality of these compounds. Moreover, if the importance of bitter receptors in eliciting the effect of bioactive compounds is established, these proteins could become a novel way of how we can ameliorate, slow down or even prevent some dysfunctions that we will face as we age.

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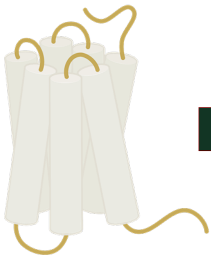
UNIVERSITAT ROVIRA I VIRGILI
TASTE PERCEPTION BEYOND FLAVOUR: BITTER TASTE RECEPTORS IN THE GASTROINTESTINAL TRACT AND THEIR ROLE IN AGEING
Florijan Jalševac

HYPOTHESIS & OBJECTIVES



HYPOTHESIS AND OBJECTIVES

As ever more countries achieve higher levels of development, a clear trend in the increased proportion of older people in the overall population is evident. With this demographic shift, the pressure on society to sustain and provide adequate support to the ageing population will continue to grow. One of the most pressing issues that societies are facing due to this trend, is the increase in the prevalence of age-related issues and diseases, such as cancer, cardiovascular diseases, and neurological disorders. While medical interventions can be adopted for the more severe disorders, delaying, or preventing the onset of the age-associated disorders, is preferable. One lifestyle method that proved to have a beneficial effect on these disorders is caloric restriction. However strict adherence to such a diet has proven to be challenging, thus, caloric restriction mimetics came to the forefront of research.

The bitter taste receptors (TAS2R) have become an interesting centrepiece of investigation in the last decades, owing to their presence in a myriad of different tissues, including the gastrointestinal tract (GIT). It has been shown that these extraoral receptors serve a great deal of distinct roles in all kinds of tissues and can have an important impact on physiological and pathological processes. In the intestine, TAS2R have been observed in different types of intestinal cells, indicating they play a role in the absorptive, protective, and hormone-secreting functions of the GIT. But the importance of intestinal TAS2R and, their specific roles in different physiological processes, such as ageing, has yet to be established.

One of the most abundant sources of bitter ligands is polyphenol extracts of plants and vegetables, such as grape seed proanthocyanidins extract (GSPE). These compounds have anti-oxidative, anti-cancerogenic, and anti-obesogenic effects. Recently, these compounds have been proposed as natural sources of caloric restriction mimetics. However, it remains unclear what is the effect of these compounds on the ageing process itself, and whether they elicit their effects through TAS2R.

Considering the above facts, we **hypothesize that bitter taste receptors may play a role in biological processes such as ageing, and that by using natural bitter compounds, we can modulate the receptors directly in order to have a positive impact on the ageing process itself.**

To demonstrate our hypothesis, we designed the following objectives:

1. **To review the current knowledge on bitter receptors and investigate their presence and abundance throughout the human gastrointestinal tract and explore the changes in these receptors that occur during the ageing process and their relationship with enterohormone secretion.**

   It has been established that bitter taste receptors appear in a wide range of extraoral locations and that they serve a certain role in individual tissues. However, overall knowledge of the receptors and the exact map of expression throughout the gastrointestinal tract, the impact of biological factors such as gender, and the role of ageing on the expression of these receptors has yet to be described. Hence, we aimed to inspect the overall literature that exists, create a complete picture of how bitter taste receptors are expressed in distinct parts of the gastrointestinal tract, explore the changes due to ageing, and investigate their role in the modulation of metabolism, due to their effects on some enterohormone secretions.

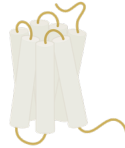
2. **To establish important connections between the human gastrointestinal bitter taste receptors and the ageing process itself, by characterizing the changes taking place during ageing, and exploring the network of global parameters with the bitter receptors.**

   It has been well established that during the ageing process, the human body experiences a wide range of changes in basically all the physiological systems, which can lead to different health issues. As bitter receptors are important sensors present in the body, they have a key role not just locally where they are present, but further down the stream as well. Hence, we decided to explore the intricate connection between the bitter taste receptors, and the global plasma parameters, to establish possible effects these receptors have on the changes that occur during the ageing process.

3. **To analyse the changes induced in the expression levels of the bitter taste receptors produced by the subchronic treatments of female rats by the GSPE extract and to establish which component of the extract could be responsible for the elicited effect.**

   It has been shown that GSPE, a natural extract, has a myriad of different positive effects on humans. GSPE is a complex mixture of different compounds, with monomeric, dimeric, and oligomeric structures being present, some of them proved as ligands for bitter receptors. Their positive effects on overall health and ageing have been observed, and some have long-lasting effects. However, how this more chronic treatment affects the profile of the chemosensory mechanisms in the GIT remains unreported. Hence, we decided to investigate the effect of a subchronic treatment with GSPE on the intestinal TAS2R and tried to establish which component of GSPE is responsible for the observed effect.

RESULTS



Part 1:

Study on the Current Insights of the Bitter Taste Receptors, Analysis of Their Presence in the Gastrointestinal Tract and the Description of Their Evolution With Ageing

Manuscript 1:

The Hidden One: What We Know About Bitter Taste Receptor 39

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Manuscript 2:

Profiling Bitter Taste Receptors (TAS2R) along the Gastrointestinal Tract and their Influence on Enterohormone Secretion. Gender- and Age-Related Effects in the Colon

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The Hidden One: What We Know About Bitter Taste Receptor 39

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Over thousands of years of evolution, animals have developed many ways to protect themselves. One of the most protective ways to avoid disease is to prevent the absorption of harmful components. This protective function is a basic role of bitter taste receptors (TAS2Rs), a G protein-coupled receptor family, whose presence in extraoral tissues has intrigued many researchers. In humans, there are 25 TAS2Rs, and although we know a great deal about some of them, others are still shrouded in mystery. One in this latter category is bitter taste receptor 39 (TAS2R39). Besides the oral cavity, it has also been found in the gastrointestinal tract and the respiratory, nervous and reproductive systems. TAS2R39 is a relatively non-selective receptor, which means that it can be activated by a range of mostly plant-derived compounds such as theaflavins, catechins and isoflavones. On the other hand, few antagonists for this receptor are available, since only some flavones have antagonistic properties (all of them detailed in the document). The primary role of TAS2R39 is to sense the bitter components of food and protect the organism from harmful compounds. There is also some indication that this bitter taste receptor regulates enterohormones and in turn, regulates food intake. In the respiratory system, it may be involved in the congestion process of allergic rhinitis and may stimulate inflammatory cytokines. However, more thorough research is needed to determine the precise role of TAS2R39 in these and other tissues.

Keywords: TAS2R39, bitter taste, catechin, GPCR, food intake, respiratory system, TAS2R39 agonist, TAS2R39 antagonist

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INTRODUCTION

Humans have five primary tastes: bitter, sweet, sour, salty, and umami (1), which are identified as membrane receptors. These receptors enable animals to analyze the chemical structure of food as soon as it hits the taste buds located on their tongue before any harmful components are digested. Although on the surface the role of these receptors may look the same, they are anything but alike. Though ubiquitous to us all, sensing salty taste remained a mystery until recently when researchers identified specialized cells that recognize this taste through the sodium ion channel (2). Other types of ion channels are used to detect the sour components of food (3), while the other three tastes are mediated differently, i.e. through specialized G protein-coupled receptors (GPCRs) known as taste receptors (TASRs) (4). Although these receptors sense different compounds, they share several structural and functional attributes. These glycoprotein receptors consist of seven transmembrane

α-helices. Upon binding of a ligand, interaction with heterotrimeric G proteins occurs, which activates the propagation of signaling cascade in the cell (5). To identify umami, sweet and bitter tastes, two families of GPCRs have been identified: TAS1R and TAS2R. The first family of receptors contains three members: TAS1R1, TAS1R2 and TAS1R3 (6). This family is responsible for detecting both sweet and umami tastes. This is done by dimerizing different members into one receptor: the TAS1R1-TAS1R3 heterodimer is responsible for umami, while TAS1R2-TAS1R3 complex is responsible for sweet (7). On the other hand, the TAS2R family is made up of a much more diverse group of receptors, which in humans consists of 25 functional genes, as well as 8 pseudogenes, though the number varies greatly from species to species (8, 9). Researchers have also discovered that although we possess a relatively low number of bitter taste receptors, we are able to detect as bitter thousands of compounds from a wide range of families. We can categorize bitter receptors into four groups based on their specificity to ligands: receptors that detect a broad spectrum of ligands; more selective receptors with just a few ligands; receptors somewhere between these two groups; and receptors that recognize specific chemical motifs (10). One member of the group that detects a moderate number of bitter compounds is TAS2R39, which is the focal point of this review.

TAS2R39 EXPRESSION IN THE BODY

TAS2R39 is encoded by the *TAS2R39* gene, which is located on chromosome 7 (7q34). Interestingly, this gene sequence contains no intron, so it belongs to a small group of genes that are known as intron-less or single-exon genes (11). This bitter taste receptor is still relatively unknown, since the first ligands for it were discovered just over a decade ago (4). Another area where more concrete information is lacking are the locations where it is expressed. As with other members of the bitter-taste receptor family, TAS2R39 is expressed in the oral tissue. The little information available about its expression indicates that it is one of the TAS2Rs that are expressed in the lowest quantities (12). The extraoral presence of bitter taste receptors has been well documented. These can be found in several types of tissues, including the brain, the respiratory system, the cardiac system, and even the reproductive system (6, 13–15). However, discrepancies exist among these receptors since some are more well-known, while others remain unexplored. Studies exploring the expression of the *TAS2R39* gene have identified TAS2R39 in the colon (16), bronchi (17), nasal mucosa (14), arteries (18), and skin (19). Additionally, cell lines used in research have been identified as expressing this gene, including the HuTu-80 and NCI-H716 intestinal cells (16), the hTERT-HM myometrial cells (20), and lung macrophages (21), while the protein has been detected in the cells of choroid plexus of the nervous system (22). Further data are constantly being added to databases such as the Human Protein Atlas, while new locations for TAS2R39 expression are being discovered, including the pancreas and spleen, and even the brain, testes and ovaries (11, 23). Although TAS2R39 appears to be widely present in human tissues, it is important to note that in all these tissues its gene expression is low and, that, as is reflected by the relatively little knowledge we have about this bitter taste receptor, detecting it is a challenge.

STRUCTURE AND SIGNAL TRANSDUCTION

TAS2R39 is a 338 amino acid long protein that, as we mentioned earlier, has seven transmembrane domains (numbered from I to VII). Based on computer analysis using modelling software, a structure-based pharmacophore model was created (24). This model suggests that the binding pocket of a TAS2R39 ligand is located extracellularly between transmembrane helices III, V, VI and VII, with a hydrophobic interaction between the ligand and receptor that covers most of the interaction area. Additionally, hydrogen bond acceptors and donors contribute to the binding and, potentially, π-π aromatic influences may also occur in the binding pocket. The structural differences between agonists and antagonists have also been inspected. Although at first sight they are quite similar, two key differences have been identified: as well as lacking a hydrogen donor, antagonists display stereochemical flexibility, which fills the binding pocket and, in this way, prevent a conformational change in the receptor upon its activation (24). Another study compared ligands that activate TAS2R14 and TAS2R39 by analyzing the structure-activity relationship. In that study it was observed that glycosylation had an inhibiting effect on TAS2R14, while activation of the TAS2R39 was preserved (though a higher concentration of agonists was needed to activate the receptor). The compounds tested were members of the isoflavonoid group. Alteration of the C-ring skeletal structure of these compounds did not hinder the activation of these two receptors, though their potency and efficacy may be affected. Also inspected was the effect of different substituents on the binding properties. It was found that, for a compound to be a TAS2R39 ligand, substitutes, preferably a hydroxy group, are obligatory (25).

All elements of the signaling cascade of the TAS2R signaling pathway remain unknown. However, researchers have discovered the importance of gustducin as a member of the G-coupled protein mechanism (26, 27). This is even more evident from the fact that TAS2Rs are expressed in cells containing gustducin (28, 29). The perception of taste starts on the tongue in the taste organ, i.e. the taste bud (30). Located throughout the oral cavity, these buds contain between 50 and 100 taste cells (31). Each of the five basic tastes is sensed by specific cells. Cells that detect bitter taste are called type-II cells, which can also sense sweet and umami tastes. Which taste they detect depends on the receptor that is expressed (32). Although it has not been confirmed, we can postulate that the signaling pathway in the oral cavity is similar in the extraoral locations. When a bitter ligand binds to the receptor, a conformational change is induced. This causes the dissociation of α-gustducin from the β and γ subunits of the G protein-coupled unit. The β and γ subunits of this protein then activate the β2 isoform of phospholipase C (PLCβ2), which in turn leads to the synthesis of inositol 1,4,5-triphosphate (IP3) and

diacylglycerol (DAG). IP3 binds to IP3 receptor, resulting in the release of Ca^{2+} from the endoplasmic reticulum. This change in the intracellular concentration of ions induces the activation of membrane ion channels, which transport Na^+ into the cells, thereby depolarizing the membrane. Once action potential is reached, neurotransmitter ATP is released from the cells, with the signal propagating forwards through afferent nerves (33, 34). However, this is not the only way the signaling pathway is activated. Upon ligand binding, another pathway is activated: the α-gustducin subunit stimulates taste phosphodiesterase (PDE), which hydrolyses cAMP. What happens then is not known but some theories suggest that this may disinhibit cyclic nucleotide-inhibited channels and in turn elevate the intracellular levels of Ca^{2+}, which again results in the exocytosis of neurotransmitters (**Figure 1**) (33, 35).

LIGANDS

For a long time, TAS2R39 was considered an orphan receptor, i.e. there were no known ligands that activated it. Recently, however, interest in this receptor has increased and, in 2009, its first ligands were discovered (10). These first ligands discovered were agonists, and most compounds were extracted or derived from plants. Various groups of TAS2R39 ligands also appeared, some of which were more specific, while others also bound to other bitter taste receptors. More studies exploring possible ligands, the most prevalent source of which were again plants, were then conducted. With theaflavins extracted from black tea, TAS2R39 shows a preference for theaflavin and theaflavin-3,3-O'-digalatte, while theaflavin-3-O'-galatte also activated TAS2R14 (36). Catechins derived from green tea are another group of ligands for this bitter taste receptor, with epicatechin gallate and epigallocatechin gallate demonstrating relatively strong affinity to TAS2R39, and epicatechin and epigallocatechin displaying lower affinity. These data show that the galloyl group creates important bonds for the ligand-receptor interaction. Note that these catechins also bind to other bitter receptors, and the only one that seems to be selective for the TAS2R39 is epigallocatechin (37). Soybean is another plant from which such compounds can be extracted. Genistein, a predominant isoflavone in soybeans, activated both TAS2R39 and TAS2R14, which is becoming TAS2R39's closest relative in terms of the ligands they both bind to. However, glucosylated forms of genistein do not activate TAS2R14, though they did activate TAS2R39. Further experiments with similar isoflavones were conducted and 15 other TAS2R39 agonists were discovered, with acetylgenistin, genistin, glycitin and malonyl genistin being specific to TAS2R39 (25). A more comprehensive study was conducted in which 67 flavonoid and isoflavonoid compounds were found to be ligands for this receptor, with new specific ligands being acacetin, 5,2′-dihydroxyflavone, gardenin A, genkwanin gossypetin, 6-methoxyflavonol and 4′-hydroxyflavanone (38). A recent study also noted the activation of this bitter taste receptor by vanillin, a major component of vanilla widely used as a flavouring agent (39).

Small molecules are not the only ones that can activate this bitter taste receptor, as some peptides have been also identified as TAS2R39 agonists. Amino acids, especially those exhibiting

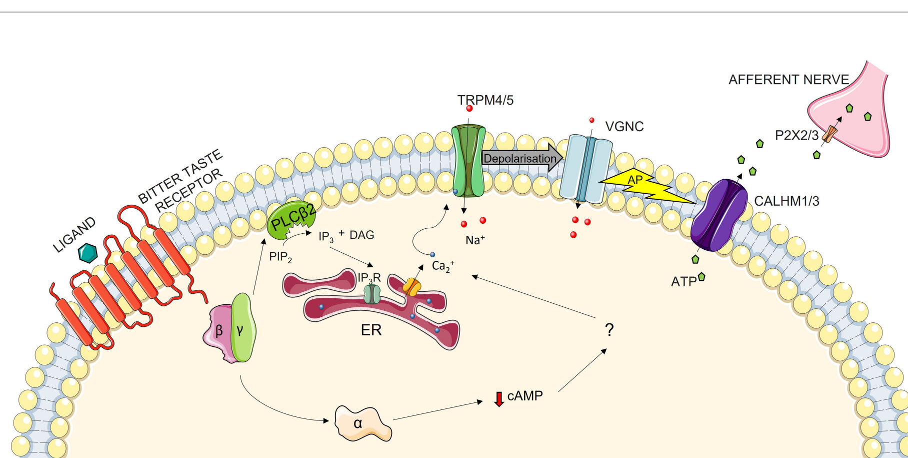

FIGURE 1 | The Bitter Taste Receptor Signaling Pathway: when the ligand binds to the TAS2R, a conformational change is induced. This in turn triggers the dissociation of the α-subunit of gustducin from the β- and γ-subunits. This dissociation of subunits signifies two divergent pathways of signal transduction. In the first of these, β- and γ-subunits activate the β2 isoform of phospholipase C (PLCβ2), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 then travels to Endoplasmic Reticulum (ER), where it binds to its receptor (IP_3R), leading to the secretion of Ca_2^+ from the ER into the cytoplasm. This increase in intracellular Ca_2^+ levels leads to the activation of sodium-selective transmembrane transporters TRPM4 and 5, thereby depolarizing the cellular membrane. Depolarization activates voltage-gated sodium channels (VGNC), thus hastening depolarization. When the action potential (AP) is reached, the calcium homeostasis modulator 1 and 3 (CALHM1/3) channel and pannexin 1 channels are activated, which leads to the transportation of ATP from cytoplasm to the intercellular space. Through P2X ionotropic purinergic receptors 2 and 3 (P2X2/P2X3), ATP is taken into the afferent nerve, thereby propagating the signal further down. The other pathway occurs through the α-subunit of gustducin, but the exact mechanism of signal propagation is unknown. It is postulated that α-gustducin lowers the level of cAMP by activating its hydrolysation though phosphodiesterase (PDE), but the next steps are still unknown. The lowering of cAMP may lead to a decrease in the levels of cNMPs intracellularly, which may regulate protein kinases and in turn regulate the ion activity in the cell. It is also possible that cNMP directly regulates cNMP-gated ion channels, thereby depolarizing the membrane and eliciting the release of the neurotransmitter.

more hydrophobic properties, show greater affinity to the receptor, since hydrophobicity is one of the major factors of ligand-bitter taste receptor interaction (40). A broad study testing all proteinogenic amino acids on all bitter taste receptors uncovered more details about the amino acid activation of bitter taste receptors. It was discovered that the most sensitive bitter taste receptors for amino acids are TAS2R1, TAS2R4, and TAS2R39, since these were activated by phenylalanine (Phe) and tryptophan (Trp). Curiously, it was observed that TAS2R39 was activated by the D-conformation amino acids, such as D-Trp. Note that these agonists expressed low levels of receptor activation. In the next stage of the study, dipeptides and tripeptides from the previously mentioned amino acids were examined. Interestingly, TAS2R39 was activated by Trp-Trp and Leu-Trp dipeptides but not by other Trp-containing combinations (like Trp-Leu), which indicates that the interaction between these kinds of ligands and receptors cannot be described solely by the presence or position of the activating amino acid in a dipeptide. Of the tested tripeptides, Trp-Trp-Trp and Leu-Leu-Leu both activated TAS2R39 (41). Finally, larger peptides found in the cheese-maturation process also activated TAS2R39 as well as TAS2R1.

Although most ligands now discovered act as agonists of the TAS2R39, some studies also identified antagonists for this receptor (42). Like many agonists, specific blockers for these bitter taste receptors come from the flavanone group of compounds. Two of the 14 screened flavanones 6,3'-dimethoxyflavanone and 4'-fluoro-6-methoxyflavanone showed clear inhibiting tendencies against TAS2R39, while 6-methoxyflavanone displayed a less inhibiting tendency. Researchers also tested the specificity of these ligands to TAS2R39 by comparing this bitter taste receptor with the other receptor that binds many of the flavonoids, i.e. TAS2R14. Here an inhibition was also observed but to a lesser extent than with TAS2R39. Structural analysis of these inhibitors showed that for a ligand to act as an antagonist on this bitter taste receptor, a methoxy group on position 6 of the A ring is mandatory, as is the absence of a double bond in the C ring of the structure (42). All currently known TAS2R39 exclusive receptor ligands can be found in the **Table 1**, while all known TAS2R39 ligands are listed in the **Supplementary Table S1**.

What is evident from the current data is that since studies of these bitter taste receptors are still ongoing and the first specific ligands were discovered only a decade ago, we still have much to learn about the interactions between the receptor and its ligands and about how to define more ligands, specific agonists, and antagonists. More specific ligands on TAS2R39 could help us discover more specific properties of this bitter taste receptor and clarify its physiological and potential pathophysiological roles.

PHYSIOLOGICAL ROLE

Animal models, especially rat and mouse, are key to understanding protein function in humans when there are orthologues for the protein under study. It is not surprising, therefore, that, apart from in humans, our knowledge of bitter taste receptors in these two species is the broadest. Roughly 35 functional genes have been discovered in mice (45) and roughly the same number have been discovered in rats (46). However, the mystery surrounding bitter taste receptors in these animals remains. Although some have been deorphanized (47), there is much more that we do not know. We can see that – like in humans – rats and mice possess different groups of TAS2Rs, with some responding to a broad range of ligands and others being tuned more specifically. Also, just as we observed the expression of taste receptors in a myriad of human tissues, the same is true for these rodents: bitter receptors were detected, for example, in the nasal mucosa (14), vascular system

TABLE 1 | Currently described as exclusive hTAS2R39 ligands.

Ligand	Effective concentration (μM)	EC50 (μM)	Source
AGONISTS			
4'-hydroxyflavone	500	nd	(38)
5, 2'-dihydroxyflavone	500	nd	(38)
5-hydroxyflavone	500	nd	(38)
Acetylgenistin	125	nd	(38)
Epigallocatechin*	nd	395.5	(37, 43)
Fisetin	1	nd	(38)
Genistin	500	nd	(25)
Genkwanin	500	nd	(38)
Glycitin	500	nd	(25)
Gossypetin	250	388	(38)
Malonylgenistin	500	nd	(25)
Paracetamol*	3000	nd	(10)
Prolylarginine	10000	nd	(44)
Theaflavin*	nd	2.79	(36)
Theaflavin- 3, 3'-O-digallate*	nd	1.55	(36)
Tricetin	250	nd	(38)
ANTAGONISTS			
4'-fluoro-6-methoxyflavanone	nd	102	(42)
6, 3'-dimethoxyflavanone	nd	407	(42)

**These are the ligands that have been tested on all 25 human bitter taste receptors and have activated only TAS2R39 receptor. nd, not determined.*

(48), and intestinal system (49) of rats, and probably also appear in many other tissues.

Although their name appears to suggest that they have a simple assignment, bitter taste receptors are involved in numerous biological processes and are therefore an important component of homeostasis. As stated earlier, their primary function is, in conjunction with other taste receptors, to sense the nutritional constituents of ingested food, but they also have a specific role in sensing the presence of toxic components such as plant alkaloids to prevent their ingestion before they harm the organism (15). However, not all bitter compounds are dangerous and some are beneficial to our health (50). Moreover, many common foods and beverages, such as cocoa beans, coffee and beer, are bitter, and we still enjoy their taste. As well as taste sensing, numerous studies have analyzed the function of this receptor in other tissues and systems. There is reliable evidence that bitter taste receptors are involved in glucose homeostasis (51). A possible role of various bitter taste receptors (including the ortholog to human TAS2R39, TAS2R139) in the control of food intake has been studied in the gastrointestinal system of rats (52). Bitter compounds and, in turn, TAS2Rs, have been linked with controlling the secretion of ghrelin, a hormone known as the hunger hormone, which, when released, increases food intake (52). Protein YY (PYY), glucagon-like peptide 1 (GLP-1) and cholecystokinin (CCK) have also been identified as enterohormones that control the appetite (53) and can be influenced by bitter compounds. It was discovered that by activating certain TAS2Rs, such as TAS2R5, GLP-1 secretion can be increased, thus leading to a decrease in food intake in rats. However, when TAS2R39 was targeted specifically, PYY secretion increased with no influence on food intake. Also, when a combination of ligands that bind to other bitter taste receptors but are preferential to TAS2R39 was tested, an increase in food intake was noted (54).

Another area where bitter taste receptors are important is the respiratory system. Their presence in this system has been well documented (55), while it is suggested that taste receptors there are important components of the innate immune system. TAS2R39 has been detected in the human bronchi but it is unlikely to be involved in bronchial relaxation since the ligands that bind to it produced no effects (17). However, once non-specific agonists were used, bronchial relaxation was observed, so the role of other bitter taste receptors cannot be ignored. The importance of TAS2Rs in the respiratory system came out of a study that inspected nasal mucosa in patients suffering from allergies. Both healthy individuals and allergic rhinitis patients were included in the study, which showed that bitter taste receptors, including TAS2R39, are ubiquitously present in nasal tissue and that their expression is increased in allergic patients. Further experiments revealed that TAS2R39 expression in this type of tissue increases when stimulated by certain cytokines, namely IL-3, IL-5, IL-10 and TGF-β. Whereas interleukins induced the expression of several bitter taste receptors, TGF-β up-regulated only TAS2R39 (14). Moreover, stimulation of the nasal mucosa by various bitter compounds triggered constriction of the mucosa in patients, while in rats, vascular constriction of the mucosa was observed. This suggests that bitter taste receptors may have an important role in constriction of the nasal mucosa, thus improving congestion and controlling allergic rhinitis. However, as some bitter taste receptors (including TAS2R39) were up-regulated by inflammatory cytokines, it may be that these receptors also participate in the pathogenesis of inflammatory diseases. These conflicting results reinforce the need for more thorough studies to decipher the role of these receptors in the homeostasis of organisms.

CONCLUSIONS

As this is a fairly novel topic of research, the significance of TAS2R39 is yet to be determined. Though present in extraoral locations, it is yet to be fully determined in which specific tissues and at what levels it is present. A more detailed future analysis of the signaling pathway could enable us to better understand its role in extraoral tissues and in turn make it easier to modulate it. Primary research exploring its role in the control of food intake is promising, though more concrete studies are needed to decipher its role in the control of satiety. Its role in the respiratory system also remains unclear since a possible preventive role in congestion seems to be at odds with its possible pro-inflammatory effect. These discrepancies with regard to TAS2R39 show us that without further research, this receptor will remain the hidden one.

AUTHOR CONTRIBUTIONS

AA and MP conceived the idea and drafted a proposal. FJ reviewed the literature and scripted the basis of the manuscript. ER-G and RB-D elaborated the figures and tables. MB and XT critically reviewed the manuscript. All the authors reviewed the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.854718/full#supplementary-material

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Profiling Bitter Taste Receptors (TAS2R) along the Gastrointestinal Tract and their Influence on Enterohormone Secretion. Gender- and Age-Related Effects in the Colon

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Abstract

Extraoral bitter taste receptors offer intriguing potential for modulating metabolism and the gut-brain axis through dietary interventions. Our understanding of these receptors is limited, and data on their effects on ageing are scarce. The complexity conveyed by their high diversity, low expression levels and species-dependent variability challenges our comprehension. We used real-time PCR to examine the relative abundance of multiple TAS2R across different segments of gastrointestinal mucosa in four human cohorts and related them to enteroendocrine secretions at the colon site. TAS2R14 exhibited the highest expression levels in all analysed tissues. In contrast, TAS2R39, -38 and -42 consistently exhibited lower expression levels. Ageing was found to upregulate TAS2R4, -5, -13, -20 and GLP-1 mRNA in the descending colon. Stimulating TAS2R14 in Hutu-80 cells induced GLP-1 secretion, while stimulating TAS2R5 modulated GLP-1 and PYY secretion. Given the modifications TAS2R agonists may undergo along the GIT, as well as the distinctive expression patterns and possible functional roles of TAS2R receptors along the intestinal tract, our findings suggest the viability of a targeted strategy aimed at enhancing specific functions to improve health outcomes. This study offers valuable insights into the intricate interplay between bitter taste receptors, gut physiology and potential dietary interventions.

Keywords: Bitter taste receptors (TAS2R); human gastrointestinal tract; enterohormones; ageing; colon

1. Introduction

Our organism is profoundly influenced by the composition of our diet, since the foods we consume provide the building blocks for body formation and maintenance and the energy required for numerous physiological processes. Food components also convey information to our bodies about the characteristics and quality of our meals. Though gut-brain communication is well established (1), it is important to also recognise that our gut communicates with our whole body. The gastrointestinal tract (GIT) wall contains numerous proteins that participate in these functions (2). In the past decade, a new group of membrane receptors, known as taste receptors (TASR), has been identified (3). Initially, taste receptors were primarily associated with the tongue, but subsequent research has revealed their presence in several other tissues (4). One location where these extraoral receptors appear is the GIT, where TASR pose as candidates for playing a key role in the detection of food components. The largest group of taste receptors are the bitter taste receptors (TAS2R). Indeed, recent data indicate that there are 26 functional variants of TAS2R in humans [5]. As we can see from the Human Protein Atlas (http://www.proteinatlas.org), the expression levels of extraoral TAS2R tend to be lower than those of other receptors. However, when their stimulation was analysed by Meyerhoff et al. (5,6), they found that the effective concentration (EC50) for their ligands is typically in the micromolar range. Considering the movement of food components through the gastrointestinal tract from the mouth to the anus, it is therefore reasonable to consider that the TAS2R present in different cells of the intestinal wall may be stimulated by effective concentrations of ingested compounds or products of food components that have been modified along the GIT.

It is important to mention that the characteristics of bitter taste receptors vary between species. Moreover, if we compare individual species, we find that each has a larger or smaller range of different TAS2R in which, though certain similarities can be found, species-specific characteristics are also present, thus making their story more fascinating (7). The small structural but impactful differences between different TAS2R mean that they exhibit different abilities to

bind different compounds, as some show high levels of promiscuity, binding to a variety of compounds, while others show higher selectivity (8). These diverse characteristics make them highly valuable assets for precise taste perception on the tongue. It could therefore be speculated that the presence of diverse TAS2R at other locations may contribute to their specific functions there, though the role of extraoral TAS2R is not yet fully understood (9). Some studies indicate that TAS2R can modulate energy expenditure (EE) (10,11) and it is suggested that they are involved in the inflammatory intestinal functions (12–14). Furthermore, it has been shown that specific genetic modifications of different taste receptors are important factor in the development of obesity (15). Finally, procedures such as bariatric surgery result in changes in the taste receptors in the lingual taste buds, but also further down the gastrointestinal tract (16).

The gut communicates, among other mechanisms, by secreting diverse range of enterohormones along all its length that allows brain-gut communication. The stimulation of TAS2R has been shown to lead to the enterohormone secretion as summarized by Tuzim et al. (17). Since enterohormones such as GLP-1, PYY, GIP and ghrelin are key regulators of food intake and related metabolic processes, it is reasonable to consider that the TAS2R are key targets for the regulation of whole-body homeostasis.

For a more thorough understanding of the significance of TAS2R receptors throughout the GIT and their potential role in mediating the communication between our diet and our body, it is therefore essential to investigate their distribution along the entire GIT, their specific functions at each location, and the factors that modulate their expression and activity. Knowledge is currently limited regarding how the functionality of these receptors is influenced by potential gender-specific effects, ageing (18), modifications in the context of obesity (19), and other relevant factors. Further studies are therefore needed to assess how these factors influence TAS2R function.

In this study we present data on the relative abundance of several TAS2R at three human mucosal locations. Our aim for this study was to establish a profile of distribution of GIT located TAS2R and to ascertain their role as enterohormone regulators, establishing these receptors as potential targets for interventions. This analysis enables us to examine the different sensitivities to bitter sensing at these sites. More specifically, we focus on the colonic site and investigate the influence of variations in gender, age and location in this particular section of the GIT. We also explore the potential relationship between the presence of TAS2R in the colon and the key enterohormones present in this region.

2. Materials and Methods

2.1. Biopsy sample collections

Included in this study were four distinct groups of patients. The exclusion criteria are summarized in Supplementary data (Supplementary Appendix A1). Samples of human cheek mucosa were kindly provided by Joan XXIII University Hospital (Tarragona, Spain). Healthy mouth mucosa samples were collected from male patients (n=25, age=65.2 ± 10.7) diagnosed with head and neck cancer. The tissues were stabilised in RNAlater preservative (Qiagen, Hilden, Germany) and stored at -80°C. This experimental procedure was approved by the Clinical Research Ethics Committee (CEIC) of Joan XXIII University Hospital in Tarragona (PI15/02047).

Jejunum samples were kindly provided by GEMMAIR from IISPV and Joan XXIII University Hospital (Tarragona, Spain). Jejunal biopsy samples were collected from women with obesity (BMI > 40 kg/m2) who had undergone a bariatric Roux-en-Y gastric bypass (RYGB) procedure (n=21, age=46.1 ± 11). The tissues were stabilised in RNAlater preservative (Qiagen, Hilden, Germany) and stored at -80°C. This experimental procedure was approved by the Clinical Research Ethics Committee (CEIC) of Joan XXIII University Hospital in Tarragona (23c/2015).

Mucosal biopsy samples from the ascending and the descending colon were kindly provided by the Hospital Clínic (Barcelona, Spain). Colon biopsy samples were collected from healthy men

(n=23) and women (n=25) who had undergone colon cancer screening procedure. The tissues were stabilised in RNAprotect (Cat. No.: 76104, Qiagen, Hilden, Germany) and stored at -80°C. Plasma samples were also obtained. Subjects were divided into two age groups (one younger, age=38.9 ± 6; and one older, age=63.6 ± 6). This experimental procedure was approved by the Drug Research Ethics Committee of Hospital Clínic de Barcelona (HCB/2019/1115).

Another set of colon samples was kindly provided by Joan XXIII University Hospital (Tarragona, Spain). The tissues were collected from patients (12 male and 5 female) with pathologically confirmed colorectal carcinoma (age=63.4 ± 3) who had undergone colon surgery. Non-diseased tissues were excised from both the ascending colon (n=8) and the descending colon (n=9). This experimental procedure was approved by the Clinical Research Ethics Committee (CEIC) of Joan XXIII University Hospital in Tarragona (CEIm 101/2017).

The potential influence of different medications prescribed to the patients has been assessed. No medication was intestine-specific, and just a handful of patients were prescribed proton pump inhibitors for the control of stomach acid (supplementary Table S1). All participants were informed before they provided their written consent to participate in their part of the study.

2.2. Colon segment enterohormone basal secretion analysis

After resection, the colon tissues obtained from Joan XXIII University Hospital were transferred to the laboratory within 30 minutes in ice-cold KRB-D-Mannitol buffer (pH 7.4) saturated with 95% O2 and 5% CO2. The tissues were rinsed, and the serosa and outer muscular layers were removed. After a 10-minute washing period, tissue segments were placed in pre-warmed KRB-D-Glucose buffer 0.1% DMSO with protease inhibitors. The samples were treated for 30 minutes in a humidified incubator (37 °C, 5% CO2). Media were collected and stored at -80 °C.

2.3. Total RNA extraction and RT-qPCR

All samples collected from the above patients for gene expression analysis were treated uniformly. RNA extraction was performed using a RNeasy Plus Mini Kit (Cat. No.: 74134, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Frozen samples were disrupted and homogenised using a TissueLyser LT small bead mill (Qiagen, Hilden, Germany). The quality and purity of the extracted RNA were evaluated using a NanoDrop® ND-1000 spectrophotometer (Fisher Scientific, Madrid, Spain), and then stored at -80°C. cDNA was obtained using a High-Capacity cDNA Reverse Transcription Kit (Cat. No: 4368814, Fisher Scientific, Madrid, Spain).

Quantitative PCR amplification was performed using specific TaqMan probes (list of probes: Supplementary Appendix A2; Applied Biosystems, Waltham, USA) for all TAS2R and for enterohormones quantification (40 ng/μL of cDNA for TAS2R, 4 ng/μL for enterohormones). The relative expression of each gene was compared with that of the control group using the $2^{-\Delta\Delta Ct}$ method and with RPS9 used as reference. Each figure indicates the analysis performed.

2.4. Hutu cell studies

2.4.1. Cell Culture

Enteroendocrine HuTu-80 (ATCC, HTB-40) cell line was provided by LGCgroup (Barcelona, Spain). The cells were grown in culture flasks (Greiner Bio-One, Frickenhausen, Germany) at 37°C in an atmosphere of 5% CO_2 and the medium was changed every 2–3 days. The growth medium consisted of EMEM (Cat. No.: 30-2003, ATCC, Manassas, USA) supplemented with 10% v/v heat-inactivated foetal bovine serum (Cat. No.: 12103C, Sigma-Aldrich, Madrid, Spain) and 100 U/mL penicillin-streptomycin mixture (Cat. No.: DE17-602E, Lonza Bioscience, Basel, Switzerland). When confluence reached roughly 80%, the cells were harvested by treatment with

0.25% Trypsin 1 mol/L EDTA (Cat. No.: T3924, Sigma-Aldrich, Madrid, Spain) for 5 minutes, and then split and sub-cultured in fresh medium.

2.4.2. Cellular gene expression analysis

For this experiment, passages 13-17 were used. HuTu-80 cells (200,000 cells/mL) were seeded into individual culture plates (Greiner Bio-One, Frickenhausen, Germany). After 48 hours, cells were washed with cold PBS, then lysed using a buffer provided in the RNeasy Plus Mini Kit (Cat. No.: 74134, Qiagen, Hilden, Germany), and stored at -80°C. The extraction process, RNA to DNA transcription, and gene expression analysis were conducted as described above using the same TaqMan probes and the same reference gene.

2.4.3. Cellular enterohormone secretion analysis

For this experiment, passages 14-17 were used. HuTu-80 cells (200,000 cells/mL per well) were seeded into 24-well plates. After 48 hours, the cells were washed with PBS and treated for two hours with specific treatments: vanillic acid (150 µM) (Cat. No.: V0017, TCI Europe N.V., Zwijndrecht, Belgium), epicatechin (500 µM) (Cat. No.: E1753, Sigma-Aldrich, Madrid, Spain), and peptone (5 mg of protein/mL) (Cat. No.: 70175, Sigma-Aldrich, Madrid, Spain). The stock solutions of the treatments were prepared in DMSO (Cat. No.: SU01581000, Scharlab, S.L., Barcelona, Spain) and then diluted to final working concentrations in glucose-free Krebs–Ringer buffer (KRB) (120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 22 mM NaHCO3). The final DMSO concentration was 0.05%. For basal secretion, KRB supplemented with 0.05% DMSO was used. After treatment, the medium was collected and stored at -80°C.

The cells were then lysed with HEPES buffer supplemented with Triton® X-100 (0.1%; Cat. No.: T8787, Sigma-Aldrich, Madrid, Spain) and stored at -80°C. Total protein content by means of a Bicinchoninic Acid (BCA) kit (Pierce, Thermo Fisher Scientific) and Lactate Dehydrogenase

Assay (LDH) (QCA, Tarragona, Spain) quantification of cytotoxicity in the cells were both performed, as previously described (20).

2.5. Enterohormone Quantification

Enterohormone assays were performed using commercial ELISA kits. For both the media obtained from human colon segments and the cellular secretion experiment, GLP-1 (intra-assay variation: 1-2%; inter-assay variation: <12%; Cat No.: EZGLPT1-36k, Millipore, Madrid, Spain) and PYY (intra-assay variation: <10%; inter-assay variation: <15%; Cat No.: FEK-059-02, Phoenix Europe GmbH, Karlsruhe, Germany) were used in accordance with the instructions. To measure plasma GLP-1 levels of the patients participating in the ascending and descending colon biopsy cohort, a Milliplex® Human Metabolic Hormone Panel V3 was used (Cat. No.: HMH3-34K, Millipore, Madrid, Spain) in accordance with the instructions provided.

2.6. Statistical analysis

Our results are expressed as mean ± standard error of the mean (SEM). P-values < 0.05 were considered statistically significant. We used Student's T test when comparing two groups or one-way ANOVA with Bonferroni post hoc test when comparing multiple groups, for parametric data. For non-parametric data we worked with Mann–Whitney11 U test, when comparing two groups, or Kruskal–Wallis test by ranks with Dunn's Multiple Comparison post hoc test when comparing multiple groups. All calculations were performed using Lumivero XLSTAT 2023.1.5 software (Addinsoft, New York, NY, USA).

3. Results and discussion

3.1. *TAS2R14*, the most promiscuous bitter taste receptor, was one of the most abundant ones at the main GIT locations

To investigate the sensing of bitter taste receptors for modulating the gut-brain axis, a comprehensive analysis of the distribution of 26 distinct TAS2R receptors along the gastrointestinal tract (GIT) is needed. This will enable a more refined delineation of the receptors that are predominant and therefore more promising targets for activation. In this study we met this challenge by conducting a comparative assessment of TAS2R gene expression at various GIT locations. We used quantitative RT-PCR as this tool was shown to be optimal for this task (21). However, even though our approach enables comparison of the bitter receptors at each location, it does not enable comparison of abundances between the segments.

First, we analysed TAS2R expression in cheek mucosa. **Figure 1A** shows the relative abundances of 18 TAS2R in a group of men aged 65 ±11. TAS2R14 showed the highest abundance, followed relatively close by TAS2R3, 4 and 5. TAS2R1 and TAS2R38 showed the lowest abundances, with the remaining TAS2R displaying varying intermediate levels. No other studies have compared the abundances of the various TAS2R in cheek mucosa. Most studies have examined inflammatory processes in pulp tissues where TAS2R stimulation would be useful for anti-inflammatory purposes or dental pulp repair (22), oral microbiome regulation (23), or histamine regulation (24).

Next, we analysed the jejunum, assessing the relative abundance of the top 12 *TAS2R* as described previously (12) in a group of women with morbid obesity aged 46 ± 11. **Figure 1B** displays the *TAS2R* profile from this group, with *TAS2R14* and *TAS2R46* again being the most abundant, and *TAS2R39, 20, 5,* and *13* the least. This profile resembles the one analysed by Liszt et al. (12), which was obtained from cultured jejunal crypts from patients with obesity. The main difference was higher *TAS2R46* expression in our results compared to *TAS2R43*, while Liszt et al. (12) showed the opposite. The main difference between these two studies is that we worked with frozen whole jejunum while Liszt et al. (12) analysed *TAS2R* expression in the primary cultures of isolated jejunum crypts from donors with morbid obesity. Another study that compared different *TAS2R*

in the intestine is that by Mori et al. (21), who quantified the 25 *TAS2R* isoforms in commercial intestine (without clarifying which intestinal segment was used). Our results share a similar relative expression profile to theirs in ten receptors. The differences were limited to *TA2R20* and *TAS2R39*, whose relative expressions were lower in our study. One of the possibilities why these differences could be the fact that we used tissues from patients from obesity, a factor that can have an influence on the genetical expression (15). However, these observed discrepancies are minimal.

Finally, we analysed colon mucosa from both genders in a third group of subjects aged 63.6 ± 6. As done previously, we analysed the receptors already described as the most abundant ones in the colon (25). **Figure 1C** shows that, in the ascending colon, *TAS2R14* again showed the highest presence, along with *TAS2R31*, *46* and *4*, while *TAS2R42*, *38* and *39* showed the lowest. The only study available on *TAS2R* expression to compare our results with is that of Rozengurt et al. (25). However, comparison to their work was somewhat challenging, as they only showed the intensity of the bands in their quantification of mRNA, and they do not show *TAS2R14* expression. Our results differ to theirs regarding the expression of *TAS2R3, 4, 13* and *49*, as we obtained lower relative expressions. We obtained a closer result to Rozengurt et al. (25) when we compare the expression levels of *TAS2R38, 39* and *42*. **Figure 1D** shows the profile obtained for the descending colon with the same group of subjects as was used for **Figure 1C**. Here we can see that relative *TAS2R* abundance had a similar pattern in both areas of the colon. *TAS2R4* and *TAS2R14* showed the highest abundance while *TAS2R38*, *TAS2R39* and *TAS2R42* showed the lowest levels. Comparison of *TASR* abundance in the ascending and descending colon showed that only *TAS2R38* was statistically less expressed in the descending colon than in the ascending colon (**Figure 2**). Ours is also the first study to compare both colon locations in this regard. Previous studies on this topic are scarce and do not define a specific colon sampling location.

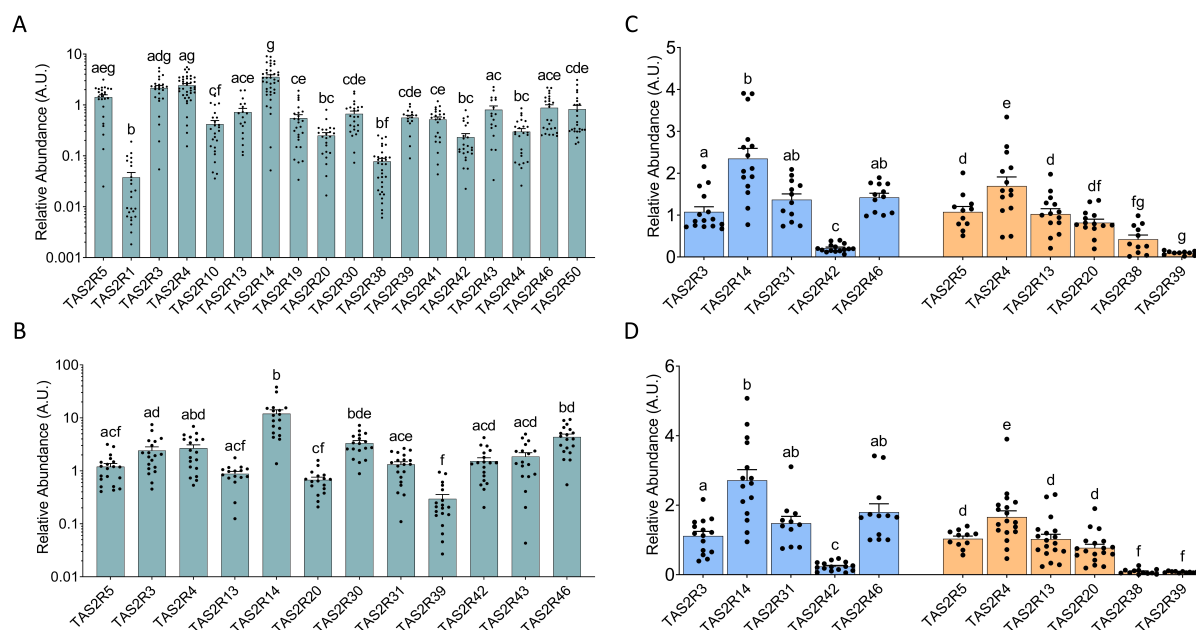

Figure 1: *Relative abundances of TAS2R along the GIT vs abundance of the first TAS2R in each graph (either TAS2R5 or TAS2R3) from mouth mucosa (**A**), jejunum (**B**), ascending colon mucosa (**C**) and descending colon mucosa (**D**). **A** and **B** show date in log scale for Y axis. The two colours used in figures **C** and **D** signify two different measurements and cannot be compared. The groups that share the same letters are not significantly different (significant p value: $p<0.05$) as determined by the post hoc test (we used either Kruskal–Wallis test by ranks with Dunn's Multiple Comparison post hoc test or one-way ANOVA with Bonferroni post hoc test; n=11-40).*

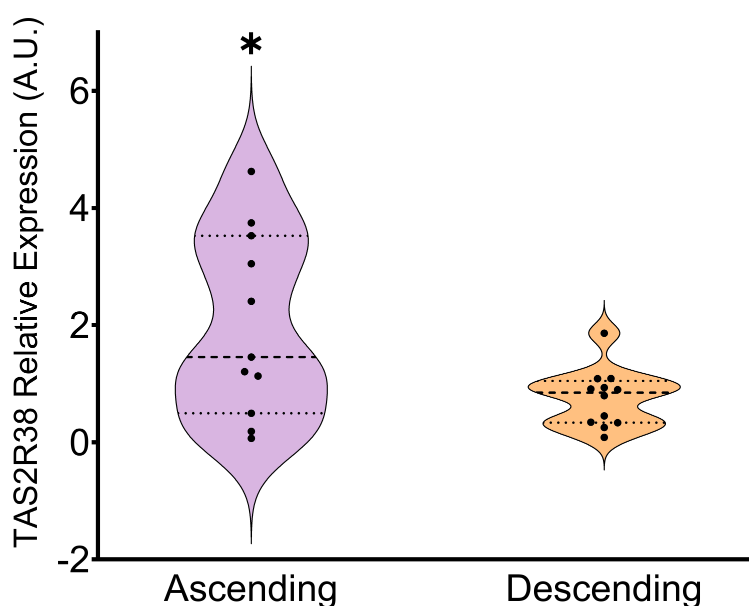

Figure 2: *Relative expression of TAS2R38 in the ascending vs descending colon. Patients from the ascending and descending colon mucosa cohort (* indicates $p<0.05$; Student's T-test; n=11-12).*

In this context we emphasise the importance of characterising the precise expression profiles of TAS2R receptors throughout the gastrointestinal tract (GIT) and highlighting the distinctions

among these profiles within specific GIT segments. By meticulously mapping the distribution of TAS2R receptors in the GIT and elucidating their roles in regulating local and broader physiological systems, it is feasible to achieve the targeted stimulation of specific GIT regions by using tailored ligands for these receptors and produce well-defined physiological outcomes. Moreover, given the diversity of TAS2R receptors, it is conceivable that targeting different receptors may yield different responses. Preliminary evidence suggests there is a connection between the activation of TAS2R receptors in the oral mucosa and improvements in oral pathologies (23). It is therefore plausible to deduce that similar beneficial effects may manifest in other GIT segments where TAS2R receptors are present.

Another crucial aspect when investigating the activation of TAS2R receptors by dietary components is the dynamic transformation these components undergo during digestion and subsequent metabolism, which include modifications induced by enzymes and interactions with the gut microbiota. These transformations can significantly alter the composition of compounds in the oral cavity compared to those in the colon (26,27). Notwithstanding the inherent complexities associated with this proposition as briefly elucidated here, **Table 1** summarises the relative abundance of the receptors analysed at each location. TAS2R14 appears at all locations as the most expressed bitter taste receptor, and interestingly, it is also the most promiscuous receptor defined to date (8,28,29). TAS2R46, a receptor that predominates in the jejunum, is also highly promiscuous (28,29). Only two of the most expressed TASR were not characterised as highly promiscuous. One of these is TAS2R3, which was highly abundant in the cheek and moderately abundant in jejunum, and reported as the least promiscuous receptor by Margulis et al. (29). The other one is TAS2R31, which was highly expressed only in the colon and was also considered to be less promiscuous by Bayer et al. (28). Though incomplete (we did not quantify all 26 TAS2R defined to date (30)), our results suggest there is a general need for a GIT response

to any bitter signal, while fine tuning and a specific response may be exerted by receptors that are not so highly expressed.

Table 1: *Coloured summary of the relative abundances of different TAS2R in different parts of the GIT. This table is a qualitative summary of figure 1. Each receptor is assigned a different colour to facilitate visualisation of the differences between locations.*

Degree of expression	Cheek	Jejunum	Ascending Colon	Descending Colon
Highest	TAS2R4	TAS2R4	TAS2R4	TAS2R4
	TAS2R14	TAS2R14	TAS2R14	TAS2R14
	TAS2R3	TAS2R46	TAS2R46	TAS2R46
	TAS2R5	TAS2R30	TAS2R31	TAS2R31
Average	TAS2R10	TAS2R3	TAS2R3	TAS2R3
	TAS2R13	TAS2R31	TAS2R13	TAS2R13
	TAS2R19	TAS2R42	TAS2R5	TAS2R5
	TAS2R43	TAS2R43	TAS2R20	TAS2R20
	TAS2R30			
	TAS2R39			
	TAS2R41			
	TAS2R46			
	TAS2R50			
Lowest	TAS2R38	TAS2R5	TAS2R38	TAS2R38
	TAS2R1	TAS2R39	TAS2R39	TAS2R39
	TAS2R42	TAS2R13	TAS2R42	TAS2R42
	TAS2R20	TAS2R20		

3.2. *TAS2R* expression was modified by ageing in the descending colon, while gender differences appeared only in the ascending colon

It is well documented that ageing modifies taste perception, leading to a loss of taste (31,32). However, most studies have not related this loss of taste to TAS2R expression. To investigate whether these effects are also reflected in extraoral TAS2R, we compared TAS2R expression in the colon samples of two groups from the same cohort used in previous experiment containing both ascending and descending colon samples: the above-mentioned group of older patients (63.6 ± 6) and a group of younger patients (38.9 ± 6).

Reduced taste perception may indicate decreased sensitivity to taste-producing molecules. Our findings indicate that ageing does not universally lead to a down-regulation of TAS2R expression. In the ascending colon, age groups showed no differences in the abundances of the TAS2R analysed (supplementary table 1). In the descending colon, there were differences between the analysed receptors only in the cases of TAS2R4, TAS2R13 and TAS2R5, which showed higher expression levels in the older group than in the younger one. The same trend was observed in the expression of TAS2R20 (**Figure 3A**). Since most of these receptors are not the promiscuous ones, we mentioned earlier (28,29), ageing might not modify the general response to bitter agonists, but it could impact specific functions of these receptors in the colon. Studies on TAS2R and ageing are limited. One study found a positive correlation between ageing and TAS2R expression in non-smokers aged 20 to 65 (33), supporting our findings of increased expression of some receptors with ageing. Another study explored TAS2R and ageing through genotype variants and longevity (34). However, most research has focused on aspects other than TAS2R expression, such as age-dependent changes in the physiology of taste receptor cells, related to taste perception loss and ageing (31). Our results therefore suggest that ageing may affect specific TAS2R in a manner distinct from down-regulation and may be specific to certain GIT regions.

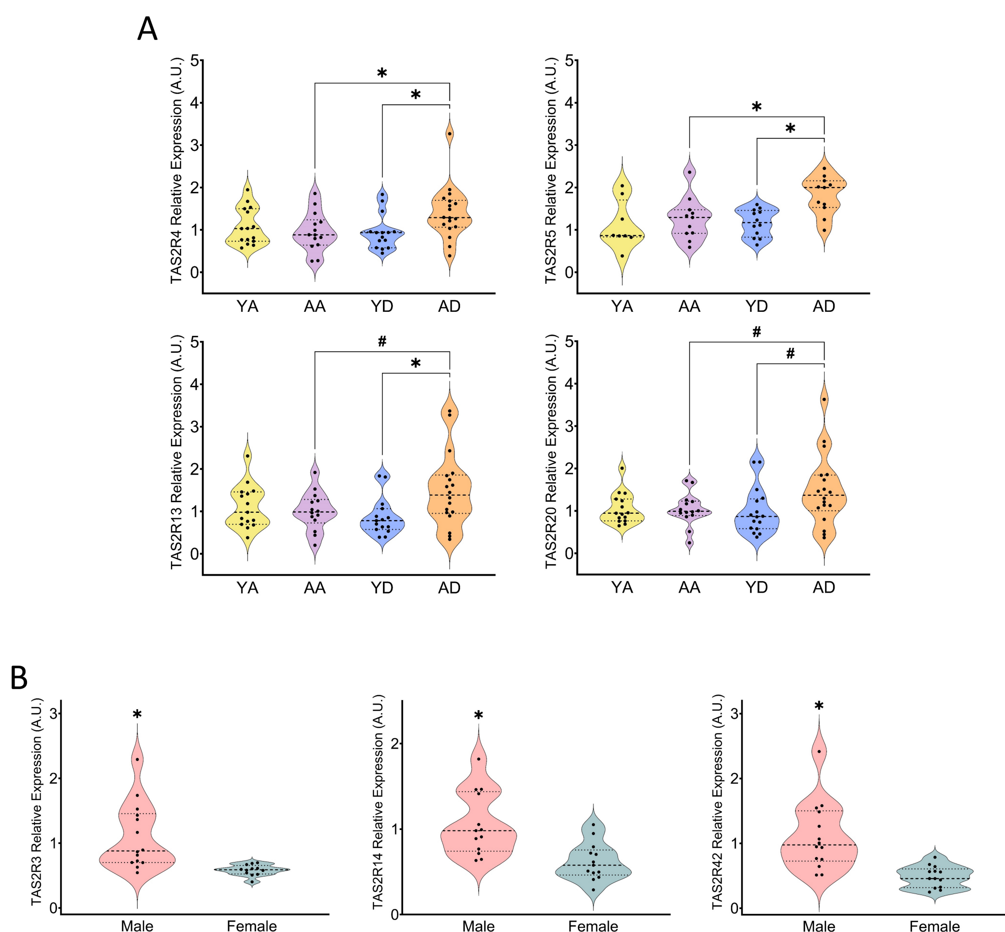

***Figure 3**: Comparison of relative expression of individual TAS2R in the young and aged groups of the ascending and descending colon (**A**) (YA - young ascending; AA - aged ascending; YD - young descending; AD - aged descending). Gender comparison of relative expressions of specific TAS2R in the ascending colon in all patients (**B**). Patients from the ascending and descending colon mucosa cohort. (* indicates $p<0.05$; # indicates $0.05<p<0.1$; tests used: Student's T-test, Mann–Whitney U test; n=8-18).*

Since this colon donor cohort included both genders, we were able to analyse the effects of gender on TAS2R expression. There was no gender effect observed in the descending colon (supplementary table 2). Figure 3B shows that, in the ascending colon (age groups merged together), we observed a higher quantity of TAS2R3, 14 and 42 in males than in females, which suggests that males are more sensitive to the stimulation of these receptors. Regrettably, due to data limitations, we cannot compare our results with other studies. Regarding taste perception, it has been shown that females are more sensitive to PTC (Phenylthiocarbamide) bitterness than males, possibly because women had more fungous papillae than taste buds (35). Regarding the

TAS2R expression, however, the only study we found described gender differences that were observed only in certain areas of the skin, only for certain receptors, and dependent upon exposure to the sun (36).

3.3. Relationship between TAS2R and colonic enterohormones

Some extraoral bitter taste receptors have been shown able to control enterohormone secretion (37–39). To explore this functionality at a deeper level, we analysed the relative presence of PYY, GCG, GLP-1R, ghrelin and ChgA by quantifying gene expression in the same samples of colonic mucosa from the human ascending and descending colon used previously to measure TAS2R expression.

In **Figure 4A** we can observe location-based differences in enterohormone expression in the younger group. GCG was more abundant in the descending colon than in the ascending colon, though the receptor for GLP-1 (GLP-1R) showed no significant differences between locations (GLP-1R data not shown). A clear location-based effect was also observed for PYY abundance (greater in the descending colon than in the ascending colon). Additionally, ghrelin and ChgA abundances tended to be higher only in the descending colon (data not shown). These higher levels of PYY expression in the descending colon than in the ascending colon was also observed in human (40), and in mouse and pig (39) tissues in addition to GCG displaying the same trend in both humans and mice (39).

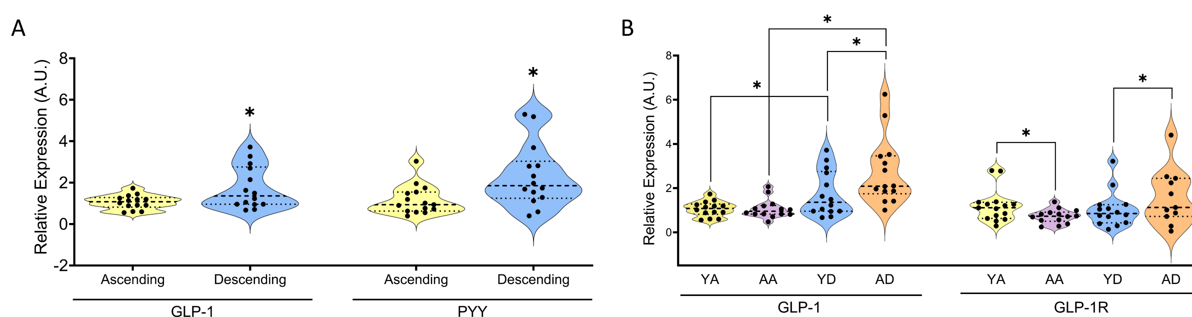

***Figure 4**: Relative expression of specified enterohormones in the colon samples of the young group of (**A**) and relative expressions of GLP-1 and its receptor in the colon samples of all groups (**B**) (YA - young ascending; AA - aged ascending; YD - young descending). Patients from the ascending and descending colon mucosa cohort (* indicates p<0.05; tests used: Student's T-test, Mann–Whitney U test; n=11-15).*

To relate expression levels with the secretion of enterohormones, we used a different group of mucosae samples (41) to analyse basal, unstimulated GLP-1 and PYY secretion ex-vivo using free explants. In colonic samples we observed that the location significantly influenced PYY secretion, with the descending colon exhibiting higher levels compared to the ascending colon (Table 2). However, the same result was not observed for GLP-1 basal secretion. Similar responses were observed for PYY and GLP-1 (results not shown) in basal, unstimulated secretion measured using an Ussing chamber system.

Table 2: *Analysis of basal PYY and GLP-1 secretion obtained from colon tissue explants (* signifies statistically significant differences between the two segments of the colon for each enterohormone in each experiment; Student's T-Test; n= 9).*

	Ascending Colon	Descending Colon
PYY (pM)	52 ± 7.23	236.17 ± 65.94*
GLP-1 (pM)	36.92 ± 10.36	41.24 ± 8.1

Since TAS2R38 was the only TAS2R sensitive to colon location, we conducted a correlation analysis between it and PYY and GCG expression levels in the descending colon (results not shown), which yielded no significant effect.

To further explore the relationship between TAS2R expression and enterohormones in the colon, we examined how ageing affects enterohormone expression. **Figure 4B** shows that GCG mRNA was affected by ageing mainly in the descending colon, increasing the differences between the ascending and the descending colon. Ageing led to a statistically significant decrease in GLP-1R expression in the ascending colon. However, ageing did not affect the levels of PYY, ghrelin or ChgA (supplementary table 3). This differential GCG gene expression was not strongly associated with the abundance of enteroendocrine cells, as indicated by ChgA expression, which showed no age-related differences in both colon locations, therefore indicating no changes in the abundance of cells. These results differ from those previously obtained by our group in a study of 24-month-

old female rats (42), where, when working in the proximal colon, we clearly found that ageing decreased GCG, PYY and ChgA expressions. The discrepancy between these results may be related to different causes. Regarding ageing effect, rats were older than the humans analysed here. As mentioned earlier, ageing increased the sensitivity of the descending colon to stimulation by TAS2R4, 5, 13 and 20. Combined with the increased GCG abundance and higher basal PYY secretion, this suggests the descending colon is more susceptible to ageing effects. In this study we analysed the correlation between plasma active GLP-1 (supplementary table 4) and measured TAS2R. **Table 3** shows the correlation analysis conducted in the older group, where we obtained positive correlations with three of the TAS2R sensitive to ageing (TAS2R4, 5, 13) and TAS2R39 in the ascending colon. This reinforces the sensitivity of these receptors to ageing and indicates a greater ability to secrete active GLP-1 when the ascending colon is more sensitive to these specific TAS2R stimulations.

Table 3*: Significant correlation results of the receptors indicated with plasma levels of GLP-1 (Spearman's Rho test; * indicates p < 0.05).*

	Correlation Coefficient	P value	N
TAS2R4	0.621*	0.024	13
TAS2R5	0.709*	0.022	10
TAS2R13	0.610*	0.027	13
TAS2R39	0.794*	0.006	10

3.4. Specific TAS2R stimulation induced GLP-1 and/or PYY secretion

Several studies that specifically stimulated TAS2R reported increased calcium mediator in various cell types (6,43) but did not report further effects on enterohormone secretion. Working with the samples we used earlier for the basal secretion experiments, we observed a clear connection between TAS2R and enterohormone secretion (41). We stimulated colon tissue samples with an extract rich in procyanidins (GSPE) containing both agonists and antagonists with different specificities for some TAS2R (43,44). We found that both the ascending and

descending colon were sensitive to GSPE stimulation, resulting in increased PYY secretion, but found no effect on GLP-1 secretion.

To clearly relate TAS2R as integral components of enteroendocrine control, it was necessary to simplify the system. To do so, we selected the HuTu-80 cell line, an enteroendocrine epithelial cell line derived from male duodenal tissue. We decided upon the use of this line as duodenum is location where, yet unmodified compounds arrive to the mucosa and interact with the chemosensory peptides located in the epithelium, and spend significant time, thus enabling the interactions between them. In **Figure 5**, which shows the relative abundance of the most abundant TAS2R in these cells, the receptors analysed were those previously reported as being the most abundant in these cells, and our results were mostly consistent with those observed by Rozengurt et al. (25). One discrepancy between their results and ours concerns TAS2R38, which they observed to be moderately expressed (closer to TAS2R13) but which we found barely detectable. Another discrepancy concerned TAS2R5, as our data suggest that the expression levels for this receptor were closer to those for TAS2R4, 13, 20 or 46, whereas Rozengurt et al. (25) reported very modest expression closer to the TAS2R39. Mori et al. (21) presented a quantitative TAS2R profile for Hutu-80 cells, showing relatively lower abundances of TAS2R4 and TAS2R46 and a higher abundance of TAS2R42 when compared to our data. If we compare the TAS2R profile obtained from our cells with those for the various intestinal segments analysed in this paper (see **Table 1**), we see that the profile is very similar to that obtained from the jejunum samples. This is as expected for cells of duodenum origin, such as those of the HuTu-80 cell line.

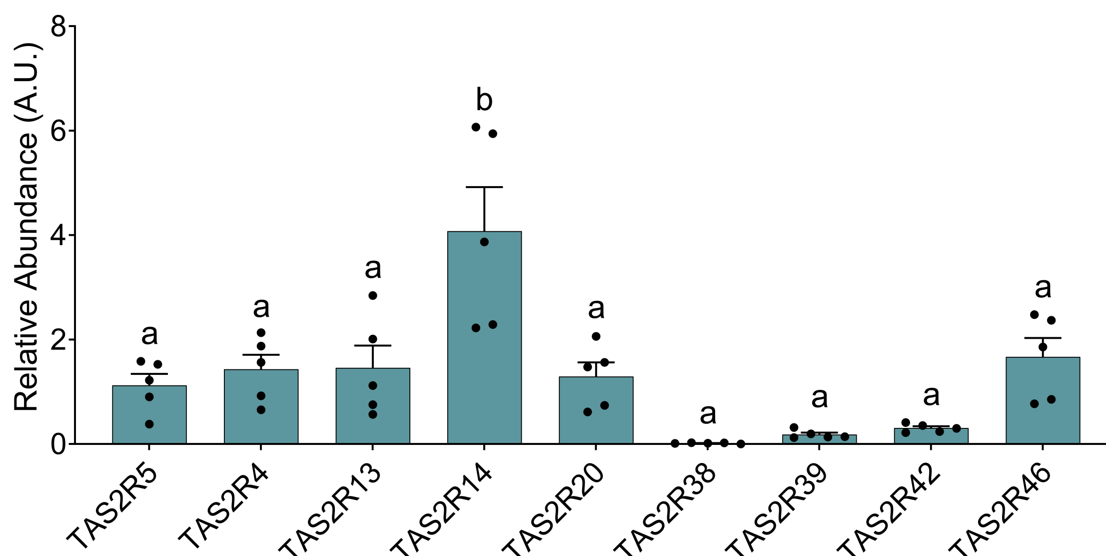

Figure 5*: Relative abundances for specific TAS2R obtained from the HuTu-80 cells under basal conditions. The groups that share the same letters are not significantly different (significant p value: p<0.05) as determined by the post hoc test (one-way ANOVA test with Bonferroni post-hoc test; n=5).*

To investigate the stimulation of specific enterohormones in the HuTu-80 cell line, we chose ligands with preferential binding to specific TAS2R. We selected vanillic acid as the specific agonist for TAS2R14 and epicatechin to stimulate TAS2R5 and TAS2R39 (45). Before conducting the experiments, we discarded the toxicity effect of the assayed doses by LDH viability assay (data not shown). **Table 4** shows that vanillic acid, at the concentration of its EC50 (44), clearly stimulated GLP-1 secretion in the cells but did not stimulate PYY. Epicatechin 500 μM, which binds preferentially to TAS2R5 (EC50= 3.2 mM) rather than to TAS2R39 (EC50= 3.8 mM) (43), showed the ability to induce GLP-1 and PYY secretion in these cells, which, according to Rozengurt et al., expressed higher levels of GCG and lower levels of PYY (25). Stimulation of the secretion of GLP-1 but not of PYY after activating TAS2R14 is a result we observed in previous studies when, interestingly, we used meat and insect hydrolysates in human colon samples (46). In fact, we obtained the same result in our cells when we used peptone as a positive control (see **table 4**). Supporting this idea are the results obtained by Kohl et al. (47) in a screening study in which they activated different TAS2R using various peptides and showed that the peptides positively stimulated only receptors TAS2R1, 4, 39, 14 and 46 but did not stimulate TAS2R5. This

reinforces the idea that specific TAS2R stimulation produces differential enterohormone secretions. Our results show that TAS2R14 stimulation induces GLP-1 secretion and that TAS2R5 stimulation can induce GLP-1 and PYY secretions.

Table 4*: Analysis of PYY and GLP-1 secretion obtained after specific TAS2R stimulation in Hutu-80 cells. The data represents the average value for each treatment of each hormone, normalised to the values of the control (* signifies statistically significant stimulation by the treatment vs control group; Student's T-Test; n= 9).*

	GLP-1 secretion (A.U.)	PYY secretion (A.U.)
Control	1.00 ± 0.08	1.10 ± 0.07
Peptone (5mg of protein/mL)	3.84 ± 0.90*	0.98 ± 0.12
Vanillic Acid 150 µM	1.97 ± 0.33*	1.12 ± 0.94
Epicatechin 500 µM	1.79 ± 0.22*	1.41 0.03*

In summary, our study elucidated variations in TAS2R expression profiles at three gastrointestinal (GIT) locations while highlighting their modulation by age and gender, especially in the colonic region. This profiling provides valuable insights into the potential dissimilar sensitivities of each location to dietary components and their ensuing metabolites post-GIT transit. Combining this knowledge with a broader understanding of TAS2R-mediated control over enteroendocrine secretion will advance our comprehension of gut-brain communication and inform the development of targeted strategies aimed at modulating these receptors in response to intestinal compounds. Notably, we observed that TAS2R14, receptor recognised as the most promiscuous one, exhibited the highest abundance in the four GIT segments analysed. However, its role in regulating PYY secretion appears to be minimal. Conversely, TAS2R5, a less prevalent but more specific TAS2R, demonstrated the capacity to stimulate both GLP-1 and PYY secretions. Moving forward, the imperative lies in establishing definitive associations between specific TAS2R and cellular functions. This would represent a pivotal milestone in characterising distinct bitter taste receptors as viable therapeutic and preventive healthcare interventions.

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CONFLICT OF INTEREST

None the authors have any conflicts of interests.

AUTHOR CONTRIBUTIONS

Conceptualization: MP, AA & XT; Data curation: FJ, MD & HS; Formal analysis: FJ, MD & CG; Funding acquisition: RB, MTB, XT, MP & AA; Investigation& Methodology: FJ, MD, TA, FXA, FB, and RJ; Project administration: XT, MP & AA; Supervision: TA, FXA, FB, and RJ; Validation; Visualization; Roles/Writing: FJ, MP and AA; and Writing - review & editing: all the authors.

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SUPPLEMENTARY DATA

Appendix A1: Exclusion criteria used in each cohort:

- Exclusion criteria for the samples of human cheek mucosa, kindly provided by Joan XXIII University Hospital (Tarragona, Spain): (1) serious systemic disease such as obesity, cancer or severe kidney or liver disease; (2) systemic diseases with intrinsic inflammatory activity; (3) history of liver disease (chronic active hepatitis or cirrhosis) and/or abnormal liver function (alanine transaminase and/or aspartate transaminase three times above the upper normal value) or altered renal function (creatinine>1.5mg/dL); (4) adherence to a vegetarian or irregular diet; (5) severe disorders of eating behaviour; (6) clinical symptoms and signs of infection in the previous month; (7) anti-inflammatory chronic treatment with steroidal and/or nonsteroidal anti-inflammatory drugs; (8) antibiotic treatment in the previous three months; (9) major psychiatric antecedents; and (10) uncontrolled alcoholism or drug abuse.

- Exclusion criteria for the jejunum samples, kindly provided by GEMMAIR from IISPV and Joan XXIII University Hospital (Tarragona, Spain): (1) intake of ethanol or other toxins above 10 g/day; (2) acute or chronic hepatic or inflammatory disease, infectious disease or neoplastic disease; (3) menopause or use of contraceptives; (4) diabetes with administration of insulin or other medication to modulate endogenous insulin levels; and (5) administration of fibrates.

- Exclusion criteria for the mucosal biopsy samples from the ascending and the descending colon, kindly provided by the Hospital Clínic (Barcelona, Spain): (1) body mass index (BMI) below 18.5 or above 35; (2) diagnosis of diabetes mellitus type 1 or 2; (3) chronic treatment with anti-inflammatory medicines; and (4) previously diagnosed illnesses.

- Exclusion criteria for the mucosa colon samples, kindly provided by Joan XXIII University Hospital (Tarragona, Spain): (1) alcohol intake above 30 g/day; (2) body mass index above 40 kg/m2; (3) use of drugs unrelated to treatment for metabolic syndrome; (4) presence of intestinal malabsorptive or inflammatory bowel disease; (5) presence of acute or chronic inflammatory or infectious disease; and (6) presence of neoplastic disease at advanced stages or requiring pharmacological treatment.

Appendix A2: List of probes tested in the study:

TAS2R1, Hs00251930_s1; TAS2R3, Hs00249942_s1; TAS2R4, Hs00249946_s1; TAS2R5, Hs01549633_s1; TAS2R10, Hs00256794_s1; TAS2R13, Hs00256781_s1; TAS2R14, Hs00256800_s1; TAS2R19, Hs00853130_s1; TAS2R20, Hs00604340_s1; TAS2R30, Hs03054740_Sh; TAS2R31, Hs00604313_sH; TAS2R38, Hs00604294_s1; TAS2R39 Hs00603443_s1; TAS2R41, Hs00603461_s1; TAS2R42, Hs00704057_s1; TAS2R43, Hs00853105_sH; TAS2R44, Hs00604313_Sh; TAS2R46, Hs00853124_s1; TAS2R50,

Hs00604351_s1; GCG, Hs01031536_m1; GLP-1R, Hs00157705_m1; PYY, Hs00373890_g1; CHGA, Hs00900370_m1; Ghrelin, Hs01074053_m1; and RPS9, Hs02339424_g1.

S-table 1: The list of medication that have been prescribed to the participants of the study; Data available for the patients of the colon samples donated by Hospital Clínic from Barcelona. Some patients were prescribed medication for more than 1 medical issue.

Medical Issues	Number Of Patients Prescribed Medicine
None	18
Heart/Circulation-related issues:	9
Control of stomach acid:	8
Lowering of Lipids:	5
Mental Health Problems:	4
Anticoagulants:	2
Antihistaminics:	2
Calcium:	1
Cancer Prevention:	1
Contraceptives:	1
Glaucoma:	1
Hypothyroidism:	1
Prostatic Hyperplasia:	1
Pulmonary Disease:	1
Smoking Cessation:	1
Vitamin D:	1

S-table 2: Evaluating the effect of Age on the Relative expression levels of analysed TAS2R in Ascending colon (either Student's T-test or Mann–Whitney U test was used).

	Young			Aged		
Receptor	Average (A.U.)	SEM	N	Average (A.U.)	SEM	N
TAS2R3	1.065	0.140	11	1.246	0.139	15
TAS2R4	1.078	0.112	15	0.955	0.122	14
TAS2R5	1.119	0.200	8	1.256	0.151	11
TAS2R13	1.109	0.132	15	1.004	0.120	14
TAS2R14	1.101	0.171	11	1.242	0.130	15
TAS2R20	1.053	0.094	15	1.031	0.103	14
TAS2R31	1.131	0.184	7	0.825	0.084	12
TAS2R38	1.196	0.336	8	1.990	0.472	11
TAS2R39	1.127	0.155	8	1.073	0.156	11
TAS2R42	1.305	0.307	12	1.372	0.161	15
TAS2R46	1.080	0.167	7	1.045	0.069	12

S-table 3: Evaluating the effect of Gender on the Relative expression levels of analysed TAS2R in Descending colon (either Student's T-test or Mann–Whitney U test was used).

	Male			Female		
Receptor	Average (A.U.)	SEM	N	Average (A.U.)	SEM	N
TAS2R3	0.734	0.075	12	0.936	0.101	13
TAS2R4	1.512	0.205	20	1.335	0.175	20
TAS2R5	1.145	0.088	10	1.412	0.195	14
TAS2R13	1.482	0.213	14	1.236	0.195	20
TAS2R14	0.843	0.073	12	0.977	0.120	13
TAS2R20	1.543	0.217	14	1.130	0.159	20
TAS2R31	0.832	0.069	10	1.255	0.345	7
TAS2R38	0.522	0.115	11	0.426	0.085	14
TAS2R39	1.039	0.107	10	1.103	0.118	14
TAS2R42	0.801	0.082	12	0.844	0.138	13
TAS2R46	0.938	0.080	10	1.176	0.251	7

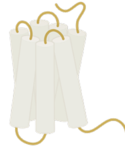
S-table 4: Evaluation of the effect of Ageing on the Relative expression levels of analysed Enterohormones in Each individual location of the colon (either Student's T-test or Mann–Whitney U test was used).

		Young			Aged		
Receptor	Colon Location	Average (A.U.)	SEM	N	Average (A.U.)	SEM	N
PYY	Ascending	1.161	0.180	15	0.795	0.072	14
	Descending	2.315	0.406	14	2.890	0.456	15
Ghrelin	Ascending	1.153	0.160	15	0.906	0.122	14
	Descending	0.820	0.107	13	0.798	0.094	15
CHGA	Ascending	1.104	0.129	14	0.997	0.116	14
	Descending	1.303	0.163	14	1.261	0.138	15

S-table 5: Evaluation of Plasma levels of GLP-1 in the ascending/descending colon cohort ($p<0.05$; Mann–Whitney U test).

	Young			Aged		
Enterohormone	Average (pg/mL)	SEM	N	Average (pg/mL)	SEM	N
GLP-1	10.581	1.290	21	**21.63** *	3.154	23

UNIVERSITAT ROVIRA I VIRGILI
TASTE PERCEPTION BEYOND FLAVOUR: BITTER TASTE RECEPTORS IN THE GASTROINTESTINAL TRACT AND THEIR ROLE IN AGEING
Florijan Jalševac



Part 2:

Establishment of the Most Likely Bitter Taste Receptors as Potential Regulators of General Age-Associated Processes

Manuscript 3:

TAS2R5 and TAS2R38 are bitter taste receptors whose colonic expressions could play important roles in age-associated processes

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TAS2R5 and TAS2R38 are bitter taste receptors whose colonic expressions could play important roles in age-associated processes

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ABSTRACT

Ageing disrupts how our bodies process nutrients, leading to deregulation of nutrient-sensing and increased inflammation. Dietary interventions can promote healthy ageing, which demonstrates the importance of both metabolism and the gastrointestinal tract for our health. Bitter taste receptors (TAS2R) present in the intestine are key members of metabolic regulation. TA2R are involved in controlling enterohormonal secretion, detect phenolic compounds in our diet, and potentially have a great impact on the ageing process.

Here, we aimed to analyse the potential role of the intestinal TAS2R on the ageing process and establish potential impact of these receptors on the biomarkers.

Healthy subjects were divided into two age cohorts: young (38.9 ± 6) and aged (63.6 ± 6). TAS2R expression was analysed in the colon. Analyses of metabolomics and of phenolic markers were performed in plasma. Best discriminatory parameters were obtained using three machine-learning methods. Finally, Spearman's rank correlation was performed.

The best separators of the age cohorts were docosahexaenoic acid and multiple lipoprotein fractions. Two TAS2R were also identified: TAS2R5 and TAS2R38. TAS2R5 correlated with multiple lipoprotein-derived fractions, inflammatory marker IL-6 and polyunsaturated fatty acids. TAS2R38 was much more selective, correlating with a few parameters, including membrane lipid sphingomyelin, ketone body acetone, and omega acids. Both TAS2R 5 and TAS2R38 correlated with β-hydroxybutyrate.

The parameters that correlated with TAS2R have known effects on the ageing process. This suggests that TAS2R5 and TASR38 are the bitter receptors most likely to play a role in the development and progress of ageing.

Keywords: Bitter taste receptors, TAS2R5 and TAS2R38, ageing, metabolomics, phenolic metabolites, gastrointestinal tract

Abbreviations:

CPMG Carr-Purcell-Meiboom-Gill

RF Random Forrest

PLS-DA Partial Least Squares-Discriminant Analysis

IDL Intermediate-density lipoprotein

VLDL Very-low-density lipoprotein

LDL Low-density lipoprotein

HDL High-density lipoprotein

NMR Nuclear magnetic resonance

LMWMs Low-molecular-weight metabolites

sMRM Scheduled multiple reaction monitoring

MS Mass spectrometry

PSI Pounds per square inch

Glyc-A N-acetyl glucosamine/galactosamine

GlycB Sialic acid

MCP-1 Monocyte Chemoattractant Protein-1

CCK Cholecystokinin

Ppb Parts per billion

PUFAs Polyunsaturated fatty acids

CD36 Cluster of differentiation 36

PROP 6-n-propylthiouracil

1. Introduction

Ageing is characterised as a progressive loss of physiological functions, a deterioration that poses a significant risk of developing serious health disorders [1]. One aspect that is greatly affected as we age is metabolic regulation. As we progress through life and our physiological makeup changes, a shift is observed in how our cells process nutrients and how energy production is conducted [2]. One hallmark of the ageing process is the deregulation of nutrient-sensing [3]. When food is ingested, the signalling cascades are activated by the components of our diet, such as glucose and amino acids, and can achieve an anabolic response that is characterised by changes in cellular activity. Autophagy, protein synthesis, glucose, and nucleotide and lipid metabolism are just a few noticeable effects [4–6]. However, these mechanisms are often affected by ageing-induced changes that potentially produce negative effects on our health. These changes lead to excessive anabolic signalling and, finally, increased inflammation, which is one of the most prominent causes of age-related health issues [7,8]. Further evidence of the importance of metabolism in the progression of ageing is provided by the fact that dietary interventions such as caloric restriction, which have been widely studied over the years, have displayed a positive effect in delaying the ageing process or achieving healthier ageing [9,10].

An important component of this nutrient-sensing complex are the numerous chemo-sensing receptors found along the gastrointestinal tract (GIT) that form important signalling pathways with the whole range of physiological systems [11,12]. These receptors possess a wide range of functions. One family of receptors found here are taste receptors [13]. Although their name provides a clue as to their roles, there is much more to these receptors – as we deduce from their appearance in a myriad of diverse tissues. One large group of these receptors are the bitter taste receptors, or TAS2R. We currently know of 26 different forms of TAS2R in humans, which makes them the most diverse taste receptors in our species [14]. These receptors have evolved to perform a protective function (prevent the ingestion of potentially poisonous foods), which was the first

role discovered for them. However, in the last few decades, researchers have found that they appear in a highly diverse set of tissues from the brain to the reproductive system [15]. Bitter taste receptors became a point of interest for researchers seeking to explain why they are so ubiquitous. One area in which they have been studied recently, mostly as components of the immune system, [16,17]. Evidence also exists that they are involved in numerous pathophysiological processes [18–20]. One of the most interesting TAS2R locations for researchers in recent years, however, is the gastrointestinal wall [21], where they appear in a range of cells that make up the GIT. Some of the most interesting of these cells are the enteroendocrine cells, which secrete and regulate enterohormones [22,23]. These hormones play a key role in the regulation of metabolism, nutrient uptake, insulin secretion and, therefore, glucose regulation and the body's overall systemic response to the presence or absence of nutrients. The prevalent theory in the literature is that the presence of bitter taste receptors in these cells is a key factor in their function. Beyond the fact that many components of our daily diet contain polyphenolic compounds that bind to these receptors, it is clear that the interplay between food and TAS2R is important for our well-being.

However, the novelty of this research area means that much is yet to be established. The interplay between the bitter receptors located in the GIT, the metabolic pathways, and the consequences of this network for our health are yet to be determined. Moreover, although we know that phenolic compounds do interact with TAS2R, we lack overall knowledge regarding how these compounds from our daily diet influence TAS2R and their expression in the intestine. Importantly for us, we also lack knowledge on the role that TAS2R play in the metabolic changes that take place during ageing. Although we have some data on ageing and TAS2R [24,25], and there is evidence that bitter agonists have anti-ageing benefits [26,27], our overall knowledge in this area is wanting.

To shed light on these uncertainties, and possibly create a starting point for further research, we designed the present study in which, working with two age cohorts, we analyse the potential role of intestinal bitter receptors on the ageing process. First, we present a comprehensive metabolomic analysis accompanied by an analysis of enterohormonal secretion, anti-inflammatory markers, and food-derived phenolic markers in plasma. Then, building on our previous results for the expression of TAS2R present in the GIT (Submitted results), we used machine-learning models and computer analysis to determine the importance of these receptors and establish their possible connection with changes in the parameters that occur during the ageing process.

2. Materials & Methods

2.1.Study Cohorts

All the samples in this study were kindly provided by the Gastroenterology Department of the Hospital Clínic of Barcelona, Spain. 23 healthy men and 25 healthy women participated in this study. Subjects were divided into one a young cohort (range 20-50 years: mean age ± SD =38.9 ± 6 years) and one an aged cohort (range 50-80; mean age ± SD =63.6 ± 6 years). The exclusion criteria for participation in the study were: 1) a body mass index (BMI) below 18.5 or above 35; 2) a previous diagnosis of diabetes mellitus type 1 or 2; 3) chronic treatment with anti-inflammatory medicines; and 4) previously diagnosed illnesses. This experimental procedure was approved by the Drug Research Ethics Committee of Hospital Clínic in Barcelona (HCB/2019/1115). All participants were informed before they provided their written consent to take part in the study. The procedures performed were in compliance with local laws.

2.2.Colon Mucosa Samples

All participants underwent a colonoscopy performed for several indications (i.e. colorectal cancer screening, polyp surveillance or investigation of gastrointestinal symptoms) where

colonic mucosa sampling was performed. Samples from the ascending and descending colon were collected. All individuals had no colorectal cancer at the time of colonoscopy. Colonic samples were stored at -80 °C until processing. Briefly, RNA extraction was performed using a RNeasy Plus Mini Kit (Cat. No.: 74134, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The quality and purity of the extracted RNA were evaluated using a NanoDrop® ND-1000 spectrophotometer (Fisher Scientific, Madrid, Spain). cDNA was obtained using a High-Capacity cDNA Reverse Transcription Kit (Cat. No: 4368814, Fisher Scientific, Madrid, Spain). Quantitative PCR amplification was performed using specific TaqMan probes (list of probes: supplementary appendix A1; Applied Biosystems, Waltham, USA). For further processing and subsequent analysis, relative gene expression was calculated as $1/^{\Delta}Ct$, where RPS9 was used as reference.

2.3.Plasma Samples

Plasma samples were collected from the participants on the same day as the tissue samples. The samples were stored at -80°C until further use. Various kits were used to measure enterohormones and inflammatory markers. CCK (intra-assay variation: <10%; inter-assay variation: <15%; Cat No.: EKE-069-04, Phoenix Europe GmbH, Karlsruhe, Germany) and PYY (intra-assay variation: <10%; inter-assay variation: <15%; Cat No.: FEK-059-02, Phoenix Europe GmbH, Karlsruhe, Germany) were measured individually whereas the other parameters were analysed together using Milliplex® Human Metabolic Hormone Panel V3 kit (Cat. No.: HMH3-34K, Millipore, Madrid, Spain). All analyses were performed strictly in accordance with manufacturers' instructions.

2.4.Analysis of metabolomic biomarkers in plasma

Metabolomic analysis was performed by Biosfer Teslab (Reus, Spain) using 1H-NMR spectroscopy to analyse the samples (306 K, proton frequency 600.20 MHz, 14.1 T).

Lipoprotein profile was analysed using the NMR-based Liposcale® test. The lipid concentrations, size and particle number of the four main classes of lipoproteins (intermediate-density lipoprotein or IDL, very-low-density lipoprotein or VLDL, LDL and HDL) as well as the particle number were determined as previously described [28]. Glycoprotein profile was determined by analysing the glycoprotein-specific region of the 1H-NMR spectrum using several analytical functions according to previously published procedures [29,30]. Low-molecular-weight metabolites (LMWMs) were identified and quantified in the 1D Carr-Purcell-Meiboom-Gill (CPMG) spectra using an adaptation of Dolphin [31]. After 1H-NMR metabolomic characterisation, the diluted serum samples were lyophilised and then diluted with 100 µL of 50 mM PBS at pH 7.4 before lipid extraction using the BUME method with slight modifications [32]. The upper lipophilic phase was completely dried in Speedvac until the organic solvents evaporated. This phase was then frozen at -80 °C until NMR analysis. Lipid signals in 1H-NMR spectra were quantified using LipSpin [33], an in-house program based on Matlab.

2.5.Analysis of markers of intake of phenolic compounds in plasma

The plasma samples were subjected to a protein precipitation procedure with minor modifications using a Sirocco Plate (Waters, Milford, MA, USA) as previously described [34]. Briefly, 100 µL of plasma samples were spiked with 10 µL of 1 mg/L myristoyl-L-carnitine d9 and ferulic acid 13C3 in water. The samples were subsequently mixed with 500 µL of cold acetonitrile (−20 °C) containing 1.5 M formic acid and 10 mM ammonium formate in the plate, vortexed for 1 min, and kept at −20 °C for 10 min to promote protein precipitation. A Waters Positive Pressure-96 Processor was used to collect the extracts in 96-well collection plates, which were taken to dryness under a stream of nitrogen gas. Finally, the samples were reconstituted in 100 µL of water:acetonitrile (80:20, v/v) containing 0.1% formic acid (v/v) and 100 µg/L of the taxifolin and caffeine 13C3 and centrifuged at 1000 g for 5 min. Clean extracts then were transferred to 96-well plates for further analysis.

Analyses were performed on a 1290 Infinity UHPLC system (Agilent, Santa Clara, CA, USA) coupled to a QTRAP 6500 mass spectrometer equipped with Ion Drive Turbo V ion source (Sciex, Framingham, MA, USA). Chromatographic separation was achieved on a Luna Omega Polar C18 column (100 mm × 2.1 mm (i.d. 1.6 µm)) using a fully porous polar C18 security guard cartridge (Phenomenex, Torrance, CA, USA). The chromatographic conditions can be found here [34]. Mass spectrometry detection was performed under positive and negative ionisation in separate runs by using the scheduled multiple reaction monitoring (sMRM) mode. The general MS parameters were as follows: ion spray voltage, +4500/−3500 V; source temperature, 600 °C; curtain gas, 30 psi; ion source gas 1 and gas 2, 50 psi each; collision-activated dissociation gas, 3 psi; entrance potential, ±10 V; and target scan time, 0.05 s.

Calibration curves were prepared at 12 concentration levels in the 0.1–4000 µg/L range. Compounds lacking the corresponding commercial standard were semi-quantified using the calibration curves of structurally similar metabolites.

2.6. *Statistical analysis*

All our results are expressed as mean ± standard error of the mean (SEM). P-values < 0.05 were considered statistically significant. For parametric data, we used Student's T test when comparing two groups of parametric data, or Mann–Whitney U test when comparing two groups of non-parametric data. All calculations were performed using Lumivero XLSTAT 2023.1.5 software (Addinsoft, New York, NY, USA). Each figure or table indicates the analysis performed.

2.6.1. Integrative Analysis and Variable Selection

The whole process of data processing, integration, variable selection pipeline and statistical analysis outlined in this section was conducted using RStudio version 2023.06.0 Build 421 (2009-2023 Posit Software, PBC).

All initial data were subjected to pre-processing. This included elimination of parameters with more than seven missing values and median imputation for missing values of the others. Once the data were processed, we continued with our analysis. To analyse the parameters that best discriminate between our groups, we applied the following procedure: for the results of gene expression, we used 1/ΔCt as the value expressing the quantity of gene expression and for the other parameters, we used the values obtained from the processing. To rank the variables, we used three machine-learning methods: Elastic Net, Random Forrest (RF) and Partial Least Squares-Discriminant Analysis (PLS-DA). For Elastic Net and PLS-DA, the data were scaled using the built-in "ScaleData" function. Briefly, Elastic Net is a linear regression method used to regularise a wide range of parameters; Random Forrest is an algorithm used for classification and regression problems; and PLS-DA is a dimensionality-reduction method. The parameters for each method underwent optimisation through 100-times-repeated 5-fold cross-validation. To avoid overfitting, we used the glmnet R package and followed a rule that adds a little flexibility. We applied the above machine-learning algorithms to compare all parameters and determine how well the parameters distinguished between the young and the aged cohorts. Each method therefore produced scores that reflect the importance of variables in this task. These scores were treated as individual values for each variable within each method. The sum of the values obtained from the three methods yielded the final value. This value indicates the overall importance of the variables in the context of our study, i.e. how well an individual parameter differentiates between these age groups. The one with the highest score was identified as the variable with the most significance in our study. With these results we conducted an integrative analysis that ranked all variables based on their order of importance. This selection method enabled us to concentrate our analysis on variables with the greatest discriminatory power between the groups.

For the correlation analysis we used Spearman rank-order correlation with all the data that underwent the above processing steps without separating the patients by age.

3. Results

3.1. The concentrations of most endogenous plasma biomarkers increased with age

Our first aim was to analyse endogenous plasma biomarkers and define the ageing-induced changes on metabolic parameters and inflammatory markers in the two age cohorts. To do so we conducted a panel analysis of various parameters in the plasma obtained from patients during biopsy sampling and compared a young group (38.9 ± 6 years) with an aged group (63.6 ± 6 years).

Figure 1 shows the results for several essential enterohormonal parameters and inflammatory markers. The enterohormones that significantly increased in the aged cohort were C-peptide, CCK, active GLP-1, PP, and glucagon (**Figure 1A**). The same trend was observed in relation to the inflammatory markers IL-6 and MCP-1, which were also elevated in the aged group (**Figure 1B**).

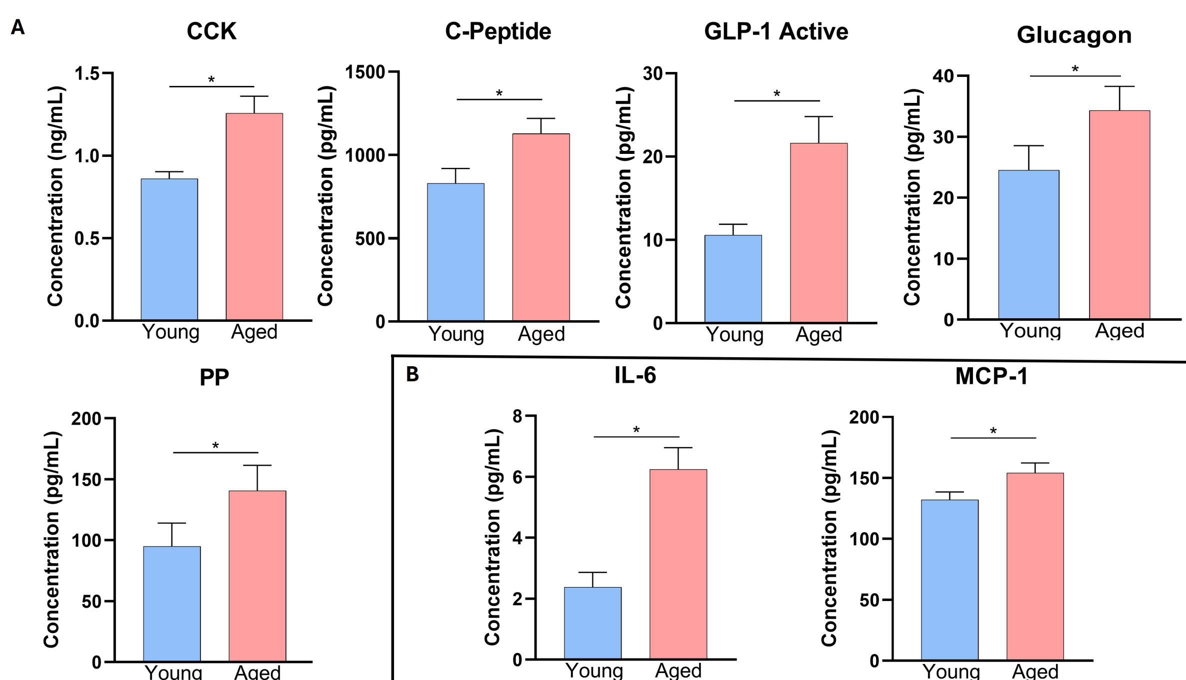

***Figure 1: Parameters analysed in plasma that were significantly modified by ageing**. A) Enterohormones; and B) Inflammatory markers (all concentrations are in pg/mL; * p<0.05; T-test or Mann–Whitney U test was used depending on the distribution of the data; n=15-25).*

To obtain a more complete metabolic picture of the changes that are produced as we age, we then conducted a comprehensive metabolomic analysis of 60 different parameters with additional focus on lipoprotein characterisation (supplementary appendix A2). **Table 1** shows the 23 parameters with statistically significant differences between the age groups.

Table 1: *Statistically different metabolic parameters analysed in plasma. Data are shown as mean ± SEM ($p<0.05$; the higher values are indicated in bold; $p< 0.1$ is indicated in italics; T-test or Mann–Whitney U test was used depending on the distribution of the data; n=20-25).*

Group	Parameter	Young	Aged
Low-Molecular-Weight Molecules	Creatine (µmol/L)	48.40 (± 2.53)	**58.99 (± 3.33)**
	Glucose (mmol/L)	3.02 (± 0.10)	**3.46 (± 0.15)**
	Glycine (µmol/L)	**155.03 (± 6.17)**	132.64 (± 5.33)
	Histidine (µmol/L)	**50.80 (± 1.36)**	45.03 (± 2.20)
	Lactate (µmol/L)	153.51 (± 10.17)	**190.48 (± 11.20)**
Lipoproteins	IDL-Cholesterol (mg/dL)	6.03 (± 0.31)	**9.71 (± 0.65)**
	LDL-Cholesterol (mg/dL)	125.80 (± 2.97)	**142.66 (± 4.37)**
	IDL-Triglycerides (mg/dL)	7.43 (± 0.28)	**9.89 (± 0.44)**
	LDL-Triglycerides (mg/dL)	10.81 (± 0.28)	**14.45 (± 0.67)**
	LDL-P (nmol/L)	1197.43 (± 27.68)	**1378.36 (± 41.35)**
	Medium LDL-Particle Number (nmol/L)	335.43 (± 11.88)	**437.97 (±21.55)**
	Small LDL-Particle Number (nmol/L)	638.86 (± 12.77)	**717.97 (± 19.78)**
	Medium HDL-Particle Number (µmol/L)	**11.41 (± 0.33)**	10.14 (± 0.26)
Glycoprotein	Glyc-B (µmol/L)	288.45 (± 7.99)	**320.18 (± 7.85)**
	Glyc-A (µmol/L)	559.25 (± 15.21)	**629.12 (± 16.85)**
	H/W Glyc-B	3.63 (± 0.1)	**4.03 (± 0.1)**
	H/W Glyc-A	12.85 (± 0.26)	**14.99 (± 0.39)**
Lipid	Free cholesterol (mmol/L)	2.39 (± 0,05)	**2.60 (± 0.06)**
	Polyunsaturated fatty acids (mmol/L)	14.72 (± 0.45)	**17.38 (± 0.58)**
	Linoleic Acid (mmol/L)	*4.15 (± 0.13)*	3.86 (± 0.10)
	omega-3 fatty acids (mmol/L)	0.30 (± 0.02)	**0.40 (± 0.02)**
	Docosahexaenoic acid (mmol/L)	0.11 (± 0.01)	**0.19 (± 0.01)**
	Arachidonic acid + Eicosapentaenoic acid (mmol/L)	1.23 (± 0.04)	**1.38 (± 0.05)**

As we can see, most of the statistically significant parameters were increased in the aged cohort. In the group of low-molecular-weight molecules, an increase with ageing was observed in the levels of creatine, glucose and lactate, while the levels of glycine and histidine were significantly lower in the aged group. In the panel of lipoproteins, the levels of IDL and LDL constituents, their

particle number and concentrations clearly tended to be higher in the aged cohort with only the number of HDL particles displaying an opposite trend. The concentrations of glycoproteins N-acetylglucosamine/galactosamine (Glyc-A), sialic acid (GlycB) and their aggregation states (height/weight; H/W) were again higher in the aged cohort. Finally, all lipid molecules analysed (free cholesterol, polyunsaturated fatty acids, omega-3, docosahexaenoic acid, arachidonic acid and eicosapentaenoic acid) except linoleic acid were significantly higher in the aged group than in the young group.

3.2. TAS2R5 and TAS2R38 were among the internal parameters that best distinguished between the young and the aged cohort

The next aim of the study was to use the results we obtained together with the expression analysis of TAS2Rs we had previously published (Submitted results) to create a list of the most relevant parameters for distinguishing between the age groups and identify whether bitter taste receptors are important in this ranking. Our analysis began with a total of 82 parameters. To do so we used three machine-learning methods to characterise the optimal parameters for achieving this goal. We then combined the results from all three methods and created a final list of the most important discriminatory parameters (**Figure 2**).

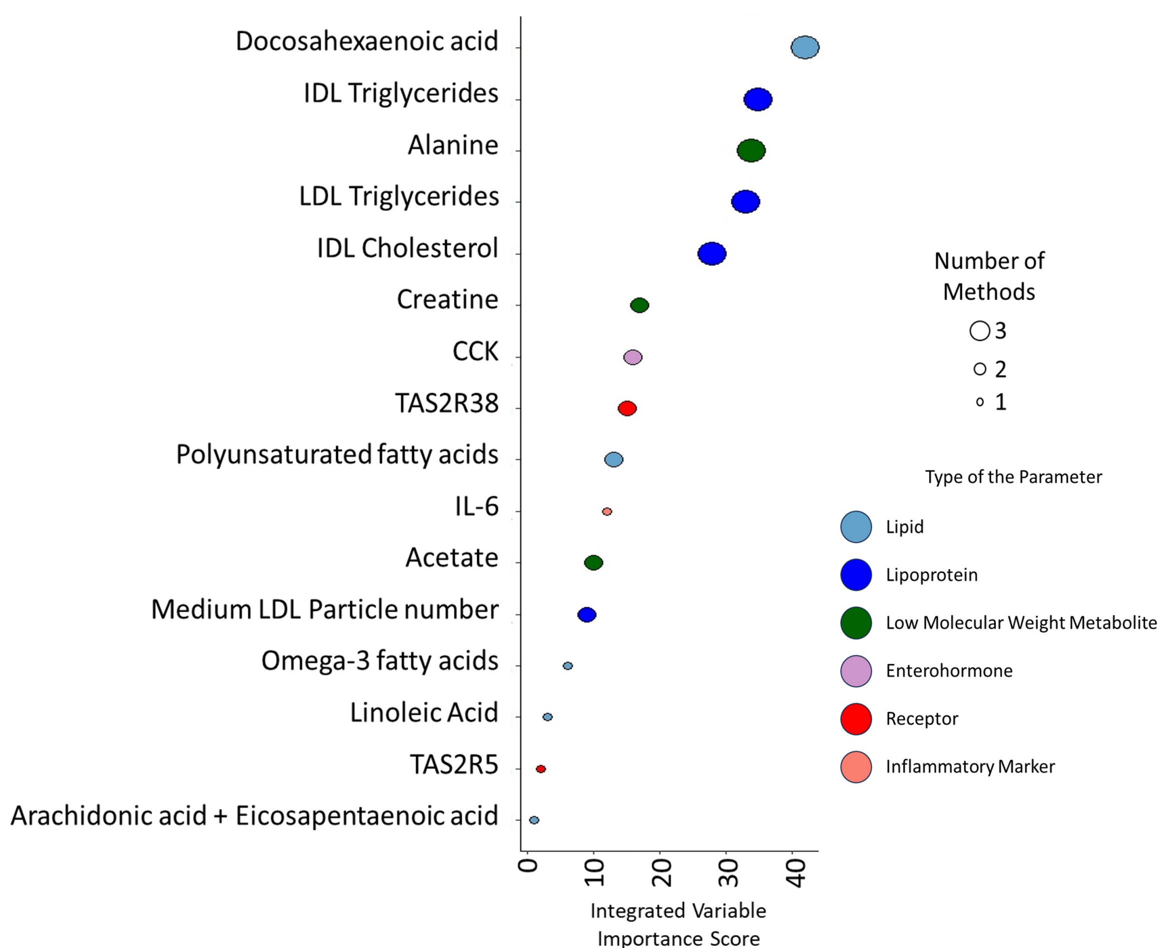

***Figure 2: Ranking of the best variables for discriminating between the age cohorts**. The methodology used to obtain this figure is explained in Materials and Methods chapter 2.6.1.*

At first glance the parameters appear to be divided into two groups depending on their importance – one with the first five parameters and one with the rest. Of all the groups of parameters we quantified (receptors, enterohormones, inflammatory markers, lipoproteins, glycoproteins, low-molecular-weight metabolites and lipids), only glycoproteins did not appear in this analysis.

If we take a closer look at the biomarkers, we see that docosahexaenoic acid is the most important distinguisher between the ages with all three methods used. In this first group of biomarkers,

some lipoproteins (IDL-Triglycerides, LDL-Triglycerides and IDL-Cholesterol) and, interestingly, alanine were the biomarkers selected.

The second observed group in the **Figure 2** contained more parameters. Especially interesting for us was the appearance of two bitter receptors (TAS2R38 and TAS2R5) that were described as significant by two methods and one method, respectively.

All the parameters selected in **Figure 2** appear as statistically significant in **Figure 1** and **Table 1**, with the notable exception of acetate, which was labelled as a good distinguishing biomarker by two of the three techniques.

3.3.All TAS2R displayed at least one significant correlation with the measured biomarkers

Once we had established which parameters were interesting for further studies in the context of ageing, we focused on the interplay between these, and other molecules and the bitter receptors found in the GIT.

At first, we aimed to establish the possible connection between the TAS2R present in the GIT and the biomarkers measured in plasma in the overall population.

To better understand this and the potential importance of interactions between bitter taste receptors and the endogenous biomarkers we measured, we conducted a correlation analysis. **Figure 3** shows the correlation heatmap of TAS2R with biomarkers that had at least one significant correlation as calculated by Spearman's rank correlation.

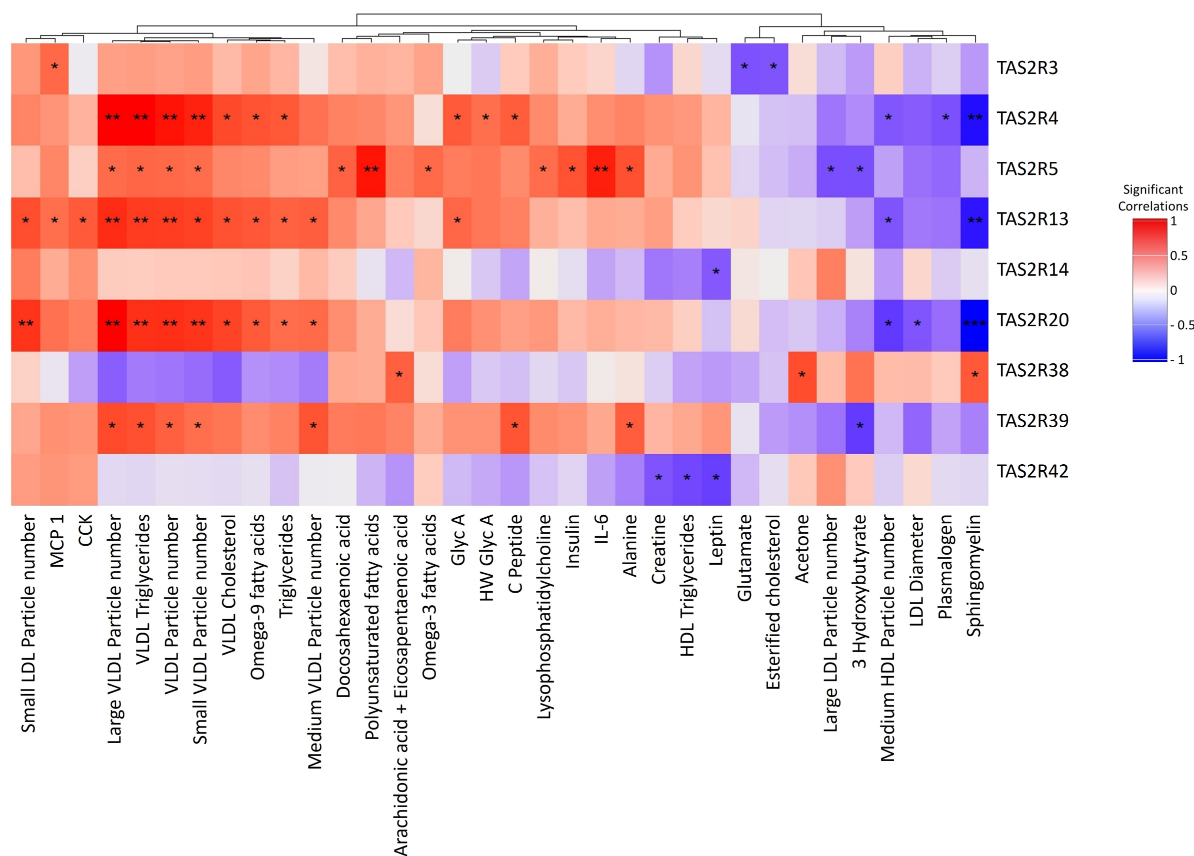

Figure 3: Correlation heatmap. *Results of the correlation analysis between the TAS2R and the endogenous biomarkers (Spearman rank-order correlation; * p<0.05; ** p<0.01; n=26).*

A closer look at this heatmap reveals that most of the significant correlations are characterised as positive (illustrated by the predominantly red colour in **Figure 3**). If we focus on the receptors, we see that all nine TAS2R analysed here displayed at least one correlation with at least one biomarker. Interestingly, TAS2R13 was the receptor with the highest number of biomarkers (14), most of which were positive (MCP-1, various lipoproteins, CCK, triglycerides) though some were significantly negative (several HDL particles and sphingomyelin). The next two receptors with the highest numbers of correlations were TAS2R4 and TAS2R5, with largely similar correlation profiles to that observed for TAS2R13. Interestingly, the receptor with the least number of correlations was TAS2R14 – which is described in the literature as being the most promiscuous [35] and which we have previously reported as the most abundant receptor in various parts of the

GIT (Submitted results) – since it only correlated negatively with leptin, a hormone released by adipose tissue.

Switching our focus to biomarkers, we found that several lipoproteins correlated with five TAS2Rs. The most important biomarker for distinguishing between the age groups (docosahexaenoic acid) only correlated with one receptor (TAS2R5), which was also one of the significant discriminators between age groups (**Figure 2**). Several other biomarkers labelled as important in that analysis (IDL and LDL triglycerides, IDL cholesterol, alanine, creatine, CCK, polyunsaturated fatty acids, IL-6, numerous medium LDL particles, omega-3 fatty acids, and arachidonic and eicosapentaenoic acid) were also found to correlate with several TAS2R, all of which were positive except with creatine, and most of which had correlations with TAS2R5.

3.4. TAS2R5 and TAS2R38 were the bitter taste receptors among the metabolite markers of phenolic consumption that best discriminated between the young and the aged cohorts

TAS2R located in intestinal mucosa are a clear target for food-derived compounds. Since phenolic molecules have been shown to be TAS2R ligands, we also aimed to investigate how different polyphenolic components in the diets of our participants may have interacted with local bitter receptors and sought to establish their potential effect on the other, more global, parameters.

Table 2: Analysis of phenolic markers in plasma*. Only parameters that were significantly different statistically are shown. Data are shown as mean ± SEM (* $p<0.05$; # $0.05<p<0.1$; the higher values are indicated in bold; T-test; n=20-25).*

Parameter	Young (ppb)	Aged (ppb)
3-hydroxyphenylacetic Acid	11.37 (± 0.96)	**16.49** # (± 1.96)
Catechol Sulphate	1540.98 (± 289.7)	**3351.34** * (±785.0)
Lactic Acid	58783.91 (± 2812.9)	**70692.80** * (± 3506.2)
Pyrogallol Sulphate 1	**2735.09** # (± 1235.7)	780.00 (± 256.6)

Our analysis encompassed 370 phenolic markers with a wide range of origins (supplementary appendix A3) [36]. The signal was detected for 78 of these markers. **Table 2** shows that both catechol sulphate and lactic acid were significantly different between the age cohorts (with higher levels in the aged group), while pyrogallol sulphate (with higher levels in the young group) and 3-hydroxyphenylacetic acid (with higher levels in the aged group) displayed a trend to increase in aged cohort.

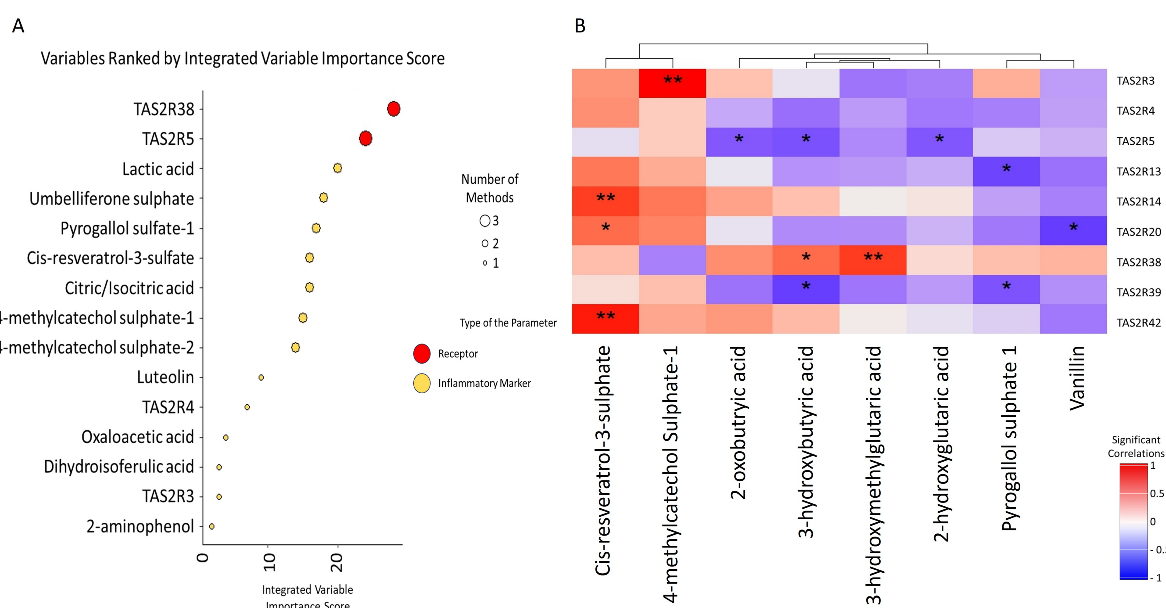

Figure 4: *Ranking* ***of the best phenolic markers for distinguish age cohort, and correlation heatmap with TAS2R.*** *A) Parameters ranked according to their capacity to differentiate between the young and aged the older groups; B) Significant correlations of the polyphenolic markers in plasma parameters (Spearman rank-order correlation; * p<0.05; ** p<0.01; n=26). How the ranking was calculated is described in Materials and Methods chapter 2.6.1.*

Since we were again interested in determining the most suitable parameters for differentiating between the two age groups, we conducted the same analysis as we did with the other markers. In this case we considered the same TAS2R as in the previous analysis plus the 78 phenolic plasma markers. **Figure 4A** shows that, this time, our analysis highlighted four receptors overall. The first two of these were again TAS2R5 and TAS2R38 and the two new ones were TAS2R4 and TASR2R3. Of the parameters considered here, TAS2R5 and TAS2R38 best discriminated between the two age groups.

To identify which TAS2R showed some correlation with these phenolic plasma markers we also ran a correlation analysis in which all groups were considered together. This analysis again highlighted TAS2R5 with three negative correlations, followed by TAS2R20, TAS2R38 and TAS2R39 with two (**Figure 4B**). Two parameters worth emphasising were cis-resveratrol-3-sulphate and 3-hydroxybutyric acid, each of which had three correlations with TAS2R.

4. Discussion

The broad approach we adopted in this study enabled us to describe the relationship between the ageing process and colonic bitter taste receptors. We ran two integrative analyses in which the gene expressions of the nine TAS2R in the colon were considered – one analysis with endogenous plasma components and the other with exogenous phenolic plasma markers – to determine the most important parameters for differentiating between the age cohorts. TAS2R5 and TAS2R38 were identified in both analyses.

TAS2R5 is one of the most explored receptors [37,38]. However, as with all bitter receptors, most of their roles in extraoral tissues are yet to be determined. We have previously shown that the expression of this receptor in the descending colon is increased by ageing (Submitted results). Now we clearly show that it plays an important role in the ageing process. Unlike previous studies in which TAS2R showed no significant correlations with HDL, LDL, and triglycerides [37], we have shown that TAS2R5 correlated with lipoprotein constituents in plasma. Kim et al. recently discovered a novel form of interaction between TAS2R and their ligands when they identified cholesterol as a key modulator of ligand recognition by TAS2R14 [39]. They also showed that cholesterol, which is abundant in the cellular membrane, binds to the extracellular binding site of this receptor, while the bitter compound binds to the intracellular allosteric binding site. Total activation of this bitter receptor was possible only when both cholesterol and the bitter ligand were bound. Together with the fact that bile acids, which are structurally similar to cholesterol

since they share common steroid motif, activates bitter receptors (-1, -4, -14, -39), cholesterol and its carriers probably play an important role in the activation and signal transduction of TAS2R [40]. Overall, these correlations between TAS2R5 and the lipoprotein particles and their constituents are interesting to further reflect on especially when we consider that, with ageing, dysregulation of lipoproteins contributes greatly to the manifestation of age-related disorders.

TAS2R5 also correlated with polyunsaturated fatty acids (PUFAs), which, interestingly, are detected by another member of the G protein-coupled receptors family, GPR120 [41]. Moreover, GPR120 shares both the type of cells in which it is expressed (enteroendocrine cells) and the function (GLP-1 regulation) with other TAS2R [42,43]. Some PUFAs, such as docosahexaenoic acid (the best for differentiating between the young and the aged cohorts), have been found to bind to TAS2R [44]. PUFAs also have multiple beneficial effects on the elderly [45,46], and it would be interesting to see whether the impact of docosahexaenoic acid and other PUFAs is due to their interactions with various TAS2R.

We also observed strong and significant correlations between TAS2R5 and inflammatory marker IL-6. In the intestine, the activation of bitter receptors induces the release of antimicrobial peptides and regulates the expression of other innate immune factors [47]. TAS2R have also been observed in tuft cells of the GIT, where such cells can detect potential helminthic and protozoan infections [48]. While evidence of the precise role of TAS2R regulation of inflammation in the GIT is still inconclusive, TAS2R5 may be an interesting point of investigation as a target for inflammatory modulation.

We found significant correlation between TAS2R5 and three markers of phenolic consumption. 2-oxobutyric acid is an endogenous molecule that is formed during the metabolism of cysteine [49]. Its conjugate base, α-ketobutyrate, has been shown to increase lifespan in C. elegans [50]. 3-hydroxybutyric acid and its conjugate base 3-hydroxybutyrate (β-hydroxybutyrate) have been proposed as anti-ageing metabolites [51]. In an animal model, a bitter compound elicited an

increase in the level of β-hydroxybutyrate [52]. Little is known about 2-hyodroxyglutaric acid, but it has been shown that its accumulation due to the mutation of isocitrate dehydrogenase leads to a hypermethylated state of DNA and histones, which activates oncogenes and ultimately leads to cancer [53]. Although initial results suggest that these markers play a role in the ageing process, whether they interact with TAS2R5 is still unknown.

The second receptor that was labelled a good discriminator of age cohorts is TAS2R38. Unlike with TAS2R5, we had previously observed no age-related differences in its expression (Submitted results). Researchers investigating the distribution of homozygous variants of this receptor in the aged population have reported a connection between the genetic variance of TAS2R38 and longevity [25]. Some studies have associated genetic variations of this specific receptor with the clinical outcomes of medical conditions such as obesity [54].

In our analysis TAS2R38 had one of the fewest significant correlations since it correlated positively only with sphingomyelin, acetone and arachidonic and eicosapentaenoic acid (measured together). Sphingomyelin is an important membrane lipid that is significant for membrane fluidity and involved in the creation of ceramide, a lipid inductor of apoptosis [55]. Sphingomyelin accumulates during ageing, which induces cellular dysfunction and some inflammatory cytokines [56]. As an important membrane lipid that regulates the function of G protein-coupled receptors such as TAS2R, sphingomyelin was studied for its potential effects on the signalling of TAS2R14, but no significant effect was found. However, its potential influence on other receptors has not been assessed [57]. Acetone, one of the ketone bodies produced by the liver, is usually elevated in diabetes, a component of the metabolic syndrome whose prevalence increases with age [58]. Interestingly, in cellular models that evaluated the response to different bacterial metabolites, it was shown that TAS2R38 was activated by acetone [59]. With regard to fatty acids such as arachidonic and eicosapentaenoic acids, in addition to the GPR120 described above, the cluster of differentiation 36 (CD36) glycoprotein has also been identified as

a possible receptor for fatty acids [60]. There is also evidence that TAS2R38 may play a role in fatty acid signalling by modulating CD36 functionality. Firstly, people with greater sensitivity to the TAS2R38 ligand 6-n-propylthiouracil (PROP) were also more sensitive to detecting fat [61]. Further studies have also shown that specific haplotypes of TAS2R38 and sensitivity to PROP are linked with the polymorphism of CD36 [62], which indicates that sensitivity to detecting bitter and sensitivity to detecting fat (such as fatty acids) are interlinked [63].

While TAS2R5 and TAS2R38 were the receptors that best discriminated between the two age cohorts, our results showed that TAS2R13 is the receptor with the highest number of correlations (fourteen) in the analysis of endogenous biomarkers. TAS2R13 was also the only receptor that correlated with one of the phenolic consumption markers that was significantly different between the age groups: pyrogallol-1-O-sulphate. This compound is the result of the microbial metabolism of catechol-moiety-containing polyphenols, which are structural motifs often found in bitter agonists (such as epigallocatechin gallate) [44,64]. Changes to the composition of microbiota are strongly affected by both internal and external factors, one of the most impactful of which is ageing [65]. It has been demonstrated that by influencing the microbiome in our gut, we can modulate and lower inflammation, which is an important factor in achieving healthier ageing [66,67]. It is also known that the secretome of the microbiota is important in controlling food intake and, therefore, body weight [68]. Researchers have also recently shown that microbes modulate the taste receptors in the GIT through the molecules they secrete and suggested that correlations exist between specific strains in the gut and taste perception [69]. Finally, an animal model of obesity showed that a high-fat diet can influence the microbiota as well as the expression of bitter receptors, which raises the idea that the luminal microbes influence the expression of bitter taste receptors [70].

TAS2R13 also correlated with CCK, an anorexigenic enterohormone that regulates the metabolic response to the intake of nutrients and whose levels increase with ageing [71,72]. The role of

TAS2R in the modulation of enterohormone secretion has been well described [73,74] and some enterohormones have been associated with the onset of age-associated diseases [75].

We have also found that several fractions of lipoproteins are good separators of the age cohorts. This is in line with previous studies in which higher levels of certain lipoprotein constituents, such as IDL cholesterol and IDL- and LDL-triglycerides, were associated with myocardial disfunction, diabetes, diabetic retinopathy and many other metabolic disorders [76–79]. The metabolic syndrome and its associated disorders are known to affect health in the aged population.

5. Conclusions

In conclusion, we have investigated the potential of bitter taste receptors present in the GIT as markers for age discrimination. TAS2R5 and TAS2R38 are identified as the most promising candidates based on their ability to distinguish between younger and older individuals. Interestingly, TAS2R5 correlated with several lipoproteins and potentially plays a role in inflammatory response, which suggests there are possible connections to age-related metabolic changes. TAS2R38, on the other hand, showed significant correlations with factors potentially linked to liver function. However, further research is needed to elucidate the precise roles of these TAS2Rs and to determine whether their expression changes are a cause or a consequence of the ageing process.

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DECLARATIONS OF INTEREST:

None.

AUTHOR CONTRIBUTIONS:

Conceptualization: FB, JGS, AA, MP; Data curation: FJ, HS, MMH; Formal analysis: FJ, TO, LAJ, CAL, MMH; Funding acquisition: RBD, ERG, XT, AA, MP; Investigation: FJ, HS, TO, LAJ, AFI; Methodology: FJ, FB, TO, RM, AFI, MMH, XT; Project administration: FB, JGS, RBD, ERG, XT, AA, MP; Resources: FB, RM, JGS, CAL; Supervision: RBD, AA, MP; Roles/Writing - original draft: FJ, HS, ERG; Writing - review & editing: All authors.

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Supplementary data

Appendix A1: List of probes tested in the study:
TAS2R3, Hs00249942_s1; TAS2R4, Hs00249946_s1; TAS2R5, Hs01549633_s1; TAS2R13, Hs00256781_s1; TAS2R14, Hs00256800_s1; TAS2R20, Hs00604340_s1; TAS2R38, Hs00604294_s1; TAS2R39 Hs00603443_s1; TAS2R42, Hs00704057_s1; and RPS9, Hs02339424_g1.

Appendix A2: The list of biomarkers analysed in the metabolomic analysis:
Very low-density lipoprotein - Cholesterol, Intermediate density lipoprotein - Cholesterol, Low density lipoprotein - Cholesterol, High density lipoprotein - Cholesterol, Very low density lipoprotein - Triglycerides, Intermediate density lipoprotein - Triglycerides, Low density lipoprotein - Triglycerides, High density lipoprotein - Triglycerides, Very low density lipoprotein - Particle number, Large Very low density lipoprotein - Particle number, Medium Very low density lipoprotein - Particle number, Small Very low density lipoprotein - Particle number, Low density lipoprotein - Particle number, Large Low density lipoprotein - Particle number, Medium Low density lipoprotein - Particle number, Small Low density lipoprotein - Particle number, High density lipoprotein - Particle number, Large High density lipoprotein - Particle number, Medium High density lipoprotein - Particle number, Small High density lipoprotein - Particle number, Very low density lipoprotein - Diameter, High density lipoprotein - Diameter, Glyc-B, Glyc-F, Glyc-A, Height/Width Glyc-B, Height/Width Glyc-A, 3-Hydroxybutyrate, Acetate, Acetone, Alanine, Creatinine, Creatine, Glucose, Glutamate, Glutamine, Glycine, Histidine, Lactate, Tyrosine, Valine, Isoleucine, Leucine, Esterified cholesterol, Free cholesterol, Triglycerides, Glycerophospholipids (except LPC), Phosphatidylcholine, Sphingomyelin, Lysophosphatidylcholine, Plasmalogen, Linoleic Acid, Polyunsaturated fatty acids, Saturated fatty acids, omega-6 & omega-7 fatty acids, omega-9 fatty acids, omega-3 fatty acids, Docosahexaenoic acid, Arachidonic acid + Eicosapentaenoic acid.

Appendix A3: The list of phenolic markers analysed in the plasma samples:
Oxaloacetic acid, citric acid / isocitric acid, cis-aconitic acid, α-ketoglutaric acid, fumaric acid, malic acid, acetoacetic acid, 3-hydroxybutyric acid, glycolic acid, lactic acid, mevalonic acid, 5-hydroxyhexanoic acid, 2-isopropylmalic acid, citramalic acid, 2-hydroxyglutaric acid, 3-hydroxymethylglutaric acid, glyoxylic acid, pyruvic acid, 2-oxobutyric acid, 4-methyl-2-oxovaleric acid / 3-methyl-2-oxovaleric acid, 2-oxoadipic acid, itaconic acid, 5-aminolevulinic acid, lipoic acid, benzoic acid, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2-hydroxybenzoic acid glucuronide, 3-hydroxybenzoic acid glucuronide, 4-hydroxybenzoic acid glucuronide, 2-hydroxybenzoic acid sulphate, 3-hydroxybenzoic acid sulphate, 4-hydroxybenzoic acid sulphate, 2,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid 3-glucuronide, 3,4-dihydroxybenzoic acid 4-glucuronide, 3,5-dihydroxybenzoic acid 4-glucuronide, dihydroxybenzoic acid glucuronide, 3,4-dihydroxybenzoic acid 3-sulphate, 3,4-dihydroxybenzoic acid 4-sulphate, 3,5-dihydroxybenzoic acid sulphate, dihydroxybenzoic acid sulphate, hippuric acid, 4-hydroxyhippuric acid, 3-hydroxyhippuric acid, 2-hydroxyhippuric acid, vanillic acid, isovanillic acid, vanillic acid glucuronide, isovanillic acid glucuronide, vanillic acid sulphate, isovanillic acid sulphate, syringic acid, syringic acid glucuronide, syringic acid sulphate, gallic acid, gallic acid 3-glucuronide, gallic acid 4-glucuronide, gallic acid 3-sulphate,

gallic acid 4-sulphate, 4-O-methylgallic acid, 3-O-methylgallic acid, methylgallic acid glucuronide 1, methylgallic acid glucuronide 2, methylgallic acid glucuronide 3, methylgallic acid sulphate 1, methylgallic acid sulphate 2, methylgallic acid sulphate 3, ethylgallate, ethylgallate glucuronide, ethylgallate sulphate, phenylacetic acid, 2-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 2-hydroxyphenylacetic acid glucuronide, 3-hydroxyphenylacetic acid glucuronide, 4-hydroxyphenylacetic acid glucuronide, 2-hydroxyphenylacetic acid sulphate, 3-hydroxyphenylacetic acid sulphate, 4-hydroxyphenylacetic acid sulphate, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid glucuronide, 3,4-dihydroxyphenylacetic acid sulphate, homovanillic acid, homovanillic acid 4-glucuronide, homovanillic acid 4-sulphate, trans-cinnamic acid, o-coumaric acid, m-coumaric acid, p-coumaric acid, cis p-coumaric acid, o-coumaric acid glucuronide, m-coumaric acid glucuronide, p-coumaric acid glucuronide, cis p-coumaric acid glucuronide, o-coumaric acid sulphate, m-coumaric acid sulphate, p-coumaric acid sulphate, cis p-coumaric acid sulphate, 3,5-dihydroxycinnamic acid, caffeic acid, 3,5-dihydroxycinnamic acid glucuronide, caffeic acid 3-glucuronide, caffeic acid 4-glucuronide, 3,5-dihydroxycinnamic acid sulphate, caffeic acid 3-sulphate, caffeic acid 4-sulphate, 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, ferulic acid, cis ferulic acid, isoferulic acid, ferulic acid 4-glucuronide, cis ferulic acid 4-glucuronide, ferulic acid 4-sulphate, cis ferulic acid 4-sulphate, isoferulic acid 3-glucuronide, isoferulic acid 3-sulphate, sinapic acid, cis sinapic acid, sinapic acid glucuronide, sinapic acid sulphate, phenylpropionic acid, 3-(2-hydroxyphenyl)propionic acid, 3-(3-hydroxyphenyl)propionic acid, 3-(4-hydroxyphenyl)propionic acid, hydroxyphenylpropionic acid glucuronide, hydroxyphenylpropionic acid sulphate, dihydrocaffeic acid, 3-(3,5-dihydroxyphenyl)propionic acid, dihydrocaffeic acid 3-glucuronide, 3-(3,5-dihydroxyphenyl)propionic acid glucuronide, dihydrocaffeic acid 3-sulphate, 3-(3,5-dihydroxyphenyl)propionic acid sulphate, dihydroferulic acid, dihydroisoferulic acid, dihydroferulic acid 4-glucuronide, dihydroisoferulic acid 3-glucuronide, dihydroferulic acid 4-sulphate, dihydroisoferulic acid 3-sulphate, 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, 3-(3,5-dihydroxyphenyl)pentanoic acid, 3-(3,5-dihydroxyphenyl)pentanoic acid glucuronide, 3-(3,5-dihydroxyphenyl)pentanoic acid sulphate, 2-(3,5-dihydroxyphenyl)ethanol sulphate, pyrogallol, pyrogallol glucuronide 1, pyrogallol glucuronide 2, pyrogallol sulphate 1, pyrogallol sulphate 2, 1-O-methylpyrogallol, 2-O-methylpyrogallol, methylpyrogallol glucuronide 1, methylpyrogallol glucuronide 2, methylpyrogallol sulphate 1, methylpyrogallol sulphate 2, methylpyrogallol sulphate 3, catechol, catechol glucuronide, catechol sulphate, 4-methylcatechol, 4-methylcatechol glucuronide 1, 4-methylcatechol glucuronide 2, 4-methylcatechol sulphate 1, 4-methylcatechol sulphate 2, 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde, vanillin, catechin, epicatechin, epicatechin glucuronide 1, epicatechin glucuronide 2, epicatechin glucuronide 3, epicatechin glucuronide 4, epicatechin glucuronide 5, epicatechin sulphate 1, epicatechin sulphate 2, epicatechin sulphate 3, epicatechin sulphate 4, epicatechin sulphate 5, 4'-O-methylepicatechin, 3'-O-methylepicatechin, methyl(epi)catechin glucuronide 1, methyl(epi)catechin glucuronide 2, methyl(epi)catechin glucuronide 3, methyl(epi)catechin glucuronide 4, methyl(epi)catechin glucuronide 5, methyl(epi)catechin sulphate 1, methyl(epi)catechin sulphate 2, methyl(epi)catechin sulphate 3, methyl(epi)catechin sulphate 4, methyl(epi)catechin sulphate 5, methyl(epi)catechin sulphate 6, epigallocatechin, epigallocatechin glucuronide, epigallocatechin sulphate, epicatechin gallate, epigallocatechin gallate, procyanidin B2, 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid, 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid 3'-glucuronide, 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid 4'-glucuronide, 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid 3'-sulphate, 4-hydroxy-5-(3',4'-

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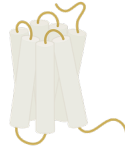

Part 3:

Evaluation of the Impact of Sub-Chronic Grape Seed Procyanidin Extract on the Presence of Intestinal Bitter Taste Receptors, and Distilling the Most Likely Candidate for the Observed Effect

Manuscript 4:

Subchronic modulation of bitter taste receptors (TAS2R) by procyanidins Unravelling the complex interplay between stimulation and expression

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Subchronic modulation of bitter taste receptors (TAS2R) by procyanidins Unravelling the complex interplay between stimulation and expression

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* Equivalent role

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Abstract

Mediated by the bitter taste receptors (TAS2R), bitter taste perception involves not just the oral cavity but various physiological systems throughout the gastrointestinal tract. The relationship between stimulation and modulation is crucial for understanding the broader implications of bitter taste signalling in health and disease. We investigated three subchronic TAS2R stimulations and assessed their impact on the gene expression of main rTas2r, their association with obesity, and their effects on GLP-1 secretion. Treating rats with procyanidins up-regulated rTas2r between duodenum and the ascending colon. Epicatechin up-regulated rTas2137, -139, -143 and -144 in jejunum. In Hutu-80 cells, epicatechin downregulated TAS2R14 after 24 hours, which limited GLP-1 secretion after acute peptone stimulation. Our results support a network effect in the role of the bitter taste receptors along the intestinal areas that must be considered to address the work with bitter agonists.

Keywords: bitter taste receptors, intestine, rat, chronic treatment, Tas2r agonism; grape-seed procyanidins, epicatechin

Introduction

The gustatory system plays a pivotal role in shaping dietary preferences and influencing overall health. Mediated by the bitter taste receptor (TAS2R) family of G protein-coupled receptors, bitter taste perception involves not just the oral cavity but various physiological systems throughout the gastrointestinal tract (GIT).[1–3] Understanding the intricate relationship between TAS2R stimulation and the modulation of TAS2R expression is essential if the broader implication of bitter taste signalling in health and disease is to be elucidated.

Grape-seed procyanidin extract (GSPE) is a complex mixture of several molecules that have proved to be agonists of several TAS2R. [4,5] After intake, they undergo slight modifications in the upper IT; subsequently, several monomeric, dimeric and trimeric forms are absorbed and partially metabolised. The other bigger structures reach the lower GIT where, together with modified forms secreted through the biliary secretions, they break down and are modified by the microbiota. [6] Our group has proved that this extract has several health-promoting effects [7–12] (for example, in a context of diet-induced obesity, it is antiobesogenic). [13,14] Some of the antiobesogenic effects can be explained by how the extract affects food intake. When administered acutely, GSPE inhibits food intake in part through the induction of GLP-1 release. [15] We have shown that some of the flavonols in GSPE modulate enteroendocrine secretion through interactions with TAS2Rs. [16] However, the antiobesogenic effect of GSPE is not limited to food intake and, although there is little information available at present, TAS2R modulation may also play a role.

The agonism of some flavonoids on human TAS2R [4,5,17] (hTAS2R) and mouse Tas2r[18] (mTas2r) has been described in studies on the functional characterization of chemosensory receptors by heterologous expression in mammalian cell lines (HEK 293T). Bioinformatic approaches have also been used to great effect to provide insight into the promiscuity and selectivity of various ligands binding to different TA2Rs.[19–21] *In vivo* studies of the administration of some mTas2r

agonists have shown that activating these receptors has beneficial effects on health.[22] However, there is little information on the effects of the chronic stimulation of bitter taste receptors. Considering their relatively high promiscuity, the possibility of heterologous desensitisation should also be considered. Likewise, studies on human airway smooth muscle (HASM) cells have shown that TAS2R14 undergoes rapid agonist-promoted desensitization and can experience up to a 50% loss of function.[23] All the tested agonists, except for one, caused subsequent TAS2R14 internalization and trafficking via early and late endosomes, leading to its down-regulation. [23]

In this study, we investigated the *in-vivo* response to subchronic GSPE stimulation on the most highly expressed rat Tas2r (rTas2r) in various intestinal regions[24], and examined the role played by rTas2r in the effects of GSPE under conditions of obesity. We aimed to elucidate the impact of subchronic epicatechin treatment on TAS2R expression and of gene expression alterations on the sensitivity of these receptors to stimulation.

Materials and methods

Animal experiments

<u>GSPE treated cafeteria animals</u>

The grape-seed proanthocyanidin extract (GSPE) was provided by Les Dérivés Résiniques et Terpéniques (Dax, France). According to the manufacturer, the composition of the GSPE used in this study (batch number 124029) includes monomers of flavan-3-ols (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5-13 units; 31.7%). 25

Thirty female Wistar rats, each weighing between 240-270g and seven weeks of age, were housed individually at a room temperature of 22°C with a standard 12-h light-dark cycle. During a week of adaptation to the environment, the rats were fed ad libitum with a standard chow diet (Panlab 04, Barcelona, Spain) and tap water. Then, the rats were randomly distributed into three

experimental groups (n=10/group) all of which were fed with a cafeteria diet. The study design is summarized in **Figure 1** and has been described in depth elsewhere.26 The three experimental groups were distributed as follows: CAFETERIA (CAF): fed with a cafeteria diet; CORRECTIVE 500 (500): fed with a cafeteria diet and given a corrective dose of 500 mg GSPE/kg for the last fifteen days of treatment; CORRECTIVE 100 (100), fed with cafeteria diet and given a corrective dose of 100 mg GSPE/kg for the last fifteen days of treatment.

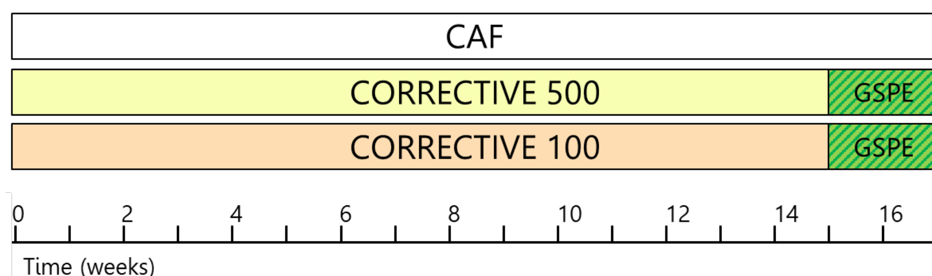

Figure 1: Schematic diagram of the experimental design with a cafeteria diet

The cafeteria diet was administered every day for 17 weeks and included bacon, biscuits with pâté, carrots, muffins, sausages, and sugared milk. Both corrective GSPE treatments were given by oral gavage daily during the last 15 days of the cafeteria intervention. The control cafeteria group received a dose of tap water as a vehicle. 25

At the conclusion of the study, the animals underwent a fasting period of 1-4 hours before being anesthetized with sodium pentobarbital (70 mg/kg BW; Fagron Iberica, Barcelona, Spain) and subsequently exsanguinated from the abdominal aorta. Blood samples were obtained using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Then plasma was obtained through centrifugation and stored at -80ºC. Finally, the tissues were rapidly collected and stored at -80ºC. All procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili (code: 0152S/4655/2015).

Epicatechin-treated animals

For this part of the study, we used eighteen 8-week-old female Wistar rats (Envigo, Barcelona, Spain). Upon arrival, rats were housed in pairs and underwent an adaptation period of 1 week, after which they were separated and again left to acclimatise for 1 week. All animals were kept under the same conditions, at a room temperature of 23 °C, with a standard 12-h light-dark cycle (lights on at 6:00) with ventilation. Animals had ad libitum access to tap water and a standard chow diet (2014 Teklad Global 14% protein rodent maintenance diet; Envigo, Barcelona, Spain). Afterwards the animals were distributed into two groups: Control, and Treatment. On the day of the treatment, rats were fasted, starting at 14:00, and the treatment was performed at 17:00, one hour before the lights were turned off. The treatment consisted of a dose of 400 mg/kg of body weight (BW) of epicatechin (Cat. No.: E1753, Sigma-Aldrich, Madrid, Spain) dissolved in water. The treatment was performed by oral gavage. The control group received water by the same method. The food was introduced at 18:00. Next day, the food intake was measured at 14:00, giving a food intake of 20 hours. This treatment was then repeated for eight more consecutive days, to give a total of nine days of treatment, while the food intake measurement was collected for the first 8 days of the treatment period. On the last day of treatment, the rats were fasted, starting at 23:00, and euthanized by decapitation next morning. The intestine and other tissues were carefully removed, measured, and weighed. Different intestinal segments were excised from the duodenum, the ileum, and the proximal colon. Samples of each segment were immediately frozen in liquid nitrogen and stored for subsequent analysis.

Cellular assays

The enteroendocrine HuTu-80 (ATCC, HTB-40) cell line was provided by LGCgroup (Barcelona, Spain). The cells were grown in culture flasks (Greiner Bio-One, Frickenhausen, Germany) at 37°C in an atmosphere of 5% CO2 and the medium was changed every 2–3 days. The growth

medium consisted of EMEM (Cat. No.: 30-2003, ATCC, Manassas, USA) supplemented with 10% v/v heat-inactivated foetal bovine serum (Cat. No.: 12103C, Sigma-Aldrich, Madrid, Spain) and 100 U/mL penicillin-streptomycin mixture (Cat. No.: DE17-602E, Lonza Bioscience, Basel, Switzerland). When confluence reached roughly 80%, the cells were harvested by treatment with 0.25% Trypsin 1 mol/L EDTA (Cat. No.: T3924, Sigma-Aldrich, Madrid, Spain) for 5 minutes, and then split and sub-cultured in fresh medium.

Basal gene expression analysis

For this experiment, passages 13-17 were used. HuTu-80 cells (200,000 cells/mL) were seeded into individual culture plates (Greiner Bio-One, Frickenhausen, Germany). After 48 hours, the cells were washed with cold PBS, and then the lysis buffer provided with the RNA extraction kit (RNeasy Plus Mini Kit; Cat. No.: 74134, Qiagen, Hilden, Germany) was added. The dishes were then frozen at -80°C, until the RNA was extracted.

Epicatechin chronic treatment

Gene expression analysis: For this experiment, passages 10-12 were used. HuTu-80 cells (200,000 cells/mL per well) were seeded into 12-well plates. After 72 hours, the cells were washed with PBS and treated for 24 hours with epicatechin at either 10 or 50 µM. The stock solutions of the treatments were prepared in DMSO (Cat. No.: SU01581000, Scharlab, S.L., Barcelona, Spain) and then diluted to final working concentration in the complete growth medium. The final DMSO concentration was 0.05%. The control group was given fresh growth medium with added DMSO at the same concentration as the treatments (0.05%). The next day, the cells were washed with cold PBS, and then the lysis buffer provided with the above-mentioned RNA extraction was added. The plates were then frozen at -80°C, until the RNA was extracted.

Enterohormone secretion analysis: For this experiment, passages 10-12 were used. HuTu-80 cells (200,000 cells/mL per well) were seeded into 12-well plates and treated with either 10 or 50 µM epicatechin for 24 hours as described above. The next day, the medium was aspirated, and the

cells were washed with PBS and treated for 2 hours with peptone (5 mg of protein/mL) (Cat. No.: 70175, Sigma-Aldrich, Madrid, Spain). This treatment was prepared in glucose-free Krebs–Ringer buffer (KRB) (120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 22 mM NaHCO3). The control group received KRB, supplemented with the same final concentration of DMSO. After the final treatment, the mediums were collected and stored at -80°C for subsequent analysis. Total GLP-1 secreted to the media was quantified using the commercial ELISA kit for GLP-1 (Cat No.: EZGLPT1-36k, Millipore, Madrid, Spain).

The cells were then washed with PBS, lysed with HEPES buffer supplemented with Triton® X-100 (0.1 %, Cat. No.: T8787, Sigma-Aldrich, Madrid, Spain) and stored at -80°C. Total protein content was determined with a bicinchoninic acid (BCA) kit (Pierce, Thermo Fisher Scientific) and cytotoxicity in the cells was quantified with a lactate dehydrogenase assay (LDH) (QCA, Tarragona, Spain) as described elsewhere.[27]

Gene expression analysis

RNA from the animal tissues was extracted using Trizol (Cat. No.: 15596018; Invitrogen, USA) and trichloromethane-ethanol (Cat. No.:141252; Panreac, Barcelona, Spain). All the samples underwent the same procedure. Frozen samples were disrupted and homogenised using a TissueLyser LT small bead mill (Qiagen, Hilden, Germany).

RNA was obtained from the cell experiments using RNeasy plus mini kit. The procedure was performed by strictly following the instructions provided by the kit manufacturer.

The extracted RNA was evaluated for quality and purity using a NanoDrop® ND-1000 spectrophotometer (Fisher Scientific, Madrid, Spain), and then stored at -80°C. cDNA was obtained using the High-Capacity cDNA Reverse Transcription Kit (Cat. No: 4368814, Fisher Scientific, Madrid, Spain).

Quantitative PCR amplification was performed using specific TaqMan probes (Applied Biosystems, Waltham, USA) for all genes (40 ng/μL of cDNA) (probes found in supplementary Table 1). The relative expression of each gene was compared with that of the control group using the $2^{-\Delta\Delta Ct}$ method. For the samples obtained from animals, *Ppia* was the reference gene, while for the cells, it was *RPS9*. Each figure indicates the analysis performed.

Statistical analysis

All our results are expressed as mean ± standard error of the mean (SEM). P-values < 0.05 were considered statistically significant. For parametric data, we used Student's T test to compare two groups or one-way ANOVA with the Bonferroni post hoc test to compare multiple groups. For non-parametric data, we worked with the Mann–Whitney U test to compare two groups, or the Kruskal–Wallis test by ranks with Dunn's multiple comparison post hoc test to compare multiple groups. All calculations were performed using Lumivero XLSTAT 2023.1.5 software (Addinsoft, New York, NY, USA).

Integrative Analysis and Variable Selection

The whole process of data processing, integration, variable selection, and statistical analysis outlined in this section was executed using RStudio version 2023.06.16 Build 446 (2009-2023 Posit Software, PBC).

The data on the parameters (biochemical, inflammation, intestinal permeability, enterohormones, and morphometrical) were collected, analysed and published elsewhere for various research purposes.[7–9,12,26,28,29]

All the initial data (91 parameters) was subjected to pre-processing, including median imputation for missing values and the removal of redundant variables to make the data simpler and more straightforward. Subsequently, the data was adjusted and scaled using the 'ScaleData' function.

Additionally, the 'RunPCA' function was used to help us to see if the samples grouped together or if any animals stood out and needed to be excluded in the next steps.

Once the data was clean, we used three machine learning methods – Elastic Net, Random Forest (RF), and PLS-DA, to look more closely at the variables in our multivariate approach throughout the selection process. Elastic Net is particularly useful for managing datasets with numerous features, especially when some are closely related. RF, a robust and adaptable algorithm belonging to the family of tree-based models, is suitable for both categorization and prediction tasks. And PLS-DA is a versatile statistical method that excels at categorizing groups in complex datasets, so it is well-suited to our goal of distinguishing various experimental groups from the variables analysed. The parameters for each of the methods were optimised using 100 times repeated 5-fold cross-validation. To avoid overfitting, we used the glmnet R package and followed a rule that adds a little flexibility.

In this case, we applied these machine-learning algorithms to compare the cafeteria group first with the corrective 500 treatment and then with the corrective 100 treatment.

Each method produced scores reflecting the importance of variables in distinguishing between groups. These scores were treated as individual values for each variable within each method. The sum of the values obtained from the three methods yielded the final value. This score acted as an indicator of the variables' overall importance in the study context. The variable with the highest score was identified as the most significant variable in the study. Using these results, we conducted an integrative analysis that ranks all variables in order of importance. Using this selection method, we were able to concentrate future analyses on those variables with the highest discriminatory power between groups.

Results

Subchronic treatment with GSPE up-regulates most rTas2r located in jejunum in cafeteria-obese female rats

In this study, we investigated the impact of two subchronic treatments of GSPE on the expression of rTas2r. Our aim was to elucidate the potential involvement of predominant intestinal taste receptors [24] in mediating the effects of GSPE as part of a diet-induced obesity paradigm. To do so, we used a well-established cafeteria diet-induced obesity animal model, which serves as a robust platform for exploring the underlying mechanisms of obesity pathology.[30]

We administered a dose of either 100 mg GSPE/kg BW or 500 mg GSPE/kg BW for 15 consecutive days to obese female rats before sacrifice.[8–10,12,29,30] The highest dose induced greater changes in the rTas2r gene expression as can be observed in **Figure 2** (rTas2r analysed without statistically significant effects can be found in Supplementary Table 2). We analysed the section between the duodenum and the ascending colon, and we found that the jejunum contained the highest number of GSPE-modified rTas2r, most of which had been up-regulated. In duodenum, the 500 dose of GSPE significantly increased rTas2r119 and rTas2r139 and tended to increase the expression of rTas2r143. In jejunum, the higher dose increased the gene expression of almost all of the analysed rTas2r, as only -138 and -140 did not show any statistically significant changes. In ileum, only rTas2r137 was up-regulated by the highest dose, while -108 tended to be down-regulated. Finally, in the ascending colon, the 500 dose increased the gene expressions of rTas2r108, -138 and -140. And although GSPE 100 was barely effective in the small intestine, it was more effective at colonic site (**Figure 2**), where it increased rTas2r138 and decreased rTas2r137 expressions. There was a trend to increase rTas2r140 and decrease rTas2r143.

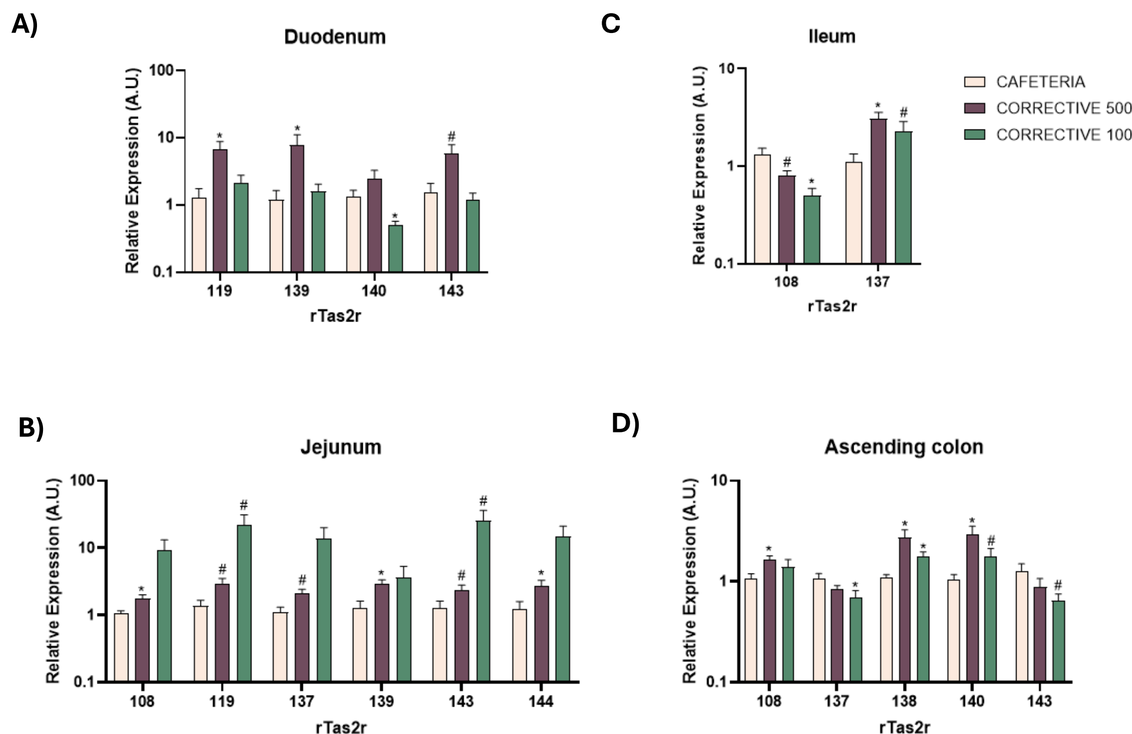

Figure 2. Relative expressions of rTas2s along the gastrointestinal tract A) duodenum, B) jejunum, C) ileum, and D) ascending colon. The Y-axis is a logarithmic scale for easier comparison. Values are mean ± SEM. * p-value < 0.05 compared with cafeteria group. # p-value < 0.1 compared with cafeteria group as control. p-value obtained using Kruskal-Wallis.

Elsewhere we have shown that these GSPE treatments are effective at improving the health status of these animals.[8–10,12,29,30] To try to understand whether rTas2r are involved in the biological effect of GSPE, we integrated this information with the parameters analysed in those studies (biochemistry, inflammation, intestinal permeability, enterohormones, and morphometry) (**Figure 3**). We used three machine-learning methods – Elastic Net, RF, and PLS-DA– to take a closer look at the variables in our multivariate approach throughout the selection process. We started with 91 parameters and after the three machine-learning methods had been implemented 34 were selected for the 500GSPE treatment and 22 for the 100GSPE treatment. The final score, obtained by aggregating the results from the three methods described in the Materials and Methods section, indicates the variable's overall importance. The variable with the highest score is the most effective at discriminating between treatments.

From this comparison of the GSPE 500 group with the cafeteria group, we found that rTas2r137 expressed in ascending colon is the bitter taste receptor that best discriminates between obese rats and those treated with the 500 GSPE dose. It is positioned 13th among the parameters, indicating that it plays a major role in distinguishing between the two treatments. Of the 34 parameters considered (see **Figure 3A**), we also observed that rTas2r108 from colon, -144, from jejunum and rTas126 from colon were in 24th, 27th and 34th position, respectively.

If we conduct the same analysis for the 100-dose group versus the cafeteria group, the first bitter taste receptor (rTas2r108 from colon) is the 15th of the 22 parameters characterised as important, (**Figure 3B**). Additionally, Tas2r137 from colon, rTas2r140 from jejunum, and Tas2r126 from colon were in the 17th, 18th and 22nd positions, respectively.

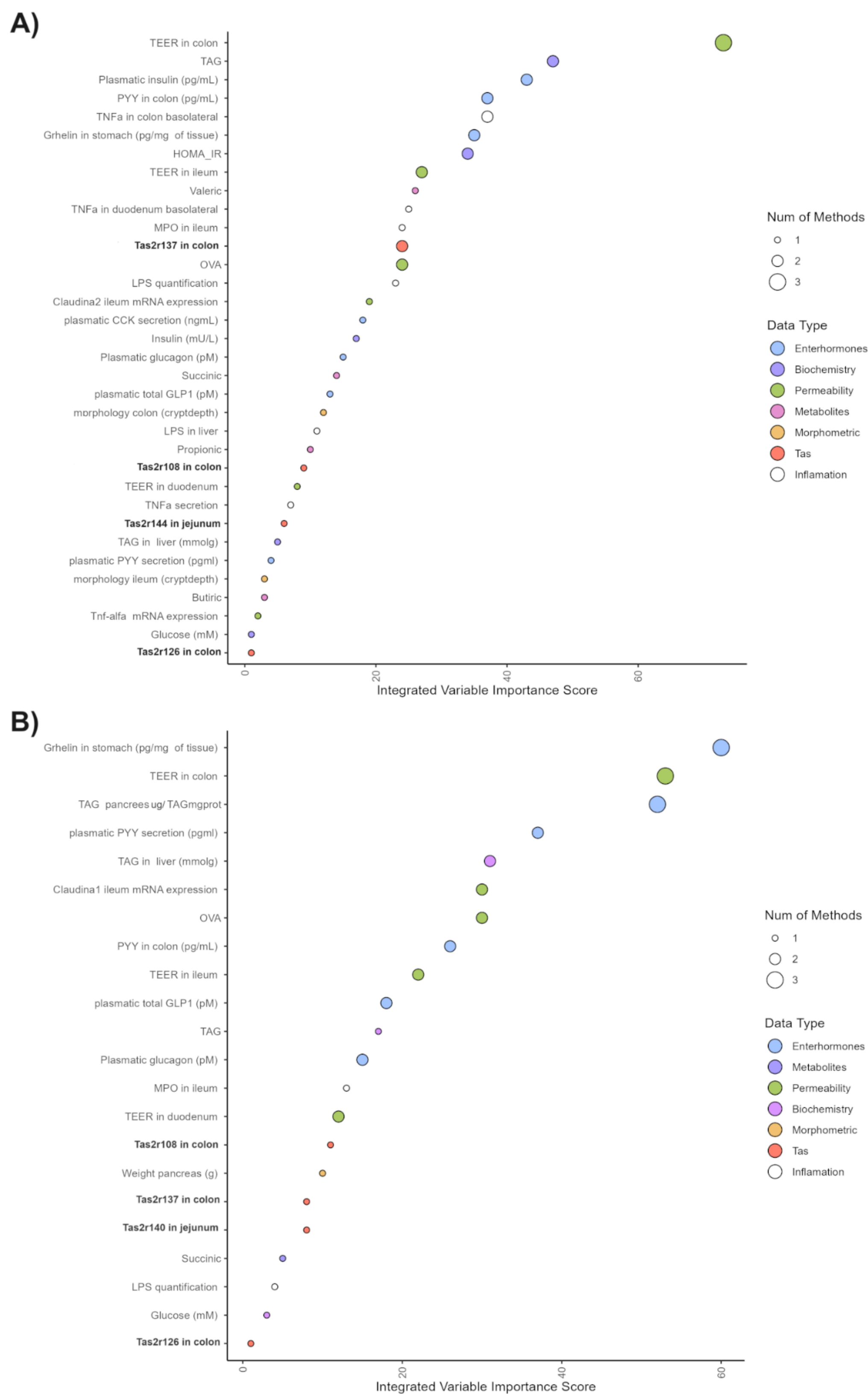

Figure 3. Principal variables distinguishing between the cafeteria group and corrective treatment groups. Integrate the analysis of selected variables using machine learning algorithms, ranking them to distinguish between A) CAFETERIA and CORRECTIVE 500, and B) CAFETERIA and CORRECTIVE 100, respectively.

Subchronic treatment with epicatechin reproduces most of the GSPE effects on jejunal rTas2r

Given that GSPE is a complex mixture of various molecules, we opted to replicate a subchronic treatment using only one of its constituents: epicatechin. This compound is one of the most abundant monomeric constituents of GSPE and the primary product of the breakdown of GSPE's oligomeric and polymeric structures.[31] It has also been identified as a specific agonist for bitter taste receptors. Specifically, in mice, at a concentration of 1 mM (threshold concentration)[18] epicatechin triggered activation of mTas2r126 and -144. And according to Soares et al.,[4] it was found to activate TAS2R4, -5, and -39 when tested against human isoforms.

Healthy female Wistar rats were administered 400 mg of epicatechin/kg BW, after which we assessed alterations in gene expression within the duodenum, jejunum, ileum, and ascending colon. We specifically targeted receptors identified as epicatechin targets, alongside their corresponding human isoforms (105, 126, 137, 140, 143, and 144).[4, 10, 32]

The nine days of epicatechin treatment modified the gene expression of only some of the receptors assayed. **Figure 4A** shows the receptors which were statistically modified by the treatment, while those not affected are shown in supplementary table 3. This treatment increased the expression of rTas2r126 in duodenum. As observed previously, the jejunum was again the intestinal segment with the highest number of receptors affected. Gene expression was increased by epicatechin treatment in rTas2r137, -143 and -144 while in rTas2r126 this increase was only a trend. The ileum and ascending colon were not sensitive to this treatment. Since some of these receptors showed different abundancies in different intestinal locations, we compared relative abundances to gain greater insight into the relative abundance of each of them (**Figure 4B**). The highest abundances were of rTas2r137 and -140, followed by -143, while rTas2r144, -126 and -105 showed lower abundances.

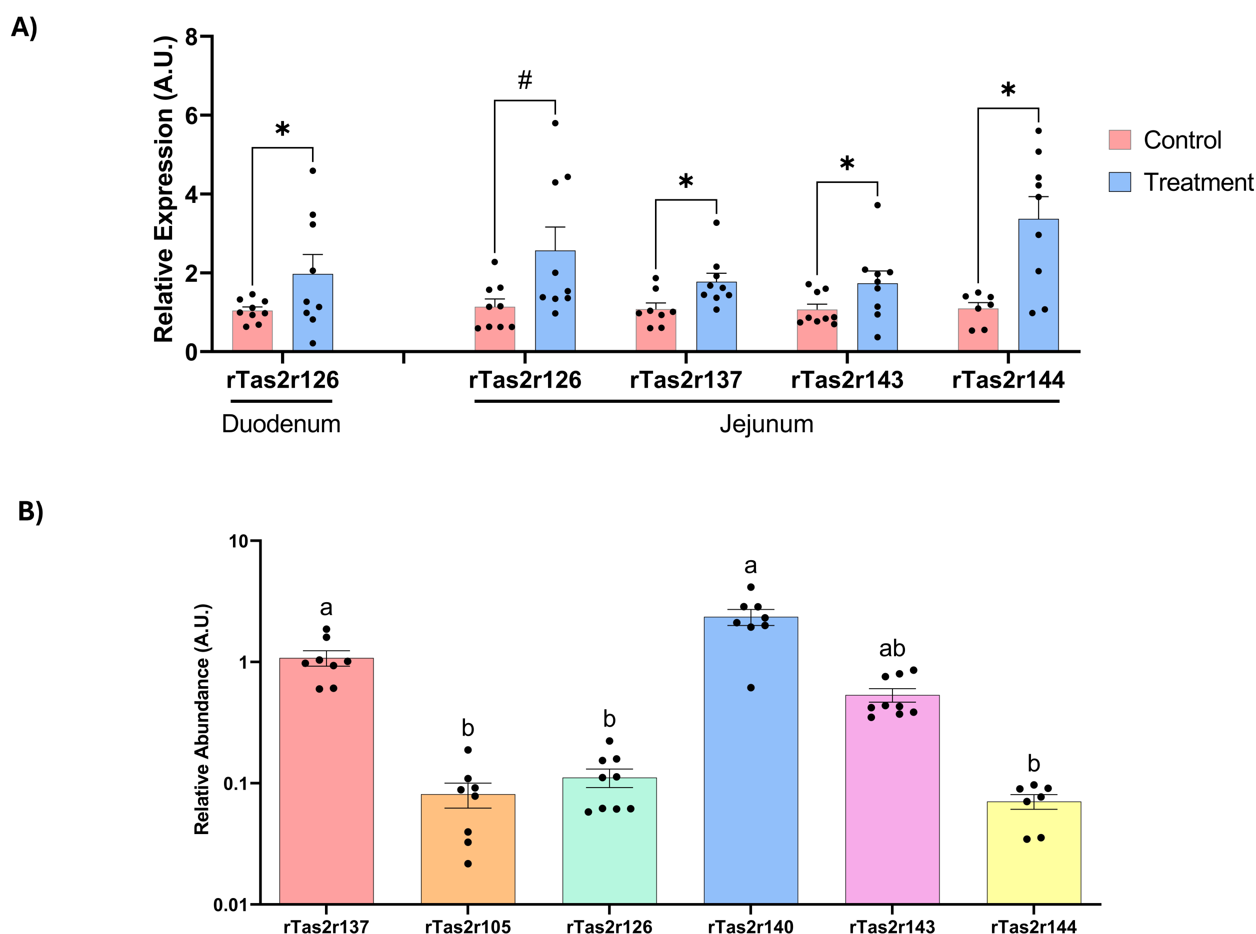

Figure 4. Relative expression of Tas2r in healthy rats. A) Bitter taste receptors which have shown significant changes in their expression after a 9-day treatment with epicatechin 400 mg/kg BW (* p<0.05; 0.05<#<0.1; either Student's T-test or Mann–Whitney U test was used, depending on the distribution of the data; n=7-9). **B)** Relative abundances of rTas2r measured in jejunum, normalised to the rTas2r137. Y-axis is a logarithmic scale for ease of representation. The different letters signify significant differences (p<0.05) between the groups (Kruskal–Wallis test by ranks with Dunn's Multiple Comparison post hoc test; n=7-9).

Given that the jejunum was highly sensitive to epicatechin treatment, we further examined the alterations in gene expression induced by epicatechin in an effort to reveal any similarities with the effects of GSPE. Subsequently, we focused on those rTas2r receptors that displayed the most significant modifications after GSPE treatment in the jejunum: rTAS2r108, -119, and -139. We observed that rTas2r139 was increased by the treatment (Control: **1.18** ± 0.27; Epicatechin: **2.36** ± 0.41; p< 0.05 by Student's T-test), while there were no significant effects on the expression of rTas2r108 (Control: **0.77** ± 0.11; Epicatechin: **1.19** ± 0.30; p> 0.1 by Student's T-test). Given the

significantly lower expression levels of rTas2r119 in these rats, this receptor cannot be considered for further analysis.

Finally, to investigate the effect of subchronic epicatechin treatment on healthy female rats *in vivo*, we measured two physiological parameters: body weight and daily food intake. After nine days of treatment, we observed that the epicatechin-treated group tended to gain less weight than the control group (% decrease vs initial body weight: **-1.02%** ± 0.34% control vs **-1.75%** ± 0.21% epicatechin; p = 0.093 by Student's T-test). However, there was no statistically significant effect on the daily or accumulated food intake after eight days of treatment (20-hour food intake (kJ): **1452.3** ± 20. 2 control vs **1440.5** ± 29.6 epicatechin; p= 0.747 by Student's T-test).

In vivo subchronic effects differ from *in vitro* effects in the Hutu-80 human cell line

Previous *in vivo* investigations revealed an up-regulation of most bitter receptors after a 9-day treatment with bitter agonists. To establish a direct correlation between agonist stimulation, gene expression and biological activity, we transitioned to a cell-line approach. Specifically, we used the human Hutu-80 cell line, which had a distinct GLP-1 secretory capacity upon specific stimulation and expressed several intestinal TAS2R receptors.[33] To initially characterize our Hutu-80 cells, **Figure 5** depicts their higher abundance of hTAS2R14, moderate presence of hTAS2R3 and hTAS2R5, and lower abundance of hTAS2R39.

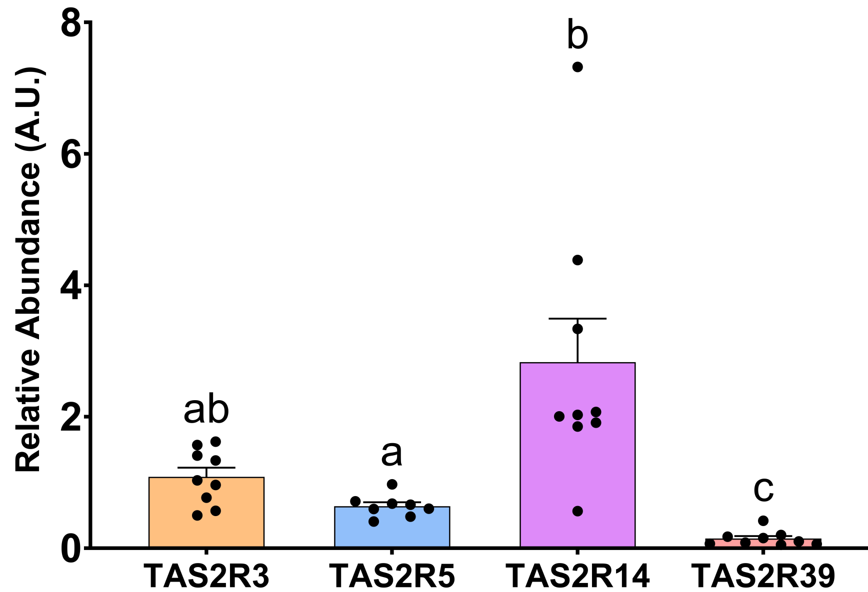

Figure 5: Relative abundances of four TAS2R in HuTu-80 cells, normalised to the TAS2R5. The groups that share the same letters are not significantly different (p-value: p<0.05) as determined by the post hoc test (one-way ANOVA with the Bonferroni post hoc test; n=8-9).

After discounting that the treatments were in any way toxic (Supplementary Table 4), we treated the cells for 24 hours with two doses of epicatechin (10 and 50 μM). We analysed the changes in the gene expression induced by these treatments. **Table 1** shows that the lowest dose of epicatechin down-regulated the receptors that, according to the literature, do not bind epicatechin (hTAS2R3 and hTA2R14). Additionally, the two known receptors that do bind epicatechin, hTAS2R5 and hTAS2R39, displayed no differences in expression when treated.

Table 1. Changes in hTAS2R gene expression after chronic treatment with two doses of epicatechin in Hutu-80 cells. A total of 14-18 wells from at least 3 passages were used for each case. Student T- test. *: p<0.05 vs negative control; #: p<0.1 vs negative control; $: p<0.1 vs epicatechin 10.

TAS2R	Treatment	Gene expression vs negative control
hTAS2R3	Negative Control	**1.082 ±** 0.100
	Epicatechin 10	**0.789 ±** 0.083*
	Epicatechin 50	**0.907 ±** 0.097
hTAS2R5	Negative Control	**1.061 ±** 0.092
	Epicatechin 10	**1.006±** 0.071
	Epicatechin 50	**1.081 ±** 0.101
hTAS2R14	Negative Control	**0.968 ±** 0.104
	Epicatechin 10	**0.758 ±** 0.060#
	Epicatechin 50	**0.928 ±** 0.067$
hTAS2R39	Negative Control	**1.249 ±** 0.221
	Epicatechin 10	**1.088 ±** 0.150
	Epicatechin 50	**0.975 ±** 0.094

To test the importance of these down-regulations on the stimulation of GLP-1, after the chronic treatment we acutely stimulated the cells with peptone as a positive control for GLP-1 secretion.[33] The 24-hour treatment with 10 mM epicatechin reduced the basal, non-stimulated, GLP-1 secretion by 6% ± 2% (p<0.05). However, this treatment also produced desensibilization to peptone stimulation, displaying only 69%± 4% of its ability to stimulate GLP-1 secretion without the chronic treatment. It has been reported that some peptides are ligands of hTAS2R14.[34] Thus, our results suggest that the effect of epicatechin down-regulating hTAS2R14 expression (table 1) could explain the lower stimulation shown by peptone.[35]

Discussion

Our objective was to investigate the relationship between the chronic stimulation of bitter taste receptors and its implications for metabolic health. We also sought to examine the specificity of this stimulation and its effects on metabolic outcomes. To do so, we worked with three experimental designs: a subchronic study with a mixture of several agonists of Tas2r in an obesity animal model, a subchronic study with a specific agonist (epicatechin) in a healthy animal, and chronic epicatechin administration to a human enteroendocrine cell line.

After the subchronic treatment with a complex mixture of several agonists as GSPE, we found mainly an up-regulation of rTas2r assayed in jejunum. Our results show that the rTas2r most sensitive to GSPE 500 mg treatment were rTas2r108, -119, -137, -139, -140 and -144, in jejunum and duodenum. This response in the upper intestine suggests that the original compounds found in the extract have an effect, as can be expected from the transit of these compounds along the GIT.[36,37] These compounds are mainly effective at the higher dose in the upper GIT, where they remain for a relatively short period of time. Once the GSPE reaches the colon, it is modified by microbiota and remains there for a longer time period. Here, both the doses of GSPE acted upon rTas2r138 and -140, suggesting that these two receptors are more sensitive to GSPE compounds that have been modified along the gastrointestinal tract.

To test the hypothesis regarding the effectiveness of pure compounds in the upper GIT and the modified ones in colon, we used epicatechin in a healthy rat model. By comparing the rTas2r modified by the GSPE and epicatechin treatments, we can identify those targets that are most likely to be up-regulated by epicatechin in each location. Jejunum showed the highest number of upregulated receptors: rTa2r137, -139, -143, and -144. Thus, our results support the idea that epicatechin in GSPE could be responsible for some of effects of the GSPE 500 mg treatment through its interaction with TAS2R. If we consider all the treatments assayed in jejunum, we can

conclude that rTas2r137, -139, -143 and -144 are the most sensitive to epicatechin treatments in the rat jejunum, all of them being up-regulated.

There is little information available on how chronic treatment with TASR agonists can affect gene expression. For sweet taste receptors, it has been shown that chronic treatment with sweeteners (for 12 weeks, much longer than ours) increased intestinal expression.[38] As far as bitter taste receptors are concerned, ellagitannins from black raspberry seeds orally administered for 21 days to mice with DSS-induced colitis increased the colonic gene expressions of mTas2r108, -119, -126, -131, -138, and -140. In parallel, there was an alleviation of colonic inflammation, regulated inflammation-related cytokine levels in the mice with colitis and increased total GLP-1 secretion.[39] These studies are in agreement with the increase we found in Tas2r gene expression after agonist treatment and the improvement in the inflammatory profile of these animals.[8,9]

To relate the effects on gene expression and the effects on metabolic health,[8,9,12] we ran an integrative analysis that identified some of the bitter taste receptors that played a role in the beneficial effects of GSPE against obesity. We ran two integrative analyses, one for each GSPE dose, and, although conducted separately, they consistently identified similar key parameters (TEER colon, ghrelin abundance in stomach, triglycerides in pancreas, PYY in colon). This reinforces the idea that these variables are effective at distinguishing between groups treated with GSPE and those exposed to a cafeteria diet. Since we analysed the same parameters for both doses, the differences between them could be related to the dose of GSPE. The bitter taste receptors modified by these treatments showed relatively low positions in these analyses, which identified three Tas2r as important in colon: Tas2r108, -126 and -137. This suggests that these receptors are important for GSPE effects in colon. The important Tas2r in the jejunal was different for the two doses, however. For 500 GSPE, it was Tas2r144 while for 100 GSPE it was Tas2r140. Very few studies have been done on rats and bitter taste receptors, but the limited information available can help to understand our results. Only mTas2r108 has clearly been shown to be a good target

for counteracting metabolic derangements[22] and TAS2R38 seems to be important for the stimulation of GLP-1 secretion in enteroendocrine L-cells.[40]

As all the previous analyses and published works unexpectedly showed an up-regulation of bitter taste receptors after chronic stimulation, we were interested in gaining greater insight into the relation between the defined agonisms and gene expression changes. In the study with epicatechin *in vivo*, the two binding receptors were up-regulated. rTas2r126 showed an up-regulation in duodenum and jejunum, and rTas2r144 in jejunum, suggesting that they were more sensitive to stimulation by their defined ligand in the upper GIT where epicatechin has not yet been modified.[6,41,42] We also found that several other rTas2r which have not been defined as targets for epicatechin had been up-regulated.[18] But this is not a common mechanism after the chronic stimulation of receptors. The cell-line study showed that chronic epicatechin stimulation down-regulated receptors, as expected, but surprisingly did not modify the gene expression of the hTAS2R, for which epicatechin has been shown to be an agonist. We found that epicatechin, an agonist of hTAS2R4, -5 and -39, downregulates hTAS2R3 and -14. And, in this case, down-regulation induced a lower stimulation by peptone suggesting heterologous desensitization. These effects have also been found by experiments conducted in human airway smooth muscle (HASM) cells, which have also shown that hTAS2R14 undergoes rapid agonist-promoted desensitization that can lead to a 50% loss of function (although some agonists caused minimal desensitization).[23] These experiments also assayed long-term desensitization with HASM cells in real time. The cells were treated with vehicle or hTAS2R agonist for 18 h and then challenged with an additional higher final dose of agonist with an immediate readout of [Ca^{2+}].[35] Molecular docking studies showed that deep within the binding pocket of the hTAS2R14 there were specific interactions between the agonists that induced desensitization and the one that did not.[35] The results we report here, however, show desensitization by compounds that are not defined ligands of hTAS2R3 and -14. It can be explained as heterologous desensitization. This does not provide an explanation for our *in vivo* effects, but the complexity of the system can induce a myriad of

mechanisms that produce the up-regulations. We cannot discard specific species differences between the two models assayed either, but the differences in the degree of the complexity of the systems (*in vivo* versus *in vitro*) is the most likely reason for both effects.

In summary, subchronic bitter taste stimulation mainly induces an up-regulation of several bitter taste receptors *in vivo* in the upper GIT. Treatment with epicatechin up-regulated its own specific rTas2r, but also some others, which suggests an increased sensitivity to stimulation. Working on the cell line, we also found heterologous desensitization, reinforcing the idea that there is a "network effect" in the role of the bitter taste receptors in the upper GIT which should be considered when addressing effects of bitter agonists.

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Conflict of interest

None of the authors have any conflict of interest.

Author contributions

Conceptualization: MP, AA & XT; Data curation: FJ, MD, HS & AV; Formal analysis: FJ, MD, ERG & AV; Funding acquisition: RB, MTB, XT, MP & AA; Investigation & methodology: FJ, MD, AV and HS; Project administration: XT, MP, ERG & AA; Supervision: RB, ERG ; Validation; Visualization; Roles/Writing: FJ, MD, MP and AA; and Writing - review & editing: all the authors.

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Supplemental Data

Supplementary Table 1: Names and reference numbers of the TaqMan probes for rats and humans used in the gene expression analysis.

Rat		Human	
Receptor	TaqMan Ref. Num.	Receptor	TaqMan Ref. Num.
rTas2r105	Rn00577003_s1	*TAS2R3*	Hs00249942_s1
rTas2r108	Rn02396427_s1		
rTas2r119	Rn00576950_s1	*TAS2R5*	Hs01549633_s1
rTas2r126	Rn00595098_s1		
rTas2r137	Rn01500928_s1	*TAS2R14*	Hs00256800_s1
rTas2r139	Rn04218919_s1		
rTas2r140	Rn01492598_s1	*TAS2R39*	Hs00603443_s1
rTas2r143	Rn02585801_s1		
rTas2r144	Rn02585844_s1	*Rps9*	Hs02339424_g1
Ppia	Rn00690933_m1		

Supplementary Table 2: Relative expressions of individual bitter taste receptors in each individual segment of the intestine, where no statistical differences were observed between the control and GSPE treated groups. Data represented Average Relative Expression (normalised to the control group for each individual receptor) ± SEM.

	Receptor	Control		Corrective 100		Corrective 500	
		Relative Expression (A.U.)	N	Relative Expression (A.U.)	N	Relative Expression (A.U.)	N
Duodenum	*rTas2r108*	1.648 (± 0.52)	10	0.729 (± 0.11)	9	2.080 (± 0.60)	8
	rTas2r137	1.300 (± 0.44)	9	0.647 (± 0.12)	9	2.385 (± 0.83)	8
	rTas2r144	1.521 (± 0.55)	8	0.944 (± 0.23)	9	4.453 (± 1.50)	9
Jejunum	*rTas2r119*	1.377 (± 0.29)	7	21.671 (± 9.61)	7	2.975 (± 0.56)	6
	rTas2r137	1.105 (± 0.21)	6	13.790 (± 6.32)	7	2.081 (± 0.35)	6
	rTas2r138	1.338 (± 0.35)	6	17.492 (± 7.02)	7	2.105 (± 0.34)	6
	rTas2r144	1.243 (± 0.35)	6	14.963 (± 6.26)	7	2.691 (± 0.62)	6
Ileum	*rTas2r119*	1.34 (± 0.30)	3	0.035 (± 0.01)	4	0.238 (± 0.01)	6
	rTas2r126	1.082 (± 0.17)	4	0.935 (± 0.33)	4	1.749 (± 0.40)	7
	rTas2r138	1.000 (± 0.11)	3	0.108 (± 0.02)	5	0.349 (± 0.11)	7
	rTas2r139	1.003 (± 0.15)	4	0.055 (± 0.01)	4	0.238 (± 0.10)	6
	rTas2r140	1.250 (± 0.12)	4	1.864 (± 0.52)	5	2.323 (± 0.62)	7
	rTas2r143	1.157(± 0.24)	4	0.316 (± 0.06)	4	0.994 (± 0.14)	7
	rTas2r144	1.085 (± 0.20	4	0.032 (± 0.01)	4	0.198 (± 0.07)	6
Ascending Colon	*rTas2r119*	1.117 (± 0.17)	10	1.274 (± 0.25)	8	1.623 (± 0.44)	7
	rTas2r126	1.210 (± 0.24)	10	0.799 (± 0.10)	10	1.279 (± 0.23)	8
	rTas2r139	1.161 (± 0.18)	10	1.065 (± 0.08)	8	1.173 (± 0.13)	8
	rTas2r144	1.218 (± 0.20)	9	1.737 (± 0.34)	8	1.567 (± 0.15)	9

Supplementary Table 3: Relative expressions of individual bitter taste receptors in each individual segment of the intestine, where no statistical differences were observed between the control and treated group. Data represented Average Relative Expression (normalised to the control group for each individual receptor) ± SEM.

	Receptor	Control		Treatment	
		Relative Expression (A.U.)	N	Relative Expression (A.U.)	N
Duodenum	*rTas2r105*	1.201 (± 0.33)	6	1.196 (± 0.32)	6
	rTas2r137	1.023 (± 0.10)	9	1.153 (± 0.13)	9
	rTas2r140	1.207 (± 0.23)	9	0.983 (± 0.22)	8
	rTas2r143	1.030 (± 0.08)	8	1.494 (± 0.34)	9
	rTas2r144	1.182 (± 0.20)	9	0.880 (± 0.16)	8
Jejunum	*rTas2r105*	1.253 (± 0.29)	8	1.574 (± 0.28)	8
	rTas2r140	1.118 (± 0.17)	8	6.206 (± 2.66)	9
Ileum	*rTas2r105*	1.525 (± 0.44)	9	2.074 (± 0.48)	9
	rTas2r126	1.103 (± 0.18)	9	1.601 (± 0.37)	9
	rTas2r137	1.239 (± 0.22)	9	1.090 (± 0.11)	9
	rTas2r140	1.148 (± 0.20)	8	1.827 (± 0.50)	9
	rTas2r143	1.170 (± 0.22)	9	1.401 (± 0.24)	9
	rTas2r144	1.158 (± 0.23)	9	1.166 (± 0.18)	9
Ascending Colon	*rTas2r105*	1.188 (± 0.42)	4	0.269 (± 0.11)	4
	rTas2r126	1.223 (± 0.22)	8	1.065 (± 0.15)	9
	rTas2r137	1.104 (± 0.14)	9	1.114 (± 0.12)	9
	rTas2r140	1.041 (± 0.11)	7	1.377 (± 0.09)	7
	rTas2r143	1.080 (± 0.18)	9	0.743 (± 0.12)	9
	rTas2r144	1.166 (± 0.19)	9	0.902 (± 0.16)	9

Supplementary Table 4: The percentage of toxicity ± SEM of different treatments of the 24-hour treatments of the HuTu-80 cells. This assay measures the activity of the enzyme Lactate dehydrogenase, which is rapidly secreted from the damaged or dead cells. The assay compares the activity in the cellular medium and in the lysate of the cell, and the ration of these 2 measurements is processed and the percentage of toxicity is obtained. No significant differences have been observed between the treatments.

	Toxicity %	N
Control	9.40 ± 0.43	6
Epicatechin 10μM	10.09 ± 0.27	6
Epicatechin 50μM	10.40 ± 0.30	6

GENERAL DISCUSSION

General Discussion

Ageing is a multifaceted problem, with numerous causes and consequences, which can be broadly separated into the age-associated disorders experienced by individuals, and the overall pressure on society to burden the consequences of the ageing population [1]. While we associate age-related diseases with the later stages of life, the mechanistic changes that produce these disorders are set off much earlier but are compensated for, at least for a time [2]. Medical interventions for more severe issues are available, but many can be prevented without medical attention [3]. Control of caloric intake is one of the most effective ways how healthier longevity can be achieved, however, patient compliance with this method is low. Due to this, caloric restriction mimetics have come to the forefront of research [4]. Some natural bioactive compounds, which are proposed as these mimetics, are also known bitter compounds, interacting with the bitter sensing system headed by bitter taste receptors (TAS2R) [5].

TAS2Rs serve a multifaceted protective role in the organism. First, upon their stimulation, the aversive sensation that is produced makes it less probable that the bitter food will be ingested [6]. The second protective role is less clear, and it has to do with the peculiar nature of TAS2Rs, which are expressed all over the organism [7]. Primarily studied in the respiratory system as modulators of the immune response, it was shown that TAS2R regulate bronchodilatation, promote mucus clearance, have an antimicrobial role, and regulate the macrophage production of cytokines [8]. But TAS2R have also a high presence in the gastrointestinal tract (GIT), where they play other roles than that protection, such as the secretory regulation of EECs [9]. This discovery brought the idea that by stimulating these receptors, regulation of satiety and metabolism could be possible, and thus, could be a novel mechanism of decreasing caloric intake in the overall population. But there is room to clearly understand the role of TAS2R in the GIT, their response to ageing and therefore whether they could be targeted to promote healthier ageing.

One interesting characteristic of TAS2R is their diversity: in humans, 26 different active isoforms have been detected. This variability inevitably leads to diversity in their functionality, making some more compelling for certain roles. For example, our group has identified certain receptors which seem more involved in the regulation of food intake [9]. One of those was TAS2R39, thus we decided to review the available knowledge about this specific receptor in the literature (**Manuscript 1**). The review of the literature showed that this receptor is still quite unknown, as the first ligands for this receptor were discovered a little over a decade ago, but now it is a receptor with one of the biggest repertoires of ligands [10,11]. Interestingly, TAS2R39 shares quite a few

common agonists with TAS2R14, but from the structural analysis of these molecules, an interesting trend was noted. For example, soy isoflavone genistein interacted with both receptors, but TAS2R14 seemed more sensitive to it than TAS2R39. However, with glucosylation of genistein, the affinity for TAS2R14 was completely lost, but not for TAS2R39 [12]. Similarly, bigger polyphenols extracted from tea, namely theaflavin-3,3-O'-digallate solely activated TAS2R39, while theaflavin-3′-O-gallate interacted with both [13]. Finally, the esterification of epigallocatechin with gallic acid to epigallocatechin gallate significantly increased its affinity to TAS2R39 [14]. The binding site of this receptor likely differs from the TAS2R14's in its size, as it seems there is space for additional receptor-ligand interactions.

While TAS2R39 has been established as a receptor appearing in extraoral locations, one noticeable characteristic is that it is a receptor with one of the lowest levels of expression [15]. Nonetheless, our group has previously observed its thiamine had an inhibitory role in CCK secretion in ex vivo experiments [9], while simultaneous activation of TAS2R5 and -39 (with stronger activation of -39) induced the food intake in an animal model.

Overall, the limited knowledge of TAS2R39, in combination with its sparse expression, represents a significant obstacle in its research. Yet, on its own or in conjunction with other receptors, it is likely TAS2R39 is involved in enterohormonal regulation, something that could prove to be useful in the regulation of caloric intake and its impact on healthier ageing.

Apart from low abundances, another limiting factor for elucidating the exact roles of different TAS2R is the large diversity in their active forms (26 different ones in humans) [16]. Moreover, these receptors express heterogeneous expression profiles between different tissues, cell types, and even individual cells [17]. This represents a challenge as if we intend to target a specific receptor to elicit the desired effect, the targeted receptor has to be present at that site. However, the knowledge about how different TAS2Rs are expressed throughout the GIT is incomplete. Thus, we deemed it imperative to describe the overall presence of the TAS2R in different parts of the GIT (**Manuscript 2**). TAS2R expression was analysed in 4 different mucosae (oral, jejunum, ascending colon, descending colon) obtained from different patient cohorts, a strategy that allows us to obtain information on all these human locations. Overall, TAS2R14 was shown to be the receptor which was most abundant in all analysed tissues. While the profiles of these tissues did share a resemblance, we did observe some unique characteristics for each tissue, especially when comparing the intestinal mucosa of the jejunum and colon. Jejunum abundance of TAS2R42 was noticeably higher than in both colon locations, while TAS2R5, -13 and -20 were noticeably higher in colon mucosa. These observed differences, however small, could be of

significance. Thus, if we want to elicit a stronger effect in one particular part of the GIT while trying to minimise secondary effects, we could achieve it by targeting a specific receptor which is more present in one location and not others. It was interesting to observe that the receptors which are predominately more expressed (TAS2R14, -46), are also more promiscuous TAS2Rs [18]. This suggests that activation of bitter receptors is an overall necessity in the GIT, as receptors that detect the widest range of ligands are also the most expressed ones. On the other hand, more specialised receptors, which detect only some molecules, could be involved in a more fine-tuning role in the intestine, eliciting specific responses depending on the stimulation.

Ageing affects every single cell in our body, so it is important to evaluate how expression changes throughout our lives. The colon cohort discussed above represents data obtained from participants with an average age of around 65 years. Additionally, we also were able to obtain samples of colon mucosa from the younger cohort, aged around 38 years. This allowed us to inspect how the profile of TAS2R changes with ageing. Our analysis revealed that the expression of four out of eleven investigated TAS2Rs was age-dependent. TAS2R4, -5, and -13 showed significantly higher expression in the older cohort, while TAS2R20 displayed a trend towards higher levels of expression with ageing. Accompanying this expression increase was an increase in enterohormonal expression (proglucagon and PYY) as well. The increased expression of some of the TAS2R was somewhat surprising, but it is something that has been described before, as Aoki et. al. saw a positive correlation between age and TAS2R expression in the tongue [19]. While the underlying mechanisms responsible for the increase, it could be a consequence of a handful of elements. Diet has a proven influence on the expression of taste receptors, and a mouse model has shown that a diet low in cholesterol upregulates the expression of some bitter receptors in individual segments of the intestine [20]. Low-cholesterol diet is likely to be more prominent in the aged cohort, as cholesterol levels usually increase as we age [21]. In this particular study, the levels of free cholesterol were higher in the aged cohort, we cannot exclude the possibility that participants with higher cholesterol are adhering to a low-cholesterol diet. Another factor that cannot be ignored is the presence and evolution of our microbiome as we age. Changes in the gut microbiota composition have been associated with inflammation [22], while bitter receptors have been shown to be upregulated in animal inflammatory models [23]. This could partially explain our observations, as measurements of IL-6 and MCP1- in these participants showed significantly higher levels in the aged cohort. Cellular senescence, that is accumulation of non-dividing cells with ageing, could potentially have an impact as well. The senescence-associated secretory phenotype is responsible for the secretion of cytokines, immune modulators, and many other components, which could again impact the expression of bitter receptors [24]. While one or all

of these factors could have a part to play in the observed expression profile of the TAS2R in the older cohort, it is difficult to pinpoint the exact factor that is at play.

Overall, the GIT bitter receptors showed some interesting characteristics. We were able to establish a map of TAS2R in different segments of the GIT, and while the profile was quite similar overall, some receptors were location-specific, making them a potentially viable objective for eliciting a targeted effect along the GIT. Additionally, it was observed that ageing, bitter receptors and metabolic homeostasis are intertwined, but it remains to be seen in which way.

Although metabolic processes significantly impact the ageing process, and the GIT is crucial for metabolic homeostasis, ageing is a systemic phenomenon affecting all tissues. This makes it challenging to establish precisely the most affected homeostatic system, and consequently, which dysfunctions are responsible for the appearance of age-associated disorders. While laboratory analyses have advanced, a universal biomarker panel for assessing age-related changes remains elusive. The analysis of a wide spectre of metabolites has been made available with the development of metabolomic analysis and was quickly adopted as a useful tool for the analysis of ageing [25]. The absorptive and endocrine roles of the intestine are important regulators of metabolism, and changes in the chemosensory molecules in the intestinal wall have a role in metabolic changes [26]. Thus, it is conceivable there are correlations between the sensory mechanisms of the GIT and the changes in the metabolite profile. To investigate it, we performed a multivariate analysis of the metabolomic parameters and the TAS2R expression from the human colon (**Manuscript 3**). Among the 2 receptors that were deemed interesting in our analysis, TAS2R5 was the more compelling one. TAS2R5 correlated with lipoprotein constituents in plasma. Kim et al. recently discovered a novel form of interaction between TAS2R and their ligands when they identified cholesterol as a key modulator of ligand recognition by TAS2R14 [27]. They showed that cholesterol, which is abundant in the cellular membrane, binds to the extracellular binding site of this receptor, while the bitter compound binds to the intracellular allosteric binding site. Total activation of this bitter receptor was possible only when both cholesterol and the bitter ligand were bound. Together with the fact that bile acids, which are synthesized from cholesterol and share a common steroid motif, activate bitter receptors (-1, -4, -14, -39), cholesterol and its carriers probably play an important role in the activation and signal transduction of TAS2R [28]. It is of note that TAS2R14 and -5 are quite distinct receptors. While the former is the most promiscuous human receptor, TAS2R5 is one of the most selective receptors [29]. Nonetheless, they do share some similarities, chiefly they both are targets for epicatechin and epigallocatechin gallate [30]. While we are still in the initial stages of understanding its uniqueness, parameters correlating with TAS2R5 have a noticeable impact on

the ageing progress, presenting TAS2R5 as a factor worthwhile keeping in mind in further studies in the physiology of ageing.

By establishing possible connections between TAS2R and ageing processes, and mapping out their profile in the GIT, it is also prudent to inspect how bitter receptors are affected by their ligands. As TAS2R have evolved as a defensive mechanism, it comes as no surprise that they can detect a myriad of different compounds, much of them being of plant origin [31]. But not everything bitter is toxic. One natural extract that has well-documented beneficial effects on overall health is grape seed proanthocyanidin extract (GSPE) [32–34]. GSPE is a mosaic comprised of numerous different polyphenols, including proposed caloric restriction mimetics catechin, epicatechin, and epigallocatechin gallate being just some [35]. The most prevalent theories on the underlying mechanisms involve the regulation of pathways of cellular regulation through the AMPK pathway and mTORC1 complex. By activating the former and suppressing the latter, the cell repairs the accumulated damage more efficiently, utilises nutrients more carefully, and activates the mechanism of cellular recycling or autophagy [36]. However, the information is scarce, and how polyphenols could trigger these mechanisms remains unclear. We postulate that bitter receptors, as sensory proteins for bitter compounds, represent an important cog in the signalling pathway. One piece of evidence that supports this potential effect is the study previously conducted by our group. We observed that 10-day GSPE treatment had multiple beneficial effects on age-associated parameters. Namely, food intake during the treatment was significantly lower in the treated animals. Moreover, the rats displayed lower visceral adiposity and had a lower incidence of carcinogenesis [37]. Interestingly, the effect of GSPE was observable 11 weeks after the treatment, a fascinating development. With the highly dynamic machinery that is the chemosensory proteins on the intestinal epithelium, it is highly conceivable that these receptors are partially responsible for the detected effects. What is less clear is what are the consequences of these longer-term treatments on intestinal detectors such as TAS2R. To investigate if a more long-term treatment impacts the expression of bitter receptors, we assessed the influence of two chronic treatments with different dosages of GSPE on the intestinal Tas2r in rats. These animals were fed with a model of a Western diet for a multitude of reasons: the Western diet simulated the diet that many people are accustomed to; it causes dysfunctional homeostasis; and finally, the previously observed positive effect of 10-day GSPE treatment on age-related processes we observed in aged healthy animals, so we decided to observe the changes in more pathological settings (**Manuscript 4**). We inspected the expression of the receptors in different intestinal segments (duodenum, jejunum, ileum, and ascending colon) of rats that were treated with either 100 mg GSPE/kg BW or 500 mg GSPE/kg BW for 15 days. Jejunum was the

segment with the greatest number of changes in the expression of Tas2r, as 6 out of 9 receptors (rTas2r108, -119, -137, -139, -143, and -144) were more expressed by either one or both treatments, with the higher dose appearing more effective. In the ascending colon, we also observed changes in the expression of 5 receptors, however, the effect was much more mixed: rTas2r137 and -143 are less expressed with treatments, while the other three (rTas2r108, -138, and -140) were increased. Additionally, overall, it resembles that the lower dose is more effective in ascending colon. Thus, the small intestine, specifically the jejunum, seems to be the tissue where the treatments have the most noticeable effect. The jejunum is the second segment of the small intestine and is the location where many nutrients are absorbed, so it comes as no surprise that some changes are apparent [38]. We also observed a different effect more downstream, in the colon, which could be linked to the microbiome-related transformation of the polyphenols. The nature of human physiology (slower transit time, less extreme pH) established that the colon is where we can encounter the most abundant and variable microbial environment [39]. The vast majority of polyphenols that are absorbed by the small intestine are in their monomeric and dimeric forms, which leaves bigger molecules to travel to the colon, where their digestion by the present microbiota takes place, and colonic catabolites can then be absorbed or act locally, and then finally excreted [40]. The exact mechanism of how bitter receptor expression is induced here remains unclear. Some previous experiments with chronic treatment of sweeteners observed the same increase in the expression of sweet receptors, which are members of the TAS GPCR family [41]. More importantly, the treatment of mice with colitis with ellagitannins from black raspberry seeds increased the expression of mice Tas2r in the afflicted animals [42]. In most cases, upon stimulation with agonists, especially when it comes to more chronic stimulation, there is a desensitization of the signalling cascade, as overexcitation could be harmful to the cell. Cellular mechanisms that include β-arrestins and GPCR kinases produce short- and long-term desensitization [43]. However, some GPCRs show higher resistance to agonist-induced desensitization than others, like for example, the β-3 adrenergic receptors. It was discovered that the gene transcript of this particular receptor was encompassed by cAMP response elements [44]. cAMP is created upon stimulation of adrenergic receptors, and cellular assays showed that upon stimulation of adrenergic receptors, a significant increase of β-3 was observed, while at the same time, β-1 type was downregulated. Additionally, polyphenols have been shown to have epigenetic modification properties, as resveratrol, found in grapes, inhibits the DNA methyltransferase enzyme, thus inducing the transcription of genes [45]. Moreover, non-coding micro-RNAs, another important epigenetic regulatory system, are also under the regulatory control of different polyphenols [46,47]. While we did not inspect any of these factors, a combined effect of

desensitization resistance of the TAS2R together with the potential epigenetic regulatory role of polyphenols could partially explain the observed effect.

One important factor we must bear in mind is the nature of our treatment: GSPE is a mixture of dozens of different compounds, ranging from monomeric flavonoids such as epicatechin to the dimers and trimers of these polyphenols and esters with different acids, like gallic acid or vanillic acid [48]. Each of these compounds has its own affinity for different bitter receptors. Furthermore, these compounds undergo microbial metabolism as they travel along the GIT [40]. Together with the differences in the TAS2R expressions in different parts of the GIT, it signifies that GSPE may have a wide range of effects, which sometimes could be negating each other. It would thus be prudent to elucidate which component or a handful of them are the ones responsible for the positive effect of the extract on age-associated disorders. We selected epicatechin as the one component of GSPE that could possibly have a great impact. Firstly, epicatechin is one of the most abundant components of GSPE, and importantly, it is one of the few ligands that can interact with TAS2R5, which we have previously identified as potentially important in the progress of ageing [49]. This led us to investigate the role of the epicatechin, this time in a more long-term setting (**Manuscript 4)**. We were interested in the expression of bitter receptors after 9-day treatment with 400 mg/kg BW of epicatechin and observed a result comparable to the effect of GSPE. More precisely, jejunum was again where the effect of epicatechin was most noticeable, with the highest number of differences between the receptors. Interestingly, epicatechin did not increase the expression of solely the receptor it binds to (Tas2r126 and -144), but also other receptors. However, unlike what we saw in the GSPE-treated animals, we did not observe any changes in the expression of bitter receptors in the ascending colon when solely using epicatechin. Epicatechin, as one of the most abundant components of GSPE, seems to be one of the important factors that influence previously observed increase of small intestine bitter receptors. One of the reasons why we do not observe any significant changes in the colon could be the heavy microbial activity that is present throughout the GIT, especially in the colon. While the absorption rate of polyphenols is limited, and these compounds reach all segments of the GIT, they are also heavily modified by microbes in the intestine [40]. Certain amounts of epicatechin do reach the distal GIT, but this amount is likely insufficient to effectively influence the expression of the receptors assayed. This adds to the theory that the microbiota metabolites are the ones that influence the expression of the Tas2r in the colon. The potential mechanisms for this increase have been partially discussed above and could potentially explain the upregulation as well. Multiple flavonols, such as epigallocatechin gallate were shown to have impacts on DNA methylation [50,51]. While epicatechin does not seem to be as potent an

inhibitor of DNA methylation as epigallocatechin gallate, it still has that effect, and in addition, it has also been proposed that epicatechin induces epigenetic changes on posttranslational histone modifications [52,53]. In this specific study, it resulted in decreased NF-κB expression and lower secretion of TNF-α, thus lowering inflammation. While we have not investigated these pathways, it might be prudent in the future to evaluate this possible effect of epicatechin and other polyphenols.

While animal models have provided insights into bitter receptors, their utility has drawbacks. There are differences in the number of bitter receptors between species: 26 in humans and 35 in rodents. Human TAS2R and rodent Tas2r have partially overlapping amino acid sequences, however, these similarities are not overwhelming, making it difficult to identify orthologs [54]. This also means that some receptors are species-specific, making investigations challenging. The novelty of the area also means the lack of substantial human studies and the prominence of cellular lines. As enterohormones regulate the sensation of satiety, and thus could be proven a useful component in the regulation of food intake and restricting calory intake, the duodenal enterohormonal HuTu-80 cells represented an appropriate study model (**Manuscripts 2 and 4**). This line was a good representative of the human intestine, since, as detected in the samples of the human tissues, TAS2R14 was again the receptor with the highest levels. The profile was quite similar to the one obtained from the jejunum described previously, which is not surprising as both segments constitute the small intestine. Next, we investigated an acute treatment with 2 bitter agonists: epicatechin (binds preferentially to TAS2R5, but also -4 and -39) and vanillic acid (activates solely TAS2R14) [55,56] (**Manuscript 2**). Acute stimulation with epicatechin increased the stimulation of both GLP-1 and PYY, while vanillic acid, an agonist of TAS2R14, only increased the secretion of GLP-1. While TAS2R14 is the most abundant receptor present in the human body, our results indicate that its presence is due to a different role and not regulation of enterohormones, as it induced secretion of solely 1 measured enterohormone. Following this, epicatechin, and activation of predominantly TAS2R5 does seem to have an enterohormonal-centric role in the GIT.

We described how previously observed an upregulation of several Tas2r in rats by both GSPE and epicatechin, thus we wanted to investigate if longer treatment has the same effect in the cellular model (**Manuscript 4**). 24 hours after the treatment with 2 different concentrations of epicatechin, we interestingly observed changes in the expression of TAS2R3 and TAS2R14, 2 receptors which are not known to interact with epicatechin. Moreover, the receptors which detect

epicatechin, TAS2R5 and -39, did not display any differences. The agonist-induced downregulation of GPCR is quite a common phenomenon and desensitization and internalization of TAS2R have been described before [57,58], however, it was interesting to observe that this downregulation seems to be induced by off-target receptors. Activating a specific GPCR activates a complex signalling cascade, they can also regulate non-G protein-dependent signalling pathways [59]. This crosstalk could explain how activating TAS2R5 for example could influence the expression of other receptors, such as TAS2R3 and -14.

Considering all the results together, in this dissertation, we are presenting a compelling case that bitter receptors in their chemosensory role have a part to play in the progress of ageing, and by selectively regulating their activity by natural compounds, can elicit beneficial effects on age-associated dysfunctions. A literature review of TAS2R39 showed some particular trends in the structure of its agonists, something that could be beneficial in the design of possible ligands for its regulation, as it does seem that TAS2R39 plays a part in enterohormonal regulation. We created an expression profile of bitter receptors in major parts of the GIT, and we saw that while similarities dominate, there are some unique qualities in each segment. Especially interesting were the intestinal segments, where TAS2R42 in the jejunum and TAS2R5, -13 and -20 in the colon were more present than in other tissues. One common factor in all segments was the high promiscuity of the most expressed receptors, seemingly showcasing that bitter receptors are necessary for the overall functioning of the intestine, but the more specialised tasks are regulated by less abundant, but still present, more selective TAS2Rs. The surprising observation that some bitter receptors are upregulated with ageing potentially implicates TAS2R with important age-associated processes, such as metabolic dysfunction and creation of reactive oxygen species, inflammation and potentially senescence. One of the receptors which has shown this upregulation was TAS2R5, which coincidentally, displayed various correlations with parameters with well-documented effects on ageing. Especially notable is its correlation with some lipoprotein fractions, whose dysfunction has been long associated with the worsening of overall health as we age. How TAS2Rs interact with ageing progress and their potential use comes down to the versatility of the bitter ligands. Bioactive extracts, like GSPE, have well-established antioxidative and anti-inflammatory effects, which potentially could be elicited through TAS2Rs. We showcased that sub-chronic GSPE treatment has a positive effect on the expression of the rat Tas2r in multiple intestinal segments and have identified that epicatechin is most likely responsible for the effect in the small intestine, while the changes observed in the colon are most likely the result of microbiome metabolites of the polyphenols. The limited knowledge and novelty of the field necessitate the utilization of cellular models, such as the here-presented

HuTu-80 line, which proved to be a suitable way to study the regulatory role of TAS2R. Previously studied TAS2R5 was again one of the more compelling receptors, as it was responsible for more complete enterohormonal secretion, due to its agonist epicatechin stimulating the secretion of both GLP-1 and PYY, than TAS2R14, which induced secretion of solely GLP-1. Ultimately, we demonstrated that TAS2Rs are capable of heterologous desensitization, as by utilizing epicatechin and activating the receptor it binds to, we can downregulate TAS2Rs that do not interact with epicatechin.

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CONCLUSIONS

CONCLUSIONS:

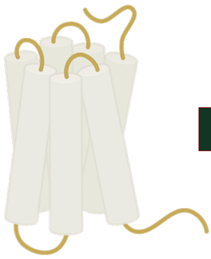
The main conclusions from this thesis are:

1. **Bitter Taste Receptors Are Differentially Expressed Throughout the Gastrointestinal Tract, and the Profile Changes with Ageing**
    - TAS2R39, while still largely unknown, is likely to have a role in enterohormone regulation, and thus could be a useful target to control caloric intake.
    - The most common bitter receptor in the analysed segments of GIT was TAS2R14.
    - Some receptors are more abundant in one segment than others: in oral mucosa – TAS2R3 and -5; in jejunum – TAS2R30 and -42; and in colon TAS2R31.
    - Ageing upregulated TAS3R4, -5, and -13 in the descending colon.
    - The increase in the expression of bitter receptors in descending colon of aged cohort was accompanied by an increase in expression of GLP-1 and its receptor (GLP-1R), while PYY expression was increased solely in the descending colon of the young cohort.

2. **Colonic TAS2Rs Might Be Involved in the Progress of Ageing**
    - Changes in the expression of some bitter receptors correlated with a higher number of well-known parameters impacted by ageing.
    - TAS2R5 and -38 are likely to be the two receptors most involved in the progress of ageing.
   - TAS2R5 displayed correlations with several fractions of the VLDL lipoproteins (their number and triglyceride content), as well as inflammatory parameter IL-6, factors that have been associated with the onset of age-related disorders.
   - TAS2R38 could be involved in the metabolic processes of the liver, due to its correlations with 2 ketone bodies: acetone and 3-hydroxybutyric acids.

3. **GSPE and its Constituents Regulate the Expression of Bitter Taste Receptors**
   - In rats, GSPE upregulated the expression of rat Tas2r in all segments of the intestine, especially in the jejunum and ascending colon.
   - Epicatechin, a prominent component of GSPE, is at least partially responsible for the changes in Tas2r expression in jejunum.
   - Enteroendocrine human Hutu-80 cell line showed that chronic stimulation with agonists for TAS2R5 and TAS2R39 downregulates the expression of non-stimulated TAS2Rs (TAS2R3 and -14)

ANNEX



Annex

Published Papers

Jalševac F, Terra X, Rodríguez-Gallego E, Beltran-Debón R, Blay MT, Pinent M, Ardévol A. The Hidden One: What We Know About Bitter Taste Receptor 39. Front Endocrinol (Lausanne). 2022 Mar 8;13:854718. doi: 10.3389/fendo.2022.854718.

Segú H, **Jalševac F**, Pinent M, Ardévol A, Terra X, Blay MT. Intestinal Morphometric Changes Induced by a Western-Style Diet in Wistar Rats and GSPE Counter-Regulatory Effect. Nutrients. 2022 Jun 23;14(13):2608. doi: 10.3390/nu14132608.

Descamps-Solà M, Vilalta A, **Jalševac F**, Blay MT, Rodríguez-Gallego E, Pinent M, Beltrán-Debón R, Terra X, Ardévol A. Bitter taste receptors along the gastrointestinal tract: comparison between humans and rodents. Front Nutr. 2023 Aug 30;10:1215889. doi: 10.3389/fnut.2023.1215889.

Segú H, **Jalševac F**, Sierra-Cruz M, Feliu F, Movassat J, Rodríguez-Gallego E, Terra X, Pinent M, Ardévol A, Blay MT. Assessing the impact of insect protein sources on intestinal health and disease: insights from human ex vivo and rat in vivo models. Food Funct. 2024 Apr 22;15(8):4552-4563. doi: 10.1039/d4fo00381k.

Segú, H.; **Jalševac, F.**; Lores, M.; Beltrán-Debón, R; Terra, X.; Pinent, M.; Ardévol, A.; Rodríguez-Gallego, E.; Blay, M.T. Intestinal Taste Receptor Expression and Its Implications for Health: An Integrative Analysis in Female Rats after Chronic Insect Supplementation. Published in Journal of Agricultural and Food Chemistry, 2nd of June 2024.

Submitted Papers

Jalševac, F.; Descamps-Solà, M.; Vilalta, A.; Segú, H.; Blay, M. T.; Beltrán-Debón, R.; Rodríguez-Gallego, E.; Terra, X.; Ardévol, A.; Pinent, M. Subchronic modulation of bitter taste receptors (TAS2R) by procyanidins. Unravelling the complex interplay between stimulation and expression. Submitted to Food & Function, 15th of May 2024.

Jalševac, F.; Descamps-Solà, M.; Grau-Bové, C.; Segú, H.; Auguet, T.; Avilés-Jurado, F.X; Balaguer, F., Jorba, R; Beltrán-Debón, R; Blay, M.T; Terra, X.; Pinent, M.; Ardévol, A. Profiling Bitter Taste Receptors (TAS2R) along the Gastrointestinal Tract and their Influence on Enterohormone Secretion. Gender- and Age-Related Effects in the Colon. Submitted to Frontiers in Endocrinology, 22nd of May 2024.

Jalševac, F.; Segú, H.; Balaguer, F.; Ocaña, T.; Moreira, R.; Gràcia-Sancho, J.; Fernandez, A.; Andres-Lacueva, C.; Martínez, M.; Beltrán-Debón, R.; Rodríguez-Gallego, E.; Terra, X.; Ardévol, A.; Pinent, M. TASR5 and TAS2R38 are bitter taste receptors whose colonic expressions play important roles in age-associated processes. Submitted to The Journal of Nutritional Biochemistry, 27th of May 2024.

Papers Under Preparation

Segú, H.; Lores, M.; Soler, O.; Colom-Pellicer, M.; Jalševac, F.; Beltrán-Debón, R; Terra, X.; Pinent, M.; Ardévol, A.; Rodríguez-Gallego, E.*; Blay, M.T. Examining the Impact of Insect-Based Diets on Intestinal and Overall Health, in Healthy and Diet-Induced Obese Rats.

Oral Presentations

Tarragona, September 2022 – NuGoweek 2022 Food bioactives for disease prevention – From mechanisms to chrononutrition – Oral presentation and Poster Presentation – Protective Effect of Grape-Seed Derived Procyanidin (GSPE) Against Ageing and Cafeteria Diet on the Expression of Bitter Taste Receptors in Rat Intestine.

Tunis, October 2023 – 9th International Conference of the Mediterranean Neuroscience Society MNS2023 – Oral Presentation - Profile of Bitter Taste Receptors in the Jejunum of Morbid Obese Patients that undergo Bariatric Surgery.

Arnes, November 2023 – IISPV Retreat 2023 – Oral Presentation - Ecnomotopic Bitter Taste Receptors as Targets for Better Health in the Obesity or the Ageing

Poster Communications

Online, September 2021 - eNuGO Week 2021: Immuno-nutrigenomics: How to feed the immune system – Online Poster: Effects of Grape seed procyanidin on gene expression of the GLP-1 system in aging in female rats.

Online, November 2021 - I Congreso Internacional en Ciencias Biomédicas: Epidemias, Endemias y Pandemias Persistentes y Emergentes – Online Poster: the Effects of Grape seed procyanidin on gene expression of the GLP-1 system in aging in female rats.

Tarragona, April 2022 – I Jornadas Sobre Nutracéutica: Compuestos Bioactivos y Nutracéuticos – Poster - Effects of Grape Seed Procyanidins on Gene Expression of the GLP-1 System in Aging in Female Rats.

Leipzig, October 2022 – 4GPCRnet International Symposium – Poster presentation and Short Oral Presentations - Different Effects of Ageing on the Expression of Bitter Taste Receptors in Human and Rat Colon.

Tarragona, April 2022 – II Jornadas Sobre Nutracéutica: Compuestos Bioactivos y Nutracéuticos – Poster Presentation - Differential Expression Profile of Bitter Taste Receptors throughout the Human Gastrointestinal Tract

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HVALA /GRACIAS / GRÀCIES / THANK YOU

The population of the world is growing ever older, and with it, the incidence of age-associated disorders is on the rise. The constant internal and external stressors are balanced by homeostatic systems, which minimise their negative effects. Dysregulation of metabolic homeostasis is one of the main drivers of many age-accompanying disorders. Chemosensory receptors of the gastrointestinal tract (GIT) can be influenced to elicit several beneficial effects. One group of these receptors are the bitter taste receptors (TAS2R). TAS2R and their ligands, such as grape seed proanthocyanidin extract (GSPE) have been proposed as novel ways to achieve healthier ageing.

In this dissertation, we discuss the diversity in the TAS2R throughout GIT, their evolution with ageing, and focus on establishing which out of 26 human receptors are more likely to have a role in the progress of ageing. Additionally, we focus on how GSPE and its constituents influence the changes in the presence of TAS2R in the GIT, and how they influence the secretory potential of the GIT.

We discovered that some receptors are more present in specific segments of the GIT, and interestingly, the ageing upregulated the expression of a handful of TAS2Rs in the colon mucosa. TAS2R5 was labelled as the most probable receptor to have important roles in the progress of ageing, due to its correlations with several parameters that are known to impact overall health. Finally, GSPE has proven to be a potent regulator of TAS2R expression, and its constituent epicatechin has been shown to impact the secretory role of the GIT cells.

In summary, this thesis presents TAS2R as interesting novel targets for potentially impacting the progress of ageing, due to their involvement in the centre of metabolic regulation and possible activation with bioactive compounds, which could elicit multiple beneficial effects.