# Intestinal Nutrient Sensing: a gut feeling for food

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Nikkie van der Wielen

**Thesis**

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# General introduction

1

## Obesity

The increasing prevalence of obesity is a major public health and economic burden (1). According to the WHO, obesity and overweight are defined as 'abnormal or excessive fat accumulation that may impair health' (2). The WHO estimates that 13% of the adult world population is currently obese, and about 39% of the adult population overweight (2). Being overweight is defined as having a body mass index higher than 25 kg/m$^2$, while obesity is defined as having a body mass index exceeding 30 kg/m$^2$ (2). Obesity increases the risk for comorbidities like type II diabetes, coronary heart disease, and cancer, and it leads to higher overall mortality rates.

The alarming increase in prevalence of obesity rates creates an urgent need for effective prevention and treatment strategies. However, achieving sustained weight loss in obese patients has proven to be very challenging, and long term life style modifications like dieting and exercise often show disappointing results (3). At the moment there are only a few approved pharmacological options available, showing only modest results (4, 5), and so far medicinal options have proven to be unsuccessful on the longer term due to lack of sustained efficacy and (or) severe side-effects. Overall, the most effective treatment for obesity today is bariatric (weight loss) surgery, which is a collective term for different surgical techniques that either reduce stomach size or bypass parts of the intestine (6). These changes result in alterations of gut hormone release, the latter playing an important role in the fast effects on glucose homeostasis, and gradual loss of body weight. Gut hormones are well-known for their effects on food intake behavior (7) and their role in weight loss after bariatric surgery is undeniable. Also, one of the recently approved pharmacotherapies for type II diabetes and obesity, liraglutide, is a gut hormone (GLP-1) analogue. This underlines why gut hormones are considered promising targets for the development of new treatment strategies for obesity and its comorbidities (8).

## Gut hormones

The gastrointestinal tract is an important endocrine organ of our body. It secretes several pivotal hormones which are released from so-called enteroendocrine cells. These cells comprise only 1% of the epithelial lining (9). Many different types of enteroendocrine cells have been described and these types were originally defined by their supposed specific product. For example, the L-cells were defined by their expression and release of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), whereas I-cells were characterized by their expression and secretion of cholecystokinin (CCK). However, it is now known that these subtypes show overlapping secretion profiles with most enteroendocrine cells actually expressing a wide array of gut hormones (10).

Enteroendocrine cells can be subdivided in closed and open cells. The closed type, which is not in contact with the lumen of the gastrointestinal tract, responds to neural and circulating signals. An example of such type of enteroendocrine cell is the histamine secreting enterochromaffin-like cell in the stomach (11). The open type of enteroendocrine cell on the other hand has direct contact with the luminal content of the gut. This type of cell can be stimulated by nutrients to secrete its hormones (12). Once secreted, these hormones can act in a paracrine manner by binding to their receptors on neighboring epithelial cells or on neurons of for example the vagal afferent fibers. These hormones can also enter the circulation and act in an endocrine manner throughout the body (13). Many gastrointestinal hormones are secreted upon food intake, and have been implicated in food intake regulation and physiological processes that underlie the control of metabolic homeostasis. As a result they are also involved in body weight management and important for the development of obesity. In the next sections the main hormones involved in satiety and satiation are discussed.

### Glucagon-like peptide-1

Glucagon-like peptide-1 (GLP-1) is one of the cleavage products of proglucagon, which is encoded by the gene GCG. There are different forms of GLP-1, the active types are GLP-1(7-36amide) and GLP-1(7-37). These active GLP-1 types can be inactivated by the enzyme dipeptidyl peptidase-4 (DPP

IV) into the inactive types GLP-1(9-36amide) and GLP-1(9-37) (14). In the intestine, GLP-1 is secreted by L-cells which are mainly located in the ileum and colon, and to a lower extent in de proximal small intestine (15, 16). Apart from the intestine, proglucagon is also expressed in the brain, tongue and pancreas (17, 18). There, different post-translational processing results in the formation of glucagon (18). Here we focus on GLP-1 produced and secreted by the intestine.

GLP-1 secretion is regulated by many physiological signals from the body, including by luminal stimuli in the gut. Upon nutrient ingestion, GLP-1 is secreted into the blood in two phases. The early rise in GLP-1, about 15-30 minutes post-prandial, is thought to be regulated by neuronal signals, including via acetylcholine. The second peak of GLP-1, 90-120 minutes post-prandial, is thought to be regulated by the detection of nutrients and their breakdown products in the lumen of the gut (19). This process will be discussed in the next section 'nutrient sensing'. Once GLP-1 is secreted, it gets rapidly degraded by DPP IV. About 25% of the secreted GLP-1 leaves the gut in its active form via the portal vein, of which another 40-50% is subsequently degraded in the liver. This results in a short half-life of only 1-2 minutes for active GLP-1 in the circulation (18). Therefore, it is conceivable that most of its effects are paracrine, including via binding to its receptors expressed on the vagal nerve endings in the proximity of enteroendocrine cells (20, 21). The GLP-1 receptor is not only expressed on enteric nerves in the intestine, but also in many other tissues, like the pancreas, cardiovascular system, hypothalamus, brainstem and liver (22). Thus, GLP-1 has a large array of effects in the body.

In relation to food intake, GLP-1 inhibits gastric emptying and motility, thereby playing an important role in the development of the so-called 'ileal brake' activation (23). These actions, together with central effects on appetite contribute to a reduction of food intake. Next to this, GLP-1 acts as an incretin hormone, increasing insulin and decreasing glucagon secretion in a glucose-dependent manner. Apart from these main effects, GLP-1 also influences blood flow, inhibits hepatic glucose production, decreases inflammation, and induces proliferation of beta cells in the pancreas (24). Currently, there are an increasing number of GLP-1 analogues appearing on the market (exenatide,

lixisenatide, dulaglutide, liraglutide etc.). Liraglutide was initially only approved for treatment of type II diabetes but its indications for use have recently been extended to management of body weight (4, 25).

**Peptide YY**

Next to GLP-1, also Peptide YY (PYY) is secreted from enteroendocrine L-cells located in the distal intestine (16). Similarly to GLP-1, PYY blood levels show an initial rise, starting 15 minutes after food intake and reaching a peak after 1-2 hours. This suggests that secretion is regulated by neuronal and gut nutrient sensing signals, similar to those for GLP-1 (26). PYY is secreted as PYY(1-36) and contrary to GLP-1, released PYY is cleaved by DPP IV into its bioactive form PYY(3-36), which binds and activates the Y2 receptor. This receptor for PYY is mainly expressed in the brain, but also on vagal nerve afferents (27). PYY has a half-life of about 9 minutes in the systemic circulation (28). Like GLP-1, PYY(3-36) also slows down gastrointestinal motility, contributing to the 'ileal brake'. Furthermore, it acts centrally by inhibiting food intake. Remarkably, the uncleaved form PYY(1-36) can also activate Y receptors other than Y2, contributing to increased food intake (29).

**Cholecystokinin**

Cholecystokinin (CCK) prepropeptide is the precursor for the many existing biologically active molecular forms of CCK, of which the most abundant are CCK8, CCK22, CCK33, and CCK58, and can exist in sulfated and non-sulfated forms (30). The most important enzyme in the synthesis of bioactive CCK is prohormone convertase 1/3 (31). CCK is produced by intestinal I-cells, which are mainly located in the upper small intestine (16). Furthermore, CCK is expressed in the brain and enteric nervous system (29). Intestinal CCK is secreted upon food intake and has a plasma half-life of about 2-3 minutes (29, 32). There are two CCK receptors, the CCK-1 receptor being mainly present in the galbladder, pancreas and gastrointestinal tract. The CCK-2 receptor is expressed in gastric tissues, and both receptors are expressed in the brain (33). The non-sulfated CCK peptide acts as agonist for CCK-2 receptor, whereas the sulfated CCK peptides are the main agonists for the CCK-1 receptor. The

activation of CCK-1 receptors causes the release of enzymes and bile acids stimulating digestion and inhibition of gastric emptying (28, 32). Moreover, CCK plays a role in the control of food intake (34).



**Ghrelin**

Ghrelin is one of the post translational processing products of preproghrelin. There are two forms of ghrelin; desacyl-ghrelin and the acylated forms, acyl ghrelin, the latter commonly designated as the bioactive molecular form(s). Acyl ghrelin is produced by attachment of a fatty acid side-chain (preferably C8 or C10) by the action of ghrelin O-acyl-transferase (GOAT). Ghrelin is expressed in many tissues, among which the pancreas and brain. Here, we focus on gastrointestinal ghrelin, which is secreted by X/A-like cells, mainly located in the stomach (35). GOAT is also expressed in many tissues among which the stomach and pancreas. Next to the alimentary system, GOAT is also present in the plasma (36, 37). Ghrelin is secreted pre-prandially by the empty stomach, and its secretion is reduced in a post-prandial state by expansion of the stomach, activation of nutrient sensing receptors, and the digestion of nutrients in the intestine (38, 39). Acyl ghrelin is a ligand for the growth hormone secretagogue receptor (GHSR)1a. Acyl ghrelin has orexigenic effects and it stimulates post-prandial gastric emptying. Furthermore, it stimulates growth hormone secretion, it influences glucose homeostasis, and stimulates adiposity (35). Although desacyl ghrelin is not able to bind to GHSR1a, it is suggested that this form of ghrelin also has a function, but these studies are still very contradictory (35).

## Nutrient sensing

As described above, the release of many of the gastrointestinal hormones is regulated by ingested nutrients and other molecules present in the gastrointestinal tract. Various chemosensory mechanisms along the entire gastrointestinal tract are continuously monitoring the concentration of nutrients, digestion products and microbial metabolites. These chemosensory processes, together with their effect on gastrointestinal hormone secretion are often referred to as "nutrient sensing". A large number of G-protein coupled receptors (GPCRs), other receptors such as PPARs, and transporters have been identified and linked to gut hormone secretion in the recent years. Here, the main receptors and transporters known to be involved in the detection of carbohydrates, proteins and lipids will be discussed.

**Carbohydrates**

In the gastrointestinal tract, ingested carbohydrates are mainly degraded to monosaccharides, among which glucose and fructose. Glucose can be transported into the cells by its sodium-glucose linked transporter (SGLT-1). Due to the cotransport of sodium, small current differences stimulate depolarization that results in hormone release from enteroendocrine cells. SGLT-1 mainly mediates the secretion of GLP-1 (40, 41). Next to SGLT-1, the sweet taste receptor, a heterodimer of T1R2 and T1R3, has been suggested to be involved in the sensing of carbohydrates and other sweet tasting compounds, including non-caloric sweeteners used in food products. Sweet taste receptors are present throughout the gastrointestinal tract, from oral cavity to colon. At cellular level they are expressed on GLP-1, GIP and serotonin-positive cells (42). Its activation has been shown to induce GLP-1 secretion (43, 44). Apart from the effect of monosaccharides via SGLT-1 or the sweet taste receptor, monosaccharides like glucose and fructose can also induce hormone secretion in a metabolism-dependent way. Metabolism of glucose results in higher ATP levels in the enteroendocrine cells, thereby affecting ATP-sensitive potassium channels (45, 46).

**Proteins**

In the gastrointestinal tract, proteins are degraded to polypeptides and amino acids. These can be detected in the gastrointestinal tract by several receptors, dependent on their size and composition. Peptones, hydrolyzed protein fragments, have been reported to activate GPR93 (GPR92 or LPAR5) and induce CCK release (47). Smaller products like di- and tripeptides are absorbed by the proton-coupled transporter Pept1. This absorption induces changes in membrane potential resulting in GLP-1 secretion (48). Furthermore, Pept1 is involved in CCK release (49). Amino acids, the smallest protein degradation products, are detected by several GPCRs, including the calcium sensing receptor (CaSR), the umami taste receptor (T1R1/T1R3) and GPCRC6a. All these amino acid receptors have been shown the play a role in gut hormone release (50-53).

**Lipids**

Ingested triglycerides are degraded to mono- or diglycerides and fatty acids. Long chain fatty acids can be detected by GPR120 and GPR40 (FFAR1). Both receptors have been implicated in gut hormone release, with GPR120 being the most extensively investigated one (54-60). Monoglycerides and fatty acid metabolites, like 2-oleoyl glycerol and oleoylethanolamide, are also known to activate GPR119 resulting in GLP-1 secretion both *in vitro* and *in vivo* (61-64). Apart from the ingested fatty acids, short chain fatty acids are also produced in the gut by microbiota. These short chain fatty acids activate GPR41 and GPR43, thereby inducing the secretion of GLP-1 and PYY (65, 66).

**Other compounds**

Next to the macronutrients discussed above, there are also other compounds in the gastrointestinal tract that can influence gut hormone secretion. Bitter compounds in our food can activate bitter receptors expressed along the intestinal tract (67). Bitter receptors belong to the taste receptor 2 family (T2R). They are G-protein coupled receptors consisting of approximately 25 subtypes in human, which exist as homo- or heterodimers. These bitter receptors are able to detect a large array of bitter compounds and most of the de-

orphanized receptors exhibit a large agonist spectrum (68). The activation of these receptors can induce GLP-1 and CCK secretion (69-72). Furthermore, bitter tastants can stimulate ghrelin secretion (39). Non-dietary compounds such as bile acids are also known to induce GLP-1 secretion via the bile acid receptor GPBAR1 (TGR5) (73, 74). Lastly, the peptide hormone somatostatin, produced amongst others in the stomach can inhibit the secretion of some other hormones, including GLP-1, PYY and ghrelin (75-78).

**Stevia**

As mentioned above, sweet compounds in the food can be detected by the sweet taste receptor and subsequently induce hormone release. This thesis will focus on a specific sweetener, namely stevia. Stevia is increasingly used in food products after its approval in the European Union in 2011 and it is marketed as a 'natural' sweetener. The active compounds derived from the plant *Stevia rebaudiana* Bertoni are steviol glycosides. The most common steviol glycosides are stevioside and rebaudioside A, but there are many more molecular forms identified, all consisting of a steviol backbone with different side groups, mostly glucose moieties (figure 1.1)(79). These steviol glycosides vary in their potencies to induce sweet taste sensations. Stevioside, rebaudioside A and rebaudioside D are reported to have the lowest sweet taste threshold compared to other common steviol glycosides when tested by a sensory panel. Comparable potencies were found in cell-based assays functionally expressing the sweet taste receptor. Apart from their sweet taste and capability to activate the sweet taste receptor, steviol glycosides have a bitter taste and they can activate the human T2R4 and T2R14 bitter receptors (80).

Upon ingestion of stevia containing products, steviol glycosides remain stable throughout the gastrointestinal tract. Only upon contact with the gut microbiota the steviol glycosides are broken down to steviol, which can be absorbed (81). Based on the stability of steviol glycosides in the stomach and small intestine, it can be assumed that intact steviol glycosides activate sweet taste receptors throughout the intestinal tract, thereby inducing gut hormone secretion. This hypothesis was tested in the current thesis.

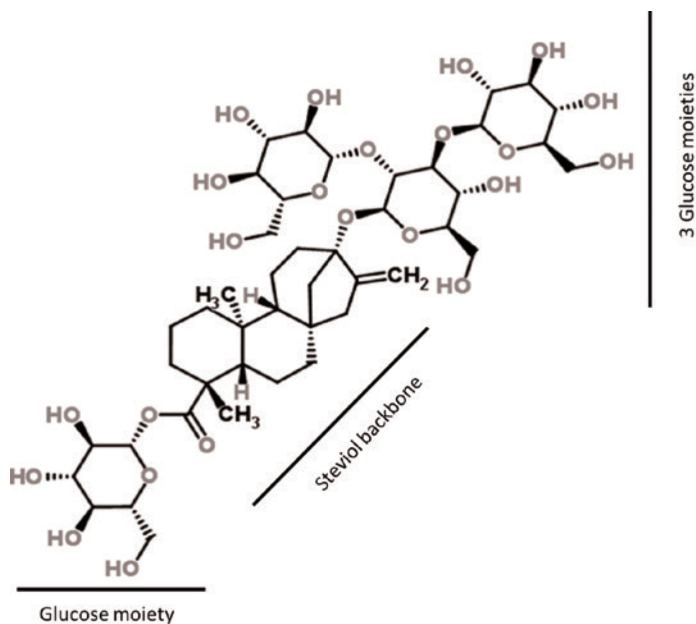


**Figure 1.1 Chemical structure of Rebaudioside A.**
Adapted from ChemSpider ID: 5294031



## Approaches to study gastrointestinal nutrient sensing

There are many strategies and models to investigate the effects of nutrients on gastrointestinal hormone release. Clearly, the ultimate goal for human nutrition is to understand the physiology of appetite regulation in man. However, options for studies in humans are limited. Therefore, a number of other options are also discussed here, among which the experimental approaches used in this thesis.

### Studies in humans: intervention studies and measurements in patients undergoing bariatric surgery

There are only a few options to study the physiological actions of nutrients in the gastrointestinal tract, and their effects on hormone release in humans. An advantage of human intervention studies is that they provide the opportunity to combine plasma hormone levels with measurements of hunger, satiety and feelings of intestinal discomfort using visual analog scales and (or) *ad libitum*

food consumption. However, effects on satiety or satiation are not always directly reflecting changes in plasma hormone levels (82).

The most basic intervention design is to study the effects of an ingested meal on plasma hormone levels. To investigate the role of specific nutrients on hormone secretion in different locations of the intestine, delivery of nutrients by intubation of the gastrointestinal tract, or by encapsulation are a better option (83, 84). However, intubation is quite invasive which limits its use for larger studies. At the same time encapsulation techniques for food products are still in their infancy. In order to study the involvement of specific nutrient sensing receptors in hormone secretion, the use of agonists and antagonists would provide opportunities. However, for human studies only a few compounds are available, and their safety is a limitation (85). In humans, it is possible to investigate the presence of these nutrient sensing receptors by obtaining mucosal biopsies. An excellent opportunity to collect these biopsies is during bariatric surgery. This gastrointestinal surgery, intended to lose weight, is mainly applied in morbidly obese patients. For this thesis, we were able to use samples from a minimally invasive form of bariatric surgery, namely the recently developed Articulating Circular Endoscopic (ACE) stapler, which reduces gastric volume by gastroplication. The feasibility, safety and efficacy of the ACE stapler procedure was assessed and this procedure resulted in a median 35 percent of excess weight loss in the first year (86).

**Animal models**

By using animal models in gastrointestinal research it is possible to gain fundamental knowledge on the effects of nutrients on gut hormone secretion. Animal models also allow fully standardized experimental procedures and there are fewer limitations to tissue sampling compared to human studies. The vast majority of the animal studies related to gut hormone research are performed in mice. *In vivo* models in mice also provide the possibility to test effects in more detail by knocking out or overexpressing certain nutrient sensing related genes. However, for other research questions an animal model that more closely resembles human gastrointestinal physiology is to be preferred. Pigs are omnivorous and show an intermittent eating pattern that to some

extent resembles that of humans. They also show a gastrointestinal physiology and intestinal transit time comparable to that of humans (87-89).

One of the limitations of *in vivo* studies is that one cannot measure local gut hormone release, the closest approximation of gastrointestinal secretion being to measure the gut hormones in the circulation. A pitfall with this measurement is that the released gut hormones in general have a very short half-life. Furthermore, many gut hormones also have paracrine functions. Measuring the effects in the circulation will most likely not accurately represent its paracrine effects . To address these issues, *in vitro* studies may be able to provide more suitable options.



### *In vitro* models

There are many *in vitro* options to study gut hormone release and the mechanisms involved, ranging from *ex vivo* tissue experiments and primary cell cultures, to organoids and cell lines.

There are several *ex vivo* models, which are mainly used to measure intestinal transport. Some of these models segregate the luminal side from the basolateral side, like the everted sac and Ussing chamber models (90, 91). Other models do not differentiate between the two sides, like the porcine *ex vivo* segment model (92). The latter was used in this thesis. For *ex vivo* tissue experiments using gut material, there is the benefit of having an intact epithelial lining and the opportunity to measure direct hormone release by excising parts of the intestinal tract. One of the pitfalls of *ex vivo* studies is the short-term viability of the tissue.

In contrast to *ex vivo* experiments, primary cells can be maintained for longer periods. Primary cells, freshly isolated from the intestine, are theoretically more similar to the epithelial cells as they exist *in vivo*, compared to immortalized cell lines. However, the intestinal epithelium only contains low numbers of enteroendocrine cells. This, together with the fact that it is difficult to keep them alive for a longer period of time makes it a difficult technique to study gut hormone secretion from primary enteroendocrine cells (40, 93). Recent advances in stem cell biology have led to a more appropriate model, intestinal organoids. Intestinal organoids, so-called mini-guts, can be grown from isolated

crypts, single Lgr5-CBC cells or pluripotent stem cells (94, 95). These organoids closely resemble the *in vivo* epithelium. It was previously shown that L-cells will develop in organoids, and that these are able to secrete GLP-1 in response to nutrient stimuli (96, 97). Therefore, intestinal organoids are an interesting model to study enteroendocrine cells and gut hormone secretion in their regular epithelial environment. However, the most commonly used three dimensional model only produces low levels of gut hormones.

The last model for studying enteroendocrine cell physiology are cell lines. There are several hormone-secreting cell lines available. The most commonly used are NCI-H716, GLUTag and STC-1. NCI-H716, a human cecal adenocarcinoma derived cell line, seems to be the best tool to study human enteroendocrine cell behavior. However, this cell line needs to undergo differentiation before use and there are doubts about its behavior reflecting human physiology (93). GLUTag and STC-1 are extensively used murine-derived cell lines. GLUTag cells are isolated from a colon tumor of a transgenic mouse which expressed a SV40 large T antigen under the control of the proglucagon promoter. The cells have been extensively studied in relation to GLP-1 secretion (98, 99). STC-1 cells are derived from a small intestinal neuroendocrine carcinoma from a double transgenic mouse (100, 101). These cells are reported to secrete CCK, GLP-1, PYY, glucose-dependent insulinotropic polypeptide, pancreatic polypeptide, GLP-2 and oxyntomodulin (100).

## Outline of thesis

As discussed above, the interactions of dietary components or their breakdown products with receptors and transporters in the gastrointestinal tract can induce the release of different gut hormones. These hormones influence food intake behavior via combinations of different paracrine and endocrine mechanisms, making signaling pathways involved in nutrient sensing interesting targets for the prevention or treatment of obesity and its comorbidities. However, many nutrient sensing mechanisms are still only partly understood and interactions between several specific nutrients and their receptors remain to be explored. Although gut nutrient sensing is often investigated in relation to appetite

control, effects of eating habits or body composition as such on these nutrient sensing mechanisms is scarcely investigated. Furthermore, much research in this field has been performed in different models and animal species, which often hampers proper comparison or translation.

In this thesis, we aimed to further elucidate gut nutrient sensing mechanisms on a cellular level. First, the expression of several gut nutrient sensing related genes along the intestinal tract was assessed in three commonly studied species, namely mouse, pig and human. Intestinal expression patterns were compared between the species, and these results are presented in chapter 2. Next, effects of a weight-loss intervention on the gastrointestinal transcriptome were investigated in humans. To this end, we analyzed gastric and duodenal biopsies of morbidly obese patients who underwent reduction of gastric volume by a gastroplication procedure. The findings of this study are described in chapter 3. In chapter 4, we sought to investigate the effects of the non-caloric sweetener rebaudioside A. This *Stevia rebaudiana*-derived compound was approved on the European market in 2011. As there is still some controversy about the effects of sweeteners in general on gut hormone release, we investigated the effects of this specific sweetener. Subsequently, in chapter 5, the underlying signaling mechanisms of rebaudioside A-induced gut hormone secretion were investigated using enteroendocrine STC-1 cells. In chapter 6, a two dimensional gut model using intestinal organoids derived from mice intestinal crypts was developed to study hormone secretion. These organoids were derived from different intestinal locations, allowing us to explore location-specific hormone release by rebaudioside A. Furthermore, in this system we were able to look at the effects of long term exposure to this compound on the development of enteroendocrine cells. The collective findings of these studies are discussed in chapter 7.

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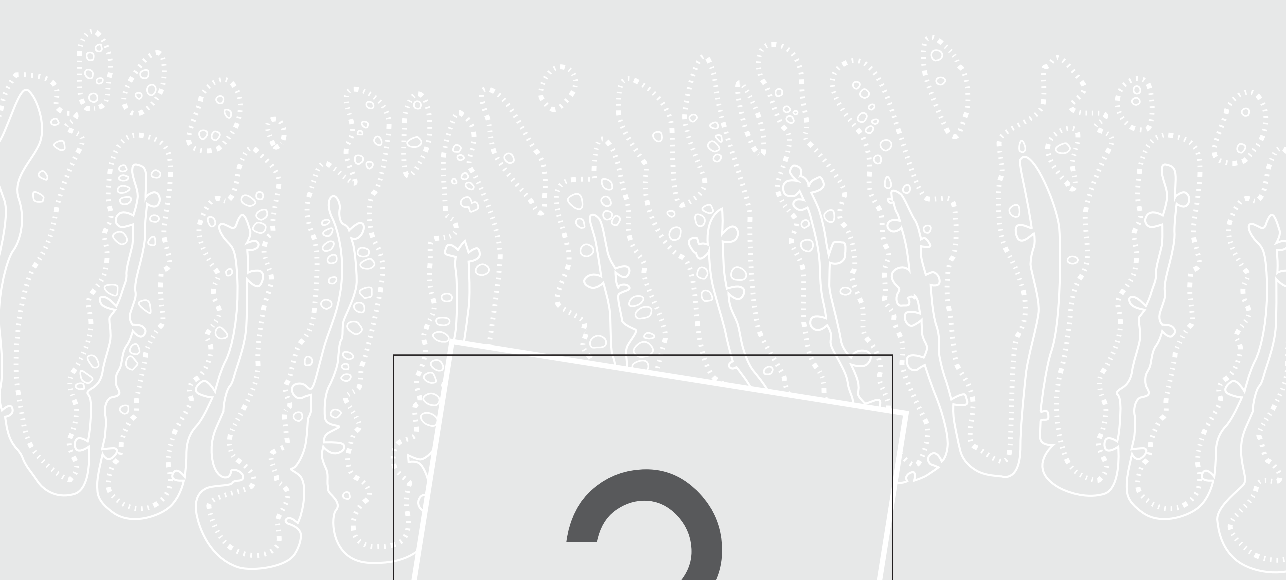

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# Cross-species comparison of genes related to nutrient sensing mechanisms expressed along the intestine

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## Abstract

**Introduction:** Intestinal chemosensory receptors and transporters are able to detect food-derived molecules and are involved in the modulation of gut hormone release. Gut hormones play an important role in the regulation of food intake and the control of gastrointestinal functioning. This mechanism is often referred to as "nutrient sensing". Knowledge of the distribution of chemosensors along the intestinal tract is important to gain insight in nutrient detection and sensing, both pivotal processes for the regulation of food intake. However, most knowledge is derived from rodents, whereas studies in man and pig are limited, and cross-species comparisons are lacking.

**Aim:** To characterize and compare intestinal expression patterns of genes related to nutrient sensing in mice, pigs and humans.

**Methods:** Mucosal biopsy samples taken at six locations in human intestine (n=40) were analyzed by qPCR. Intestinal scrapings from 14 locations in pigs (n=6) and from 10 locations in mice (n=4) were analyzed by qPCR and microarray, respectively. The gene expression of glucagon, cholecystokinin, peptide YY, glucagon-like peptide-1 receptor, taste receptor T1R3, sodium/glucose cotransporter, peptide transporter-1, GPR120, taste receptor T1R1, GPR119 and GPR93 was investigated. Partial least squares (PLS) modeling was used to compare the intestinal expression pattern between the three species.

**Results and conclusion:** The studied genes were found to display specific expression patterns along the intestinal tract. PLS analysis showed a high similarity between human, pig and mouse in the expression of genes related to nutrient sensing in the distal ileum, and between human and pig in the colon. The gene expression pattern was most deviating between the species in the proximal intestine. Our results give new insights in interspecies similarities and provide new leads for translational research and models aiming to modulate food intake processes in man.

## Introduction

Various chemosensory mechanisms along the entire gastrointestinal tract are continuously monitoring the concentration of nutrients, digestion products and microbial metabolites. These chemosensory processes together with their effect on gastrointestinal hormone secretion are often referred to as "nutrient sensing". The chemosensory mechanisms involve the action of different receptors and transporters generally located on membranes or within the cytoplasm of enterocytes, brush cells and enteroendocrine cells (1). The latter cell types comprise about 1% of the epithelial cells in the intestine (2). Nutrient sensing plays a pivotal role in the local and central regulation of food intake and gastrointestinal motility, secretion of mucus and enzymes, transport and uptake mechanisms (3). According to the most common view, stimulation of G-protein coupled receptors (GPCRs) and/or ion-dependent nutrient transporters located at enteroendocrine cells, modulate the release of gut hormones like glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK) and peptide YY (PYY) (1, 4). For example, activation of the umami taste receptor (T1R1 and T1R3) by amino acids has been suggested to induce CCK secretion (5), whereas G-protein coupled receptor 120 (GPR120) responds to fatty acids, thereby stimulating GLP-1 and CCK secretion (6, 7). In addition to receptors, several transporters for nutrients are involved in the modulation of gut hormone secretion. The sodium-glucose cotransporter member 1 (SGLT-1) has been suggested to induce GLP-1 secretion (8, 9). Recently, the peptide transporter (PepT1) was also shown to stimulate GLP-1 secretion (10). Secreted gut hormones can act via their corresponding receptors on vagal nerve afferents or via the endocrine pathway to affect food intake behavior (11). The small intestine plays a prominent role in generating this feedback to the brain during and in between meals (12).

In spite of the importance of chemosensors in relation to food intake, there are only few studies describing the distribution of various chemosensors along the human intestinal tract. More knowledge on this (regional) distribution can provide better insight in the underlying nutrient-sensing mechanisms potentially involved in individual differences in food intake and the likeliness to develop metabolic diseases. The issue of cross-species comparison is important since the

vast majority of studies in this field has been performed in rodents, such as the mouse (10, 13-15). Pigs may serve as a more suitable animal model because pigs and humans show more similarity in gut physiology than mice and humans. Pigs are omnivorous and show a meal-eating pattern in their eating behavior. They have a comparable gastrointestinal physiology and intestinal transit time to humans (16-18). However, despite these gross similarities it is not known to what extent the two species are similar with respect to gut nutrient sensing.
In the present study we extensively characterized the distribution of a number of receptors, transporters and hormones known to be involved in nutrient sensing focusing on the small intestinal tract of three species; pig, mouse and man. Next to measuring the expression of a selected set of genes involved in nutrient sensing we used Partial Least Squares (PLS) modeling to compare the three species. Lastly, the effect of fat/carbohydrate content in the diet on the expression of the selected genes was investigated.



## Methods

### Ethics statement

The use of human biopsy material for this study was approved by the Medical Ethical Committee of Maastricht University Medical Center+, the Netherlands (NCT02051881, NCT01574417). The porcine tissue was collected from control animals of a larger study, which was approved by the ASG-Lelystad Animal Care and Ethics Committee (Permit number: 2011135.c). Mice material was collected in a larger study which had been approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University (Permit number: 2010084.c).

### Tissue sampling

Human intestine: Biopsies were obtained from 40 healthy subjects (male and female between 21 and 82 years), who were referred for gastrointestinal endoscopy or participating as healthy controls in another study. Each subject gave written informed consent before participation. Exclusion criteria were as follows; the observation of any macroscopic or histologic abnormalities, history of

severe cardiovascular, gastrointestinal/hepatic-, hematological/immunologic-, or metabolic/nutritional disease, major abdominal surgery interfering with gastrointestinal functioning or/and excessive alcohol consumption. All biopsies were taken with a standard forceps and the subjects were fasted prior to the endoscopic procedure. Due to the invasiveness of the procedure, it was only feasible to obtain biopsies from one or two locations in most subjects, except for the colon where mucosal tissue samples from three or four compartments were obtained. Duodenal tissue samples were taken from subjects who underwent an upper gastrointestinal endoscopy. These biopsies were taken at approximately 10cm distal to the pyloric sphincter. Ileal and colonic biopsies were taken from subjects who underwent standard flexible colonoscopy. Ileal biopsies were taken at approximately 5cm proximal to the ileoceacal valve. Colonic biopsies were taken from the ascending, transverse and descending colon and from the sigmoid colon, respectively. In nine subjects we were able to collect mucosal tissue samples at 40-45cm distal to the pylorus, representing the proximal jejunum. All biopsies were snap frozen in liquid nitrogen and stored at -80°C until analysis.

Porcine intestine: To obtain tissue, six 10 week old male pigs (Large White x Landrace) were fasted overnight and killed by exsanguination under deep anesthesia. Immediately after this procedure, both the small and large intestine were excised and its total length was measured. From the small intestine, pieces of approximately 40cm$^2$ were cut out at 10 locations, namely at 3, 6, 20, 40, 50, 60, 70, 80, 90 and 98% of its total length (proximal to distal). These intestinal pieces were rinsed with water and scrapings were obtained. Scrapings were also taken from the cecum and at three locations in the large intestine, namely at 12.5, 37.5 and 75% of its total length. Apart from scrapings additional mucosal biopsies were taken from similar intestinal locations as mentioned above. Both the biopsies and scrapings were snap frozen in liquid nitrogen and stored at -80°C.

Mouse intestine: Male C57BL/6J mice (age 4 weeks) were housed 2 per cage in the light and temperature-controlled animal facility (12/12 (light/dark), 20°C) of Wageningen University. The mice had free access to water and received standard laboratory chow (RMH-B, Arie Blok BV, Woerden, the Netherlands)

for 3 weeks, followed by a run-in period for 2 weeks during which 4 mice received chow diet and 8 mice received a 10E% low-fat diet. Subsequently, 4 mice remained on the chow diet, 4 mice remained on the low-fat diet and 4 mice received a 45E% high-fat diet for the experimental period of 2 weeks. The composition of the low-fat and high-fat diets has been previously described by de Wit et al. (19). After the mice were fed, the small intestine of the sacrificed mice was excised. The small intestine was cut open longitudinally, divided in ten equal parts and scrapings were obtained. The colon was not sampled. These scrapings were snap frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Both the biopsy and scraping sampling methods included similar mucosal and submucosal layers of the intestine. However, with biopsies a smaller surface of the intestine is taken in comparison to the scrapings. Therefore scrapings were expected to give a more representative determination of the epithelial gene expression than biopsies. Scrapings were taken from mice and pigs. However, to exclude the possibility that interspecies differences are caused by different sampling methods, gene expression profiles were compared for biopsies and scrapings in pigs. For five genes analyzed no differences were found, only CCK and PepT1 showing about 50% lower expression in biopsies compared to scrapings (results not shown).

**RNA isolation**

RNA of the human and porcine samples was isolated by using TRIzol reagent (Life technologies, Bleiswijk, Netherlands) and further purified using the RNeasy mini kit (Qiagen) with on column DNase treatment (Qiagen, Venlo, Netherlands). The RNA isolation of the mouse scrapings was performed using the Promega SV total RNA isolation System (Promega Corporation, Madison, USA). RNA yield was measured with the Nanodrop ND-1000 Spectrophotometer and the quality of the human, mice and some porcine RNA samples was verified with an Agilent 2100 Bio analyzer (Agilent Technologies, Amstelveen, Netherlands).

**Quantitative PCR**

Subsequently, 1µg RNA was reversely transcribed using random primers with a Reverse Transcription System kit (Promega Corporation, Madison, USA) according to the manufacturer's instructions. For the negative controls, the use of the enzyme reverse transcriptase (-RT control) was omitted.

The qPCR reactions were performed on the CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, USA) using SensiMix SYBR No-ROX kit (Bioline, London, UK). Melt curve analysis and the amplification efficiency were used to verify the specificity of the amplification. Primers were designed using Beacon Designer 7.91 software, or primers were used from literature (Table S1 and S2). When using primers for Taqman analysis, the TaqMan Universal Master Mix II with UNG was used according to the manufacturer's protocol. 36B4 (*RPLP0*) was used as reference gene to normalize the mRNA abundance of each gene (20).

Glucagon (*GCG*, as precursor for GLP-1), *CCK, PYY*, GLP-1 receptor (*GLP1R*), PepT1 (*SLC15A1)*, SGLT-1 (*SLC5A1)*, T1R3 (*Tas1R3)*, GPR120 (*FFAR4)*, T1R1 (*Tas1R1)*, T1R2 (*Tas1R2)*, GPR93 (*LPAR5*) and *GPR119* were measured in all human and pig samples. However, T1R2 could not be detected by qPCR in pig and human intestine (for both species 5 primers were tested), probably due to the low level of gene expression as also reported by others (21, 22). Furthermore, despite the use of various primers T1R1 was still below detection level in the human samples and GPR119 was not detectable in the porcine samples. Lastly, GPR93 could not be quantified in pigs as the gene was not annotated. However, T1R1 was detected in mouse and pig, whereas GPR119 and GPR93 were demonstrated in mouse and man (figure S2.5).

**Microarray hybridization and analysis**

One hundred nanogram of RNA was used for Whole Transcript cDNA synthesis (Affymetrix, inc., Santa Clara, USA). Hybridization, washing and scanning of Affymetrix GeneChip Mouse Gene 1.1 ST arrays and Affymetrix GeneChip Porcine Gene 1.1 ST Arrays was carried out according to standard Affymetrix protocols. All arrays of the small intestine were hybridized in one experiment. Arrays were normalized using the Robust Multi-array Average method (23,

24). Probe sets were assigned to unique gene identifiers, in this case Entrez IDs. The probes on the Mouse Gene 1.1 ST arrays represent 21,213 Entrez IDs. The probes on the porcine gene arrays represent 17,118 Entrez IDs (25). Array data were analyzed using an in-house, on-line system (26). All microarray data have been submitted to the Gene Expression Omnibus (GSE59054).
Both microarray and qPCR techniques have been extensively studied in the past decades and evidence for a strong correlation of the measured gene expression between qPCR and Microarray analysis has been assessed and proven in several papers (27-29). Our own data were in accordance with these studies as comparisons of qPCR data with microarray data of 12 intestinal locations in pigs established that gene expression patterns were highly similar when using both techniques (data not shown).



**Statistical analysis**

Partial least squares (PLS) is a linear multidimensional fitting method. The method is used to relate sets of complex measurements X to a given external parameter Y. In this case the complex measurements are the measurement of gene expression and the external parameter is the location in the intestine for one of the species. The general formula for the method is $Y = aX + b$. Given an Y-vector and a X-matrix, PLS will calculate: a (loadings) and b (offset) which can then be used to predict the Y for any other set of data. The algorithm has many inbuilt features for scaling, filtering and cross validation (optimization of the number of factors used) of the data and is therefore very suited to be used with data where the relation between X and Y does not have to be directly linear. PLS was used to compare the intestinal expression patterns of eight genes between the species. Microarray data was used in log2 scale and subsequently all microarray and qPCR data were autoscaled to correct for the influence of the absolute intensities of the measurement. To prevent over fitting the PLS model was cross validated using a leave-one-out algorithm (30). As the porcine data consisted out of a comprehensive map of the intestinal expression patterns for the selected genes, this dataset was selected to model the relation between gene expression levels and intestinal location. For this model the data of eight genes was used, as these were measured in all three

species. Subsequently, the human and murine data were fitted into the porcine PLS model to compare the gene expression patterns for the different locations between the three species. PLS requires enough samples to cover the full range of the Y-values (locations in the intestine) to be fitted and enough samples to be able to cross validate the model. The sets used in this manuscript contain more than enough samples (pig 84 samples, human 63 samples, mouse 36 samples) to fulfill both these demands. For PLS analysis MATLAB (Version: 8.0.0.783, R2012b) and Winlin (version 1.8, TNO, Zeist, The Netherlands, (31)) were used.

## Results

### Comparison of the gene expression along the intestine between the three species

The relative gene expression pattern of each of the nutrient sensing related genes was measured at numerous intestinal locations in pig, man and mice. To compare the gene expression data of the three species, a PLS model was built for all three species. PLS analysis of the data gives a loading vector as listed in table 2.1 and 2.2. In general, high positive loading vectors reflect high distal expression, while high negative loading vectors reflect high proximal expression. From the porcine loading vectors of table 2.1 it can be seen that for example GPR120, PYY and glucagon give a positive contribution to the prediction of the location in the intestine i.e. in this model the samples at the distal intestine have relative higher expression of GPR120, PYY and glucagon than at the proximal intestine. The PLS model built on the human data, gave comparable results, with loading vectors of GPR120, PYY and glucagon being positive. To compare these two species with mouse, PLS models were built solely based on the small intestine. The loading vectors of these three species also show positive values for GPR120, PYY and glucagon and thus suggest a more distal role for GPR120, PYY and glucagon. Furthermore, the loading for CCK was negative in all three species, indicating that in all three species the relative expression of CCK is high in the proximal intestine, both in the small intestinal PLS models as well as in the complete PLS models.

**Table 2.1: Loading vectors of the pig and human PLS model.** Loading vectors obtained from PLS modeling of the complete intestinal data set of pig and human.

| | Pig | Human |
|---|---|---|
| | *5 factors* | *1 factor* |
| GPR120 | 50,9005 | 6,1034 |
| Glucagon | 16,0706 | 1,8761 |
| PYY | 11,5365 | 10,035 |
| GLP-1R | 6,1094 | -2,9701 |
| SGLT-1 | 7,534 | -3,4999 |
| T1R3 | -0,2179 | -15,0651 |
| PepT1 | -27,4127 | -6,8932 |
| CCK | -70,5 | -5,534 |



**Table 2.2: Loading vectors of the pig, human and mouse PLS model of the small intestine.** Loading vectors obtained from PLS modeling of the small intestinal data set of pig, human and mouse.

| | Pig | Human | Mouse |
|---|---|---|---|
| | *6 factors* | *1 factor* | *3 factors* |
| GPR120 | 89,5911 | 20,8624 | 7,1406 |
| Glucagon | 14,6987 | 3,129 | 9,0567 |
| T1R3 | 9,3151 | -1,1986 | 1,0031 |
| PYY | 4,6843 | 9,2374 | 3,7939 |
| SGLT-1 | 1,2987 | -1,3959 | -13,1401 |
| GLP-1R | 0,1353 | -1,1315 | -9,6277 |
| PepT1 | -17,4913 | -1,6997 | 6,533 |
| CCK | -59,3902 | -3,2496 | -6,9791 |

To further compare the gene expression data of the three species, the human en murine data were projected into the porcine model (5 factors, $R^2$=0.6541) (figure 2.1). For humans, the combined gene expression of all samples from distal ileum to colon were found to fit well to the porcine based model. The duodenal and jejunal samples, however, were more deviating from the modeled porcine samples when compared to the distal ileum and colon samples. Similar to the human proximal intestinal samples, the murine samples of the proximal small intestine are different from the modeled porcine samples. However, for the distal small intestine the difference between mice and pigs becomes less.

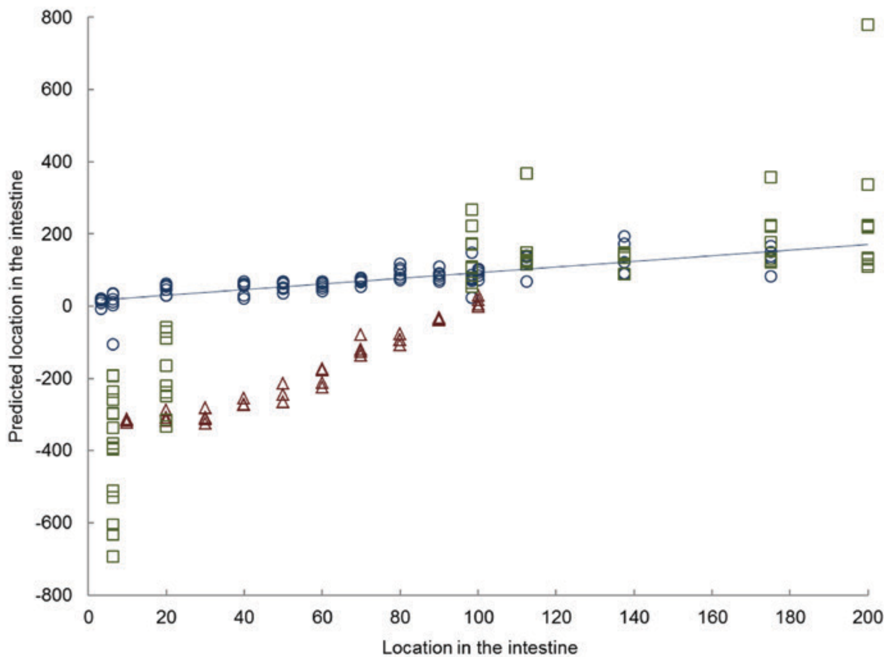


**Figure 2.1: Partial least square analysis.** Results of partial least squares (PLS) model in which porcine gene expression data (O) were used for regression analysis with locations in the intestine and the human (☐) and murine data (Δ) were projected in the model. The PLS prediction model used 5 factors and has a $R^2$=0.6541. The x-axis shows the location in the intestine, in which 0-100 resembles the small intestine from proximal to distal, 100-200 resembles the large intestine.

### Gene expression pattern along the intestine

When studying the expression patterns for the nutrient sensing genes in more detail, some general expression patterns or specific patterns could be clearly observed for several of the genes (figure 2.2). As shown in the heatmaps the gut hormones, glucagon (precursor for GLP-1), CCK and PYY and the receptor for GLP-1, all showed specific expression patterns along the intestine, which appeared similar for the three species.

Remarkably, the expression patterns of the nutrient transporters for di- and tri-peptides, PepT1, and for glucose, SGLT-1 were almost identical within each species. However, the expression patterns of both genes differed between the three species. In mice, GPR120 expression increased towards the distal small intestine, whereas in human and pigs the expression increased slightly along the small intestine. In human and pigs, the expression of this gene was more prominent in the colon.

Although the T1R family showed low to undetectable expression in the intestine, T1R3 was detected in all three species but showed a scattered expression pattern along the intestine.

Details for the expression patterns of each gene can be found in the supplemental data (figure S2.1-S2.5).

### Effect of diet on gene expression pattern in mice

To explore the effect of diet on expression of the studied genes, we also analyzed material from mice given different diets; chow, high fat-low carbohydrate or low fat-high carbohydrate diet. To analyze the effect of the three diets on the differences in gene expression of the eight genes along the small intestine, a PLS model was developed based on the data of the chow diet (using 3 factors, $R^2$=0.9681). Subsequently, the results of the high-fat and low-fat diet were fitted in this model (figure 2.3 and figure S2.6). The model showed that location in the intestine had a greater effect on gene expression level than a dietary intervention. With respect to the selected eight genes, the low-fat diet did not show a high deviation from the chow diet. Expression after a high-fat diet, however, deviated slightly from expression after a chow diet, especially in the distal part of the small intestine.

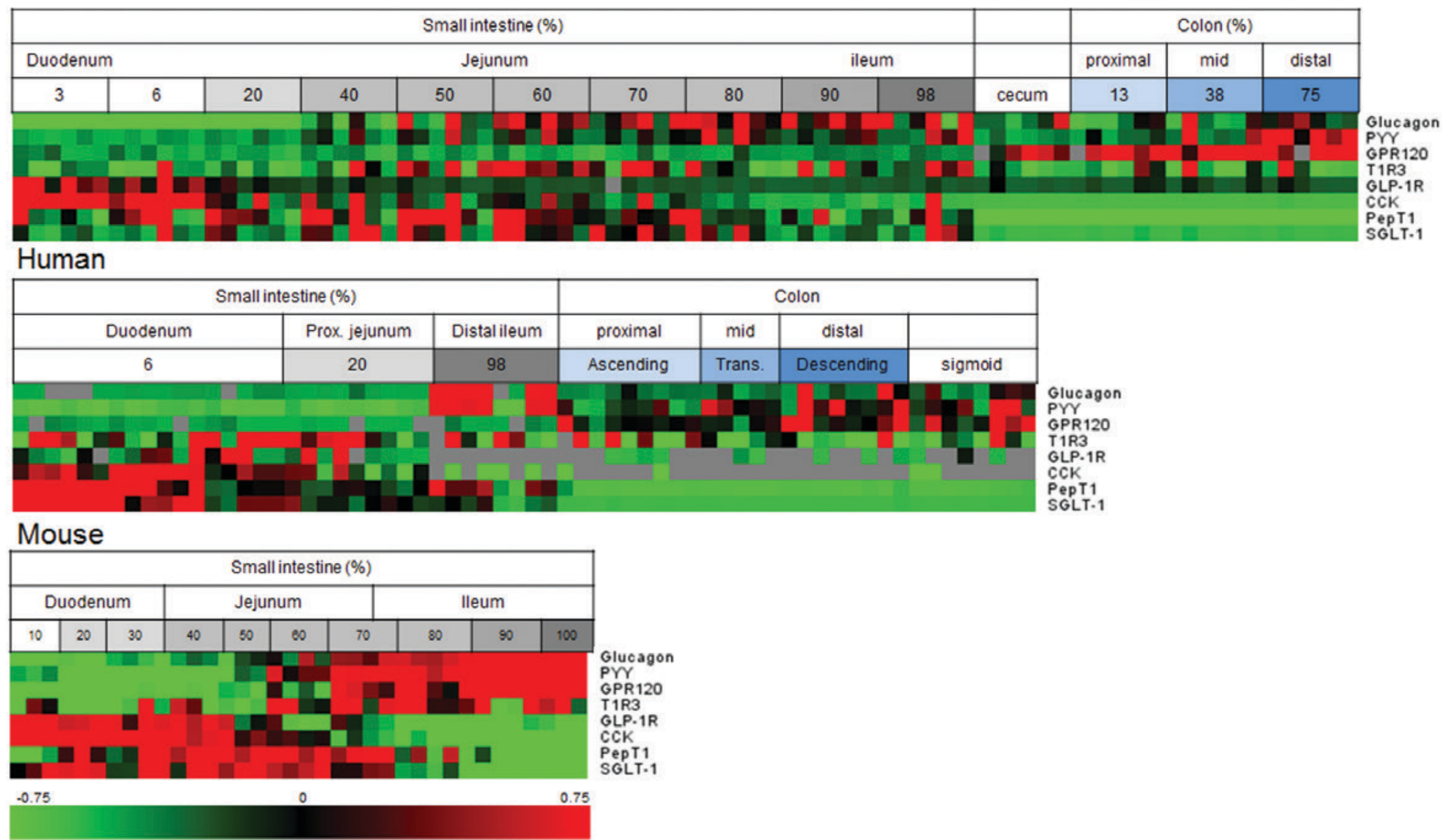


**Figure 2.2: Heatmap of pig, human and murine gene expression results.** Horizontally the individual samples of different parts of the intestine are aligned from proximal to distal and vertically the eight genes are shown. Green and red indicate low and high gene expression compared to average, respectively. Grey indicates samples that could not be analyzed/detected.

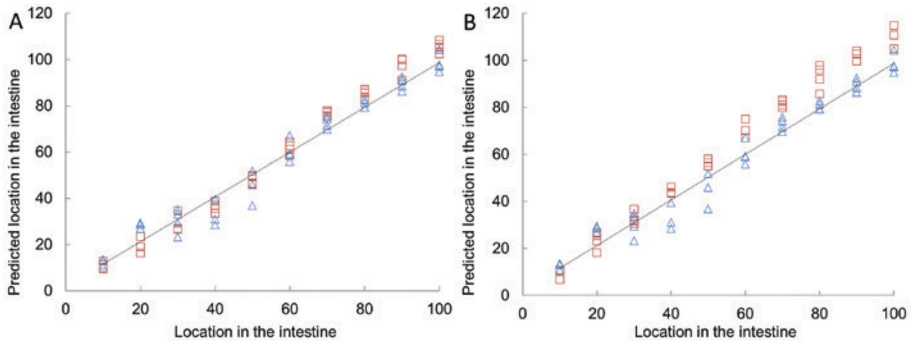


**Figure 2.3: PLS prediction of locations along the intestine based on the gene expression in a sample.** The PLS prediction model used 3 factors and has an $R^2$= 0.9681. The samples of mice fed a chow diet (Δ) were the basis of the model and the data of mice fed a low-fat (A, indicated with □) and high-fat diet (B, indicated with □) was fitted in the model.

2

## Discussion

Chemosensory receptors and transporters able to detect nutrients and other molecules present in the intestinal tract are pivotal for the regulation of food intake and other physiological responses to food ingestion. Moreover, nutrient sensing in the gut might also play a key role in maintaining metabolic homeostasis, for example of glucose. Impairment or changes of these nutrient sensing mechanisms may contribute to metabolic diseases, such as type II diabetes and obesity (1, 32, 33). It is conceivable that a time- and site dependent interaction of food and digestion products with different chemosensory and other, including stretch and osmotic, sensors is key to these processes. However, detailed studies characterizing patterns of chemosensory receptors and transporters along the intestinal tract under normal physiological conditions are scarce. Moreover, information on interspecies differences is limited. Instead, the majority of studies focused on investigating a single gene in one or two species (6, 34-36).

Our data show a strong similarity between the expression of genes related to nutrient sensing in the distal ileum of the three species studied, which is mainly explained by the large contribution of glucagon and PYY to the model.

Furthermore, the expression patterns in the colon of man and pig were highly comparable. Similarities in these locations of the intestine might be attributed to the similar high expression values of the GLP-1, PYY and GPR120 genes. Even though pigs have a higher relative volume and surface area of the large intestine than humans, we did not observe differences between the large intestine of pigs and humans as far as these genes are concerned (37). As became clear from the loading vectors of all PLS models, GPR120, glucagon and PYY were predominantly expressed in the distal part of the intestine. GPR120 is expressed in L-cells of the intestine, which are enteroendocrine cells containing both PYY and GLP-1 (38). To our knowledge the effect of GPR120 activation on PYY secretion has not been investigated yet, while a relation between GPR120 and secretion of GLP-1 and CCK has been described (6, 7, 39). Hirasawa et al. showed that in both human and mouse intestine GPR120 was abundantly expressed especially in the colon (6). This fatty acid receptor has been linked to obesity. In morbidly obese patients GPR120 expression in gastric tissue was higher compared to normal-weight individuals (40). Moreover, a GPR120 mutation, found to be associated with obesity in man, influenced the ability to secrete GLP-1 in response to α-linolenic acid in enteroendocrine NCI-H716 cells (41).

When considering all genes combined, the most pronounced differences between the species studied here were found in the proximal small intestine. In the duodenum, the expression of the transporter genes SGLT-1 and PepT1 was deviating between the species (figure 2.2 and S2.3). For PepT1, a higher gene expression in the human duodenum compared to ileum has been observed previously (42). However, this is in contrast to findings of others who did not find significant differences between its expression in the duodenum and ileum (34). In the porcine and murine intestine, the gene expression of PepT1 was highest in the jejunum, which is in agreement with findings of others (43, 44). SGLT-1 gene expression along the intestine has been investigated in rodents, showing highest expression in the jejunum, whereas our results showed highest expression in duodenum and proximal jejunum (38, 45). To our knowledge, SGLT-1 expression along the intestinal axis has not been reported previously for humans or pigs.

The basis that may underlie the different gene expression patterns in the proximal part of the intestine in the three species is unknown. However, gene expression of both transporters is known to be influenced by nutritional status or diet composition. High-protein diets are known to increase PepT1 mRNA expression and transporter activity (46, 47). However, PepT1 increases found in these studies affected the middle and distal small intestine. Furthermore, a fed or fasted state might have influenced the amount of PepT1 mRNA, but studies show contradictory results (44, 48, 49). Similarly, high-carbohydrate diets have been shown to increase SGLT-1 gene expression levels in the proximal and mid intestine but not in the distal small intestine (50, 51). This increased expression is regulated by the sweet taste receptor (52, 53). Therefore, it can be suggested that differences in dietary composition may contribute to the duodenal differences in expression patterns of these transporters in the three species. The high duodenal SGLT-1 expression in humans might be explained by a diet higher in carbohydrates compared to that of pigs and mice as the participants had no diet constrains. Interestingly, in our mice study the effect of a different fat content in the diet (at the expense of corn starch) on the gene expression of SGLT-1 was found to be much smaller than reported for effects of dietary carbohydrates in the literature. This could be due to the fact that in other studies sucrose was the main source of carbohydrates, whereas starch was the main dietary carbohydrate in the present study (53).

In spite of the fact that the expression patterns of the transporters, PepT1 and SGLT-1 along the intestine were found to differ between the species, figure 2.2 and S2.3 show a striking and species-independent similarity in gene expression pattern between the two nutrient transporters. This might be due to a similar function in the intestine in the uptake of either peptides or glucose after the digestion of proteins and carbohydrates. These macronutrients are mainly digested by the action of pancreatic and brush border enzymes, which primarily takes place in the duodenum and proximal jejunum (54).

Due to the invasiveness of the procedure, the vast majority of duodenal, jejunal and ileal biopsies were obtained from different human subjects. Gene expression in the human duodenal samples showed a high inter-individual variation compared to the other regions of the intestine. This may at least in part

be explained by different dietary habits between individuals. Additionally, genotypical differences might play a role as well.

Microarray and qPCR are two techniques for measuring gene expression and there is evidence for a strong correlation between qPCR and Microarray analysis (27-29). However as the units of the output of both techniques are not directly comparable, the data needs further appropriate processing to make a reliable comparison of the data possible. PLS is a tool that can meet this demand. PLS is commonly used in the analysis of instrumental chemical measurements. Its use with biological data is increasingly being recognized (55, 56).

Our results show a high proximal expression of the GLP-1 receptor (figure 2.2 and S2.2). This observation was remarkable as GLP-1 is mainly secreted in the distal parts of the intestine. However, it was recently shown that the GLP-1 receptor is expressed in both the small and large intestine (57). In agreement with our data, that study showed that the vagal innervation of GLP-1 is reduced along the intestinal tract (57).

In contrast to T1R3, gene expression of its heterodimer T1R2 was not detected in both human and porcine intestine. A very low gene expression of T1R2 is consistent with findings from other studies (21, 22, 58). An explanation for the much lower gene expression of T1R2 compared to the expression of T1R3 could be the potential dimerization of T1R3 with other GPRs (59). This idea is supported by the fact that tissue explants of the jejunum and ileum from T1R3 knockout mice had no GLP-1 secretion compared to explants from wild type animals, whereas ileum explants of T1R2 knockout mice still secreted GLP-1. The authors of that study suggested that T1R3 can partially compensate for the loss of T1R2 (60). T1R3 was expressed in the intestine of all three species suggesting a functional role in the intestine, possibly sensing of amino acids and/or sweet compounds.

In order to study the effect of fat content (at the expense of carbohydrate content) on expression of nutrient sensing related genes, we performed a two week diet intervention study in mice. The PLS model of these data showed slight differences in gene expression of the high-fat/low-carbohydrate diet compared to the chow diet in the distal region of the small intestine. Although

it has been shown that a high-fat diet can induce changes in gene expression in several other pathways, like lipid metabolism and cell cycle, the nutrient sensing related genes studied here were hardly influenced by the fat/carbohydrate content in the diet (19, 61).

In conclusion, this study shows that the intestinal expression pattern of genes related to nutrient sensing show the highest similarity between humans, pigs and mice in the distal ileum and a high similarity between human and pigs in the colon. At the same time, more deviating gene expression patterns between the species were found for the proximal intestine. For the proximal small intestine some prudence in extrapolation of gene expression data from one species to the other may be required with respect to nutrient sensing. Lastly, we provided detailed information on the specific expression patterns of glucagon, CCK, PYY, GLP-1 receptor, PepT1, SGLT-1, T1R3 and GPR120 over the longitudinal intestinal axis of human, pigs and mice under normal physiological conditions. To our knowledge, this is the first study where gene expression of nutrient sensing related mechanisms has been characterized in such detail along the intestinal tract, and compared for relevant species, including human. Knowledge of the expression patterns of these nutrient sensing related genes in commonly used species may contribute to a better understanding of the satiating effects of specific diets and products. Furthermore, understanding their site- (and time-) specific interactions with molecular ligands may contribute to strategies for food intake modulation.



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## Supporting Information

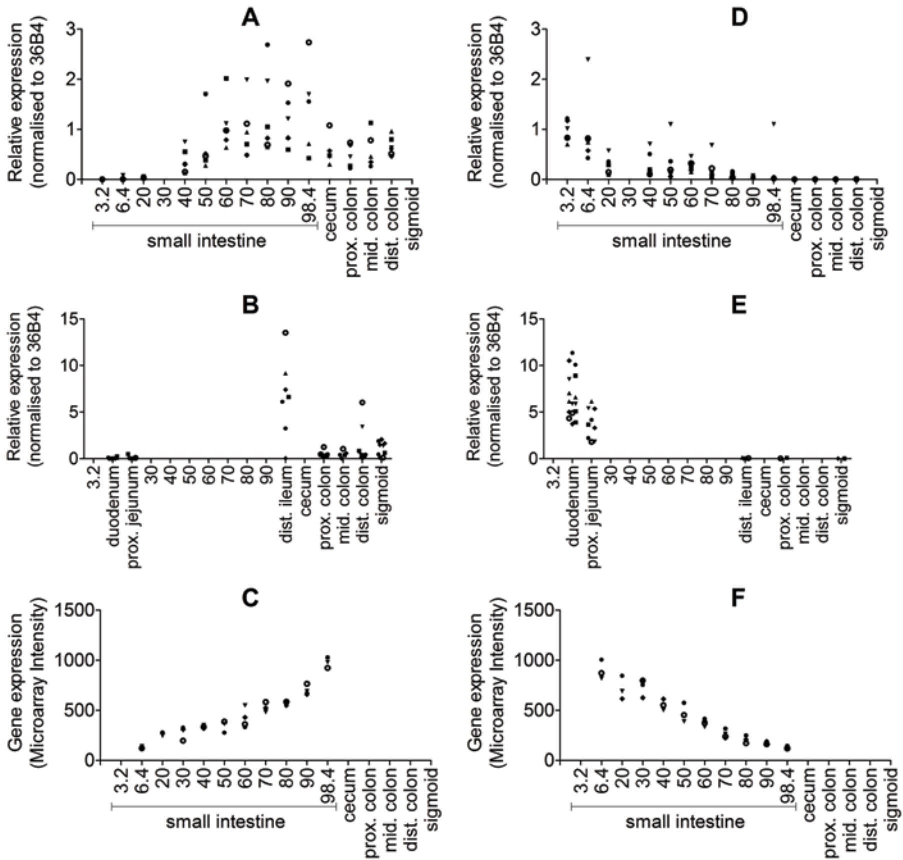



**Figure S2.1: Gene expression of glucagon and CCK along the intestine of human, pig and mouse.** Gene expression of glucagon in pig (A), human (B), mice (C) and gene expression of CCK in pig (D), human (E), mice (F) as assessed in numerous intestinal locations. Human and pig data show relative expression corrected for reference gene 36B4 determined using qPCR analysis. Mice results show microarray intensity.

2

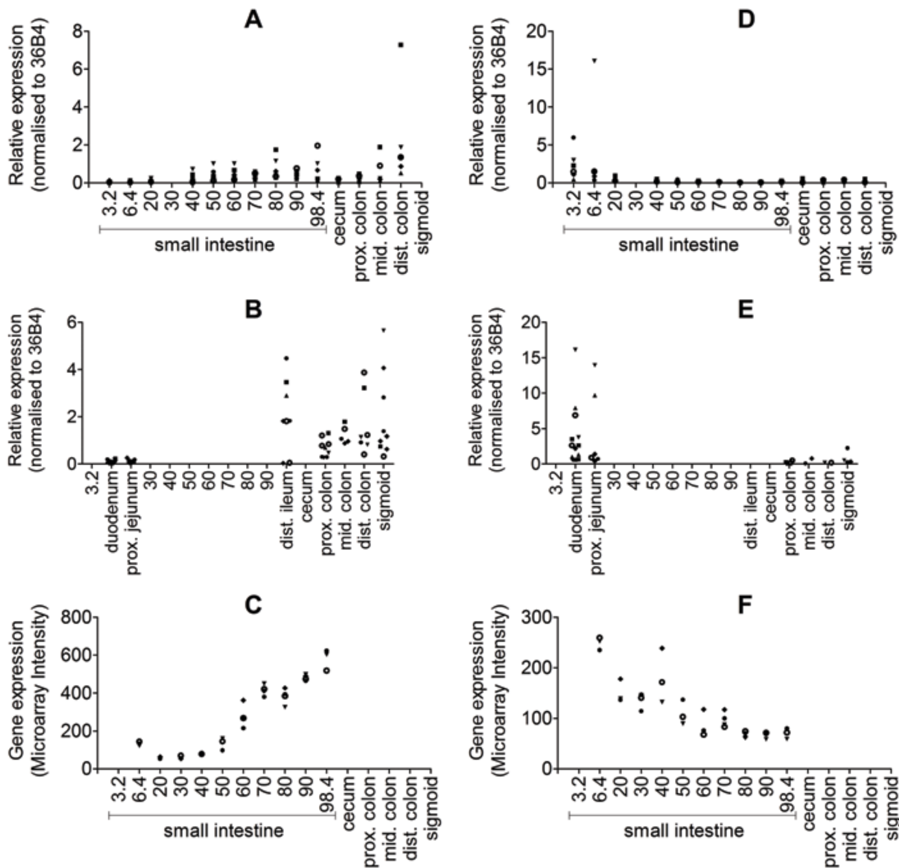



**Figure S2.2: Gene expression of PYY and GLP-1 receptor along the intestine of human, pig and mouse.**

Gene expression of PYY in pig (A), human (B), mice (C) and gene expression of GLP-1 receptor in pig (D), human (E), mice (F) as assessed in numerous intestinal locations. Human and pig data show relative expression corrected for reference gene 36B4 determined using qPCR analysis. Mice results show microarray intensity.

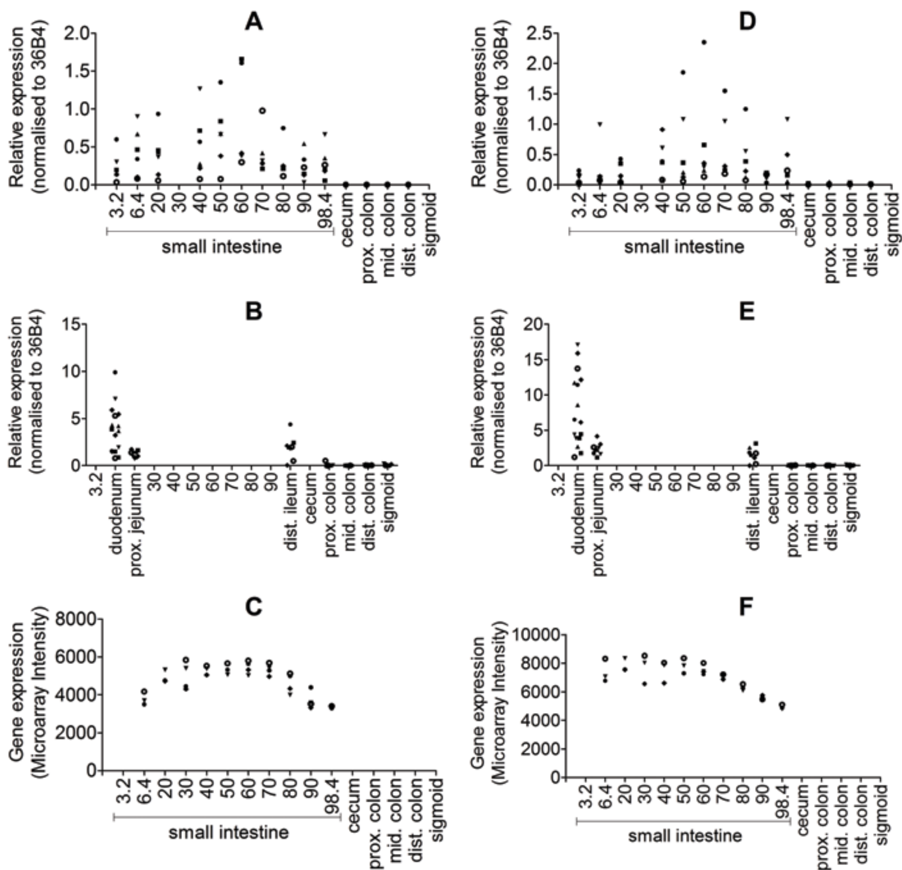



**Figure S2.3: Gene expression of PepT1 and SGLT-1 along the intestine of human, pig and mouse.** Gene expression of PepT1 in pig (A), human (B), mice (C) and gene expression of SGLT-1 in pig (D), human (E), mice (F) as assessed in numerous intestinal locations. Human and pig data show relative expression corrected for reference gene 36B4 determined using qPCR analysis. Mice results show microarray intensity. Both genes were highly expressed in all three species.

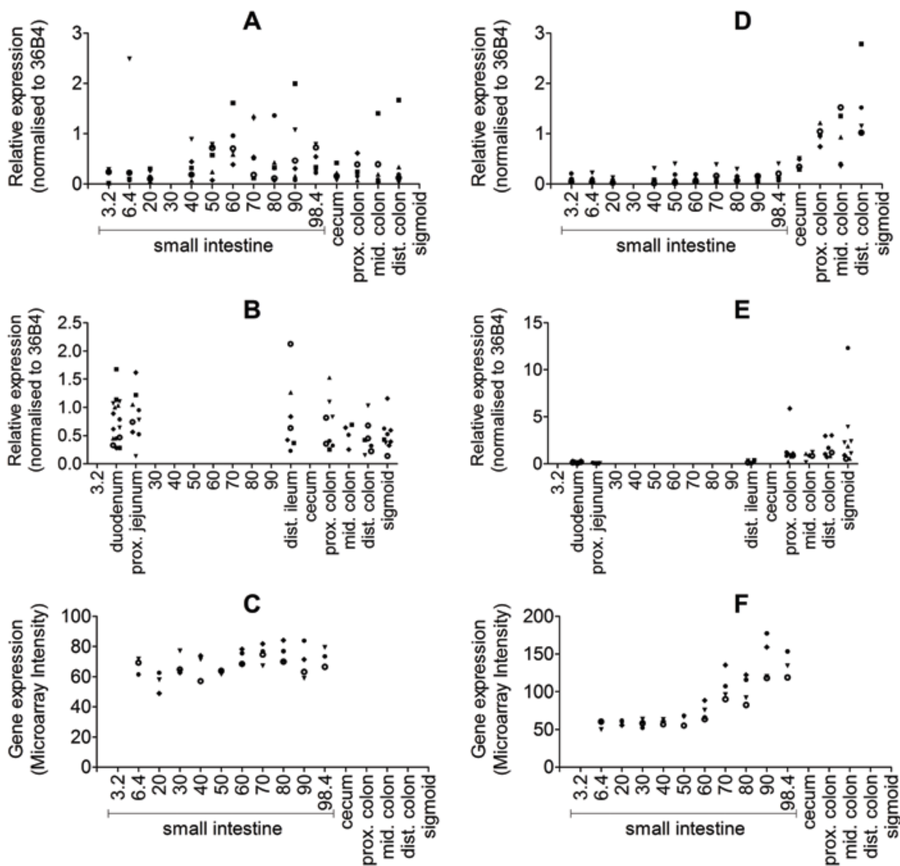
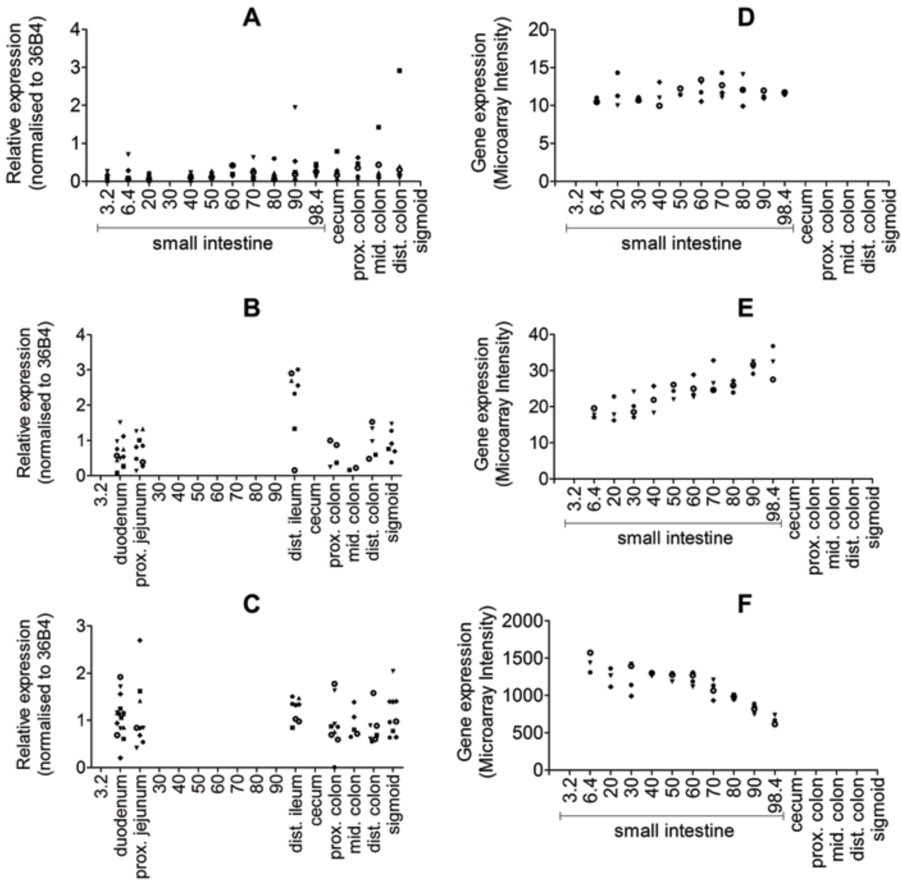



**Figure S2.4: Gene expression of T1R3 and GPR120 along the intestine of human, pig and mouse.** Gene expression of T1R3 in pig (A), human (B), mice (C) and gene expression of GPR120 in pig (D), human (E), mice (F) as assessed in numerous intestinal locations. Human and pig data show relative expression corrected for reference gene 36B4 determined using qPCR analysis. Mice results show microarray intensity.




2

**Figure S2.5: Gene expression along the intestine of human, pig and mouse.** Gene expression of T1R1 in pig (A), mice (D) and gene expression of GPR119 in human (B), mice (E) and gene expression of GPR93 in human (C), mice (F) as assessed in numerous intestinal locations. Human and pig data show relative expression corrected for reference gene 36B4 determined using qPCR analysis. Mice results show microarray intensity.

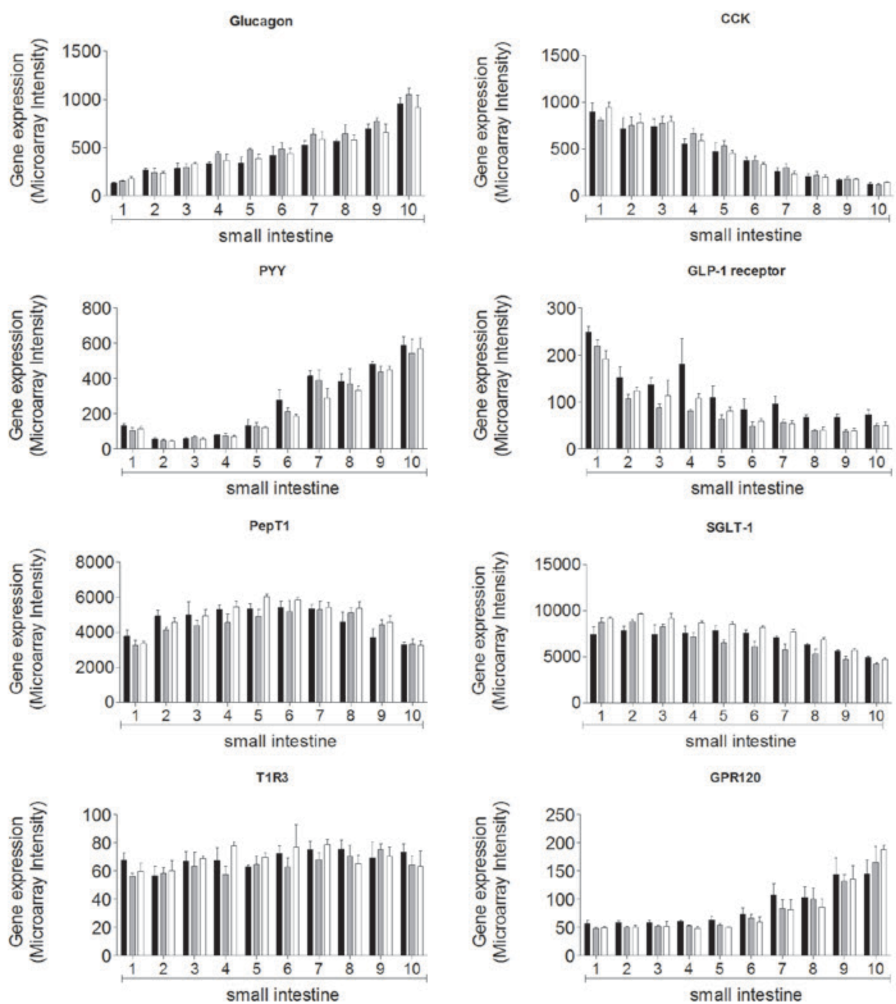



**Figure S2.6: Gene expression along the intestine of mice on chow, high-fat and low-fat diet.** Black bars show chow diet, grey bars show high fat diet and white bars show low fat diet. Results show mean microarray intensity of 4 mice per group and the standard deviation.

**Table S2.1: Porcine primers used for qPCR analysis.**

| Name | Forward primer | Reverse primer |
|---|---|---|
| GCG | CAAGAGGAACAAGAATAACAT | AAGAACTTACATCACTGGTA |
| CCK | TCAGAGGAGGCAGAAGAA | TGGACAATGTTACAGACAGATT |
| PYY | AGATATGCTAATACACCGAT | CCAAACCCTTCTCAGATG |
| GLP1R | GCATCGTCAAGTACCTCT | GGATGATGAGCCAGTAGTTC |
| Tas1R1 | ATCTGTTCTCGAGGCCAAGTCT | GCGAGTCCCCACTGTCACTAA |
| Tas1R3 | GCTGGGCGACAGGACAG | TTGATTTCCTCCACAGCCAT |
| SLC5A1 | AAAGGAGAGGTCTGGGATGGTAA | ATTTCCCTAGTGGCCTGAGATTG |
| SLC15A1 | AATTGTGTCGTTGTCCAT | AAGTCTGTGAGGTCATTG |
| LPAR5 | TGGGATGTGTCGTTTGTT | CCTTGATGCCTTGGTGAT |
| RPLP0 | CTTTAGGCATCACCACTA | TGTCTCCAGTCTTAATCAG |

**Table S2.2: Human primers used for qPCR analysis.**

| Name | Forward primer | Reverse primer |
|---|---|---|
| GCG | AGCATTTACTTTGTGGCTGGAT | CGCTTGTCCTCGTTCATCTGAT |
| CCK | TATCGCAGAGAACGGATG | AGGTTCTTAACGATGGACAT |
| PYY | CTGAACCGCTACTACGCCTC | CGTCTCTTTTCCCATACCGCT |
| GLP1R | TTGGGGTGAACTTCCTCATC | CTTGGCAAGTCTGCATTTGA |
| Tas1R3 | GCTAAATCACCACCAGAC | TGCACTGAAGAGTGTTGT |
| SLC5A1 | TCTCTACCGTCTGTGTTG | GCTCTCCTGAAGATTCCT |
| SLC15A1 | CTTCTTCATCGTGGTCAA | GGTGGACAGGTTATCATC |
| FFAR4 | CAGCAATCACATCTCCTACAT | CGTCCTGAATCGGTTCTAAG |
| GPR119 | Hs02825719_s1 (Life technologies) | |
| LPAR5 | TTCTCGCATAGTGACTTGT | TTGGACTTGGATGTTGTTG |
| RPLP0 | CGGGAAGGCTGTGGTGCTG | GTGAACACAAAGCCCACATTCC |

# Effect of endoscopic gastroplication on the genome-wide transcriptome in the upper gastrointestinal tract

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* These authors contributed equally to this work

Submitted.

## Abstract

**Background/Objectives:** Bariatric surgery is an effective intervention strategy in obesity, resulting in sustained weight loss and a reduction of comorbidities. Gastroplication, using the articulating circular endoscopic stapler, has recently been introduced as a transoral bariatric technique. This procedure reduces gastric volume and induced 34.9% (IQR 17.8-46.6) of excess weight loss in the first year (1). The aim of the present study was to gain more insight in the long-term effects and underlying mechanisms of gastroplication by investigating differences in the genome-wide gastric and duodenal tissue transcriptome before, and one year after intervention.

**Subjects/Methods:** Ten morbidly obese patients (mean BMI 39.8±0.9kg/$m^2$ (±s.e.m.)) underwent gastroplication. Previous to the procedure and after one year, mucosal biopsies were taken from the fundus, antrum and duodenum. At the same time blood samples were taken. Gene expression was measured using microarray analysis. Additionally, plasma levels of adiponectin, HbA1c, IL-1β, IL-6, IL-7, TNF-α, IFN-γ, MCP-1, IL-8, TGF-1, and CRP were determined.

**Results:** Downregulation of inflammatory genes and gene sets was observed in the fundus and duodenum one year after surgery. Furthermore, gene expression of ghrelin and its activating enzyme GOAT were downregulated in the upper gastrointestinal tract. Patients showed a reduction in plasma HbA1c levels (from 6.17±0.51% to 5.32±0.14%, p=0.004) and an increase of plasma adiponectin (from 16.87±3.67µg/ml to 27.67±5.92µg/ml, p=0.002).

**Conclusions:** Individuals undergoing gastroplication displayed a striking downregulation of inflammatory tone in the stomach and duodenum, which coincided with improved HbA1c and adiponectin levels. The reduction of inflammatory tone in the upper gastrointestinal tract may be a consequence of an improved metabolic health status, or alternatively caused by the procedure itself.

## Introduction

Bariatric surgery is the most effective medical option to achieve sustained weight loss in severe obesity. Besides traditional procedures such as laparoscopic Roux-en-Y gastric bypass (RYGB), vertical sleeve gastrectomy (VSG) and adjustable gastric banding (LAGB), less invasive options are available such as the (transorally placed) duodenal-jejunal bypass sleeve (DJBS). In general, these procedures lead to a loss of body fat, a reduction of comorbidities, and improvement of long-term health risks. Remarkably, the mechanisms behind these outcomes are still poorly understood, and it is conceivable that these comprise different combinations of biological adaptations (2). This is reflected in the markedly different immediate effects on glycaemic control following different procedures (3). Recently, endoscopic gastroplication has become available as a new minimal invasive technique. The Articulating Circular Endoscopic (ACE) stapler is used to reduce the volume of the stomach without removing tissue or bypassing other intestinal regions. For this procedure no skin incisions are necessary; it is performed via a transoral route. This procedure results in a median 34.9 percent (IQR 17.8-46.6) loss of excess weight in the first year. Moreover, only mild adverse effects were reported so far (1). Although several studies have described metabolic and anti-inflammatory effects of bariatric surgery at a molecular level, studies on these processes within the gastrointestinal (GI) tract are still limited. This holds particularly true for the upper GI tract, as most studies in this field have focussed on the mid or lower gastrointestinal tract (4-6). Moreover, these studies concern effects of RYGB, a procedure extensively changing GI anatomy and physiology. The present study was undertaken to gain more insight in the long-term effects and underlying mechanisms of gastroplication in the upper GI tract: the stomach (fundus and antrum) and the duodenum, and to relate these to general health outcomes, including parameters of inflammation. To this end, transcriptome and gene set enrichment analysis was performed with biopsies obtained before and one year following gastroplication.

## Materials and methods

### ACE stapler study

This study used biopsies and blood samples obtained from ten patients who were part of the first human ACE stapler study (1). The Medical Ethical Committee of the Maastricht University Medical Center+ in the Netherlands (NCT02381340) approved the present study as a sub study aiming to further unravel underlying mechanisms. Before inclusion, written informed consent was obtained from each participant. The inclusion criteria for the Articulating Circular Endoscopic (ACE) Stapler study are described in detail by Paulus et al. (1). In brief, participants were 18 to 50 years old with a BMI of 40 to 45 kg/m$^2$ or 30 to 39.9 kg/m$^2$ in combination with one or more comorbidities expected to improve with weight loss. The ACE stapler was introduced into the stomach together with a thin endoscope. By applying vacuum to the gastric tissue, a large full-thickness (transmural) plication was drawn into the stapler head and fixed with a staple ring. Reduction of the stomach volume along the greater curvature was completed after creating a maximum of 8 plications in the fundus and 2 additional plications in the antrum of the stomach. More details on the procedure were published previously (1). Mucosal biopsies were taken from the fundus, antrum and duodenum with a standard forceps before starting the procedure. Afterwards, patients visited the outpatient clinic regularly and were stimulated to adhere to a healthy lifestyle. A follow-up endoscopy was planned twelve months after the procedure, at which the biopsy procedure was repeated. Biopsies were snap frozen in liquid nitrogen and stored at -80°C until analysis. Table 3.1 shows a brief overview of the characteristics of included patients. A more detailed description is given by van Avesaat et al. (7).

**Table 3.1:** Overview of characteristics of patients undergoing ACE stapler procedure. Measurements were performed at baseline and one year after the procedure (7). Ghrelin was measured after a 10-hour overnight fast.

| | **Baseline** | | **One year** | |
|---|---|---|---|---|
| | *Average* | *SEM* | *Average* | *SEM* |
| Age | 39 | 2 | | |
| Male:Female ratio | 6 : 4 | | | |
| BMI (kg/m2) | 39,8 | 0,9 | 33,4 | 0,9 |
| Excess weight loss (%) | | | 37,9 | 4,8 |
| Fasted ghrelin level (pg/ml) | 46,5 | 5,9 | 63,4 | 5,2 |

## RNA isolation and microarray processing

RNA of the mucosal biopsies was isolated using TRIzol reagent (Life technologies, Bleiswijk, Netherlands) and further purified using the RNeasy micro kit (Qiagen, Venlo, Netherlands). RNA yield was measured with the Nanodrop ND-1000 Spectrophotometer and the quality of the RNA samples was verified with an Agilent 2100 Bio analyser (Agilent Technologies, Amstelveen, Netherlands). One hundred nanogram of RNA was used for Whole Transcript cDNA synthesis (Affymetrix, inc., Santa Clara, USA). Hybridization, washing and scanning of Affymetrix GeneChip Human Gene 1.1 ST arrays was carried out according to standard Affymetrix protocols.

## Microarray analysis

For the analysis of the microarray results, each location (i.e. fundus, antrum and duodenum) was analysed separately. Arrays were normalized using the Robust Multi-array Average method (8, 9). Probe sets were assigned to unique gene identifiers, in this case Entrez IDs. The probes on the arrays represent 19654 Entrez IDs (10). Array data were analysed using MADMAX pipeline for statistical analysis of microarray data (11). Quality control was performed and all arrays met our criteria, except for the fundus and antrum arrays from participant 5, which were excluded. All data were filtered, and probe sets with expression values above 20 in at least 5 arrays were included for further analysis. These data were used for gene set enrichment analysis (GSEA; www.broadinstitute.org/gsea;(12)) in MADMAX. Gene sets with a false discovery rate (FDR) <0.25 were considered significantly enriched. The gene set enrichment analysis was

visualized using the enrichment plugin in Cytoscape with conservative filtering ($p<0.001$ and FDR $q<0.05$). For further analysis of individual genes, a cut-off of IQR>0.25 was used to filter out genes that showed no variation between the samples, Intensity-Based Moderated T-statistics (IBMT) was used to assess significant differences with p-value $<0.05$.

### Plasma measurements

Blood samples were collected in EDTA-coated tubes, centrifuged and stored at -80 ℃ until analysis. The measurement of plasma adiponectin and cytokine levels were performed using an in-house developed and validated multiplex immunoassay (Laboratory of Translational Immunology, University Medical Center Utrecht, the Netherlands) based on Luminex technology (xMAP, Luminex, Austin, USA). The assay was performed as described previously (13). Using heteroblock (Omega Biologicals, Bozeman, USA) aspecific heterophilic immunoglobulins were preabsorbed. Acquisition was performed with the Biorad FlexMAP3D (Biorad laboratories, Hercules, USA) in combination with xPONENT software version 4.2 (Luminex, Austin, USA). Data was analysed by 5-parametric curve fitting using Bio-Plex Manager software, version 6.1.1 (Biorad laboratories, Hercules, USA). HbA1c levels were determined routinely at the Department of Clinical Chemistry of the Maastricht University Medical Center.

### Statistical Analysis

Statistical analyses were performed using Prism 5.0 (GraphPad Software, Inc. La Jolla, USA). The effects of treatment on plasma levels of inflammatory markers and adiponectin were tested by paired t-tests for normally distributed variables and Wilcoxon's signed ranks tests for non-normally distributed variables. A p-value $<0.05$ was considered statistically significant. Data are presented as mean with standard error of the mean (SEM). Statistical analysis of transcriptome data was described above.

## Results

### Effects of gastroplication on systemic metabolic and inflammatory parameters one year after intervention

Plasma levels of glycated haemoglobin, adiponectin and several pro-inflammatory mediators before and after intervention are shown in table 2. Significant changes were found for adiponectin and HbA1c. Adiponectin showed a 1.64 fold increase (p=0.002) in the patients who underwent ACE stapler treatment. Glycated haemoglobin (HbA1c) was significantly decreased (p=0.004) by the treatment. Plasma IL-6 showed a tendency to decrease following ACE stapler treatment by a factor 1.47. MCP-1 levels also showed a decrease (1.3 fold) but this effect did not reach statistical significance.

**Table 3.2: The effect of ACE stapler treatment on fasted plasma levels of inflammatory and metabolic markers.** Plasma levels were measured before the treatment (baseline) and one year after.

| Plasma marker | | Baseline | | One year | | Treatment effect | |
|---|---|---|---|---|---|---|---|
| | *Unit* | *Average* | *SEM* | *Average* | *SEM* | *Difference* | *p-value* |
| Adiponectin | µg/ml | 16,87 | 3,67 | 27,67 | 5,92 | 10,8 | 0.002* |
| HbA1c | % | 6,17 | 0,51 | 5,32 | 0,14 | -0,85 | 0.004* |
| IL-1β | pg/ml | 1,42 | 0,06 | 1,37 | 0,08 | -0,05 | 0,244 |
| IL-6 | pg/ml | 10,90 | 1,83 | 7,41 | 1,80 | -3,49 | 0,069 |
| IL-7 | pg/ml | 11,52 | 1,24 | 10,43 | 2,01 | -1,09 | 0,18 |
| TNF-α | pg/ml | 2,14 | 0,10 | 2,00 | 0,14 | -0,14 | 0,118 |
| IFN-γ | pg/ml | 2,54 | 0,27 | 2,23 | 0,25 | -0,32 | 0,099 |
| MCP-1 | pg/ml | 60,70 | 7,33 | 46,68 | 7,74 | -14,02 | 0,088 |
| IL-8 | pg/ml | 7,03 | 1,03 | 6,55 | 1,44 | -0,48 | 0,455 |
| LAP / TGF-1 | ng/ml | 3,11 | 0,35 | 3,24 | 0,36 | 0,13 | 0,393 |
| CRP | mg/l | 12,62 | 5,66 | 8,78 | 2,75 | -3,85 | 0,248 |

### Effects on tissue gene expression mainly relate to inflammatory pathways

Gene expression changes one year after intervention compared to baseline were analysed for different locations of the upper gastrointestinal tract, namely fundus, antrum and duodenum. After intervention 727 genes (259 upregulated, 468 downregulated) were significantly changed in the fundus, 1846 (951 upregulated, 895 downregulated) in the antrum and 921 genes (480 upregulated, 441 downregulated) in the duodenum. The top 20 up- and downregulated genes in all three locations are shown in figure 3.1. In both

fundus and duodenum a considerable number of downregulated genes have been associated with immunity and inflammatory pathways. In the fundus the expression of immune-related genes like *IGHV3-33, C7, CCL21, IFI6, IFI27, C1QB* were downregulated and *CCL18, CLC, CXCR4, IGHV1-24, RSG1, IGLV3-10, IGHV3-33, IGLV7-46* were downregulated in the duodenum. In the antrum there was an upregulation of some neuroendocrine associated genes, namely *PAX6, CHGB, SCG5*.

**Gene set enrichment analysis reveals potential processes involved**

To gain more insight into the processes changed one year following the stomach volume reduction procedure, gene set enrichment analysis (GSEA) was performed. This computational method uses molecular signatures to associate changes in gene expression with known biological processes. Analysis resulted in 236 (2 upregulated, 234 downregulated) enriched gene sets for fundus, 546 (474 upregulated, 72 downregulated) enriched gene sets for antrum and 253 (182 upregulated, 71 downregulated) enriched gene sets for duodenum. In the antrum more gene sets were upregulated, whereas in the fundus most gene sets were downregulated (figure 3.2). Of these downregulated gene sets in the fundus, many were related to immune responses, mostly to the complement system, presentation and recognition of antigens (self or pathogenic) and T-cell receptor signaling. Also in the duodenum, some of the downregulated gene sets were related to the innate immunity. In the antrum, cell cycle related gene sets were strongly enriched. In the duodenum the enrichment analysis showed also a slight upregulation of cell cycle processes. Here, more metabolic pathways were apparently upregulated, including those associated with 'fat digestion and absorption' and 'metabolism of lipids and lipoproteins'. All gene sets are specified in supplemental table S3.1.

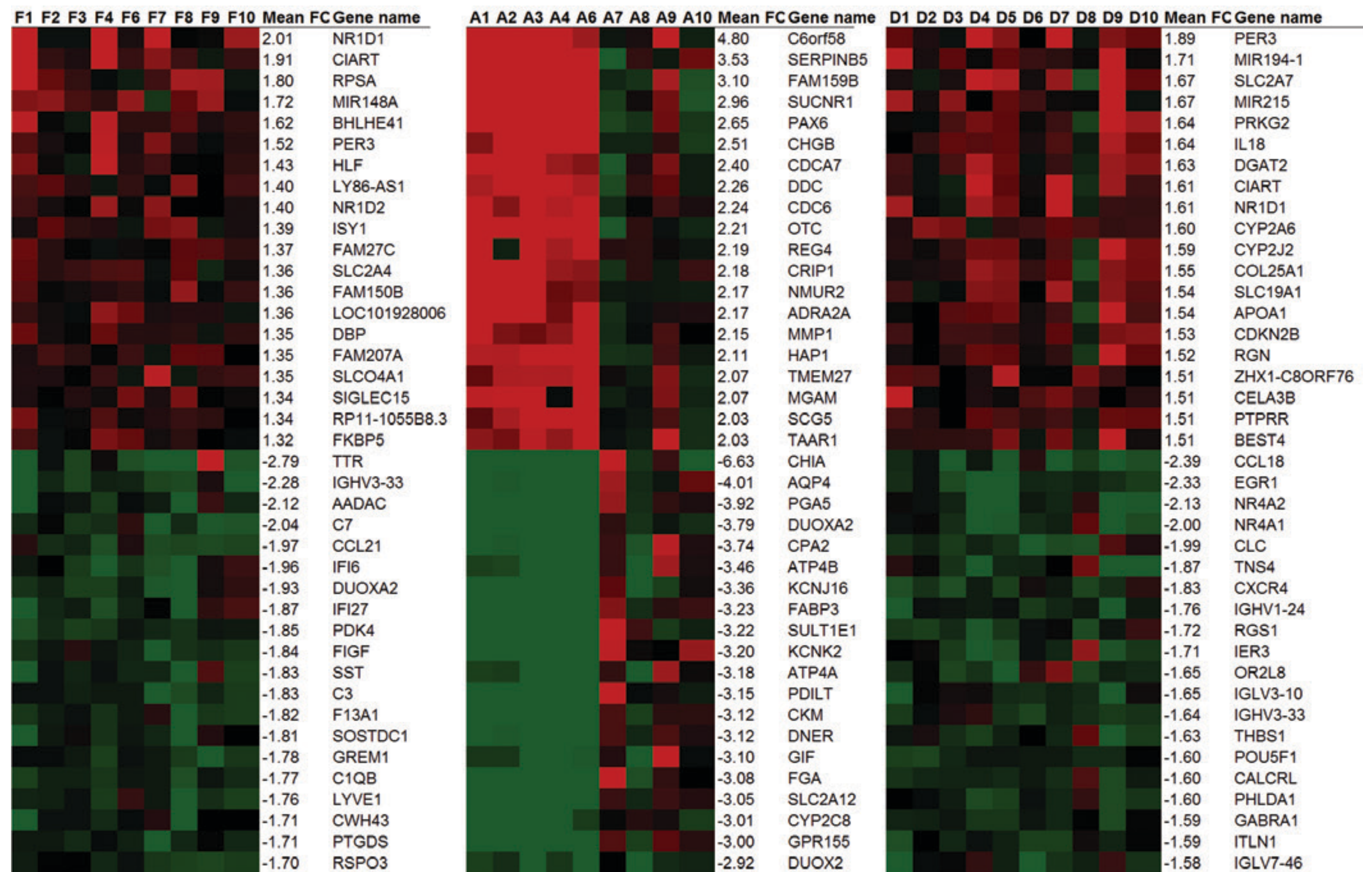


**Figure 3.1:** Expression of most highly significantly upregulated and downregulated genes in fundus, antrum and duodenum, respectively. Green is a signal log ratio of -2 and red is a signal log ratio of 2. Apart from the mean fold change (FC) of the top regulated genes by the treatment; signal log ratios are displayed to show inter-individual differences.

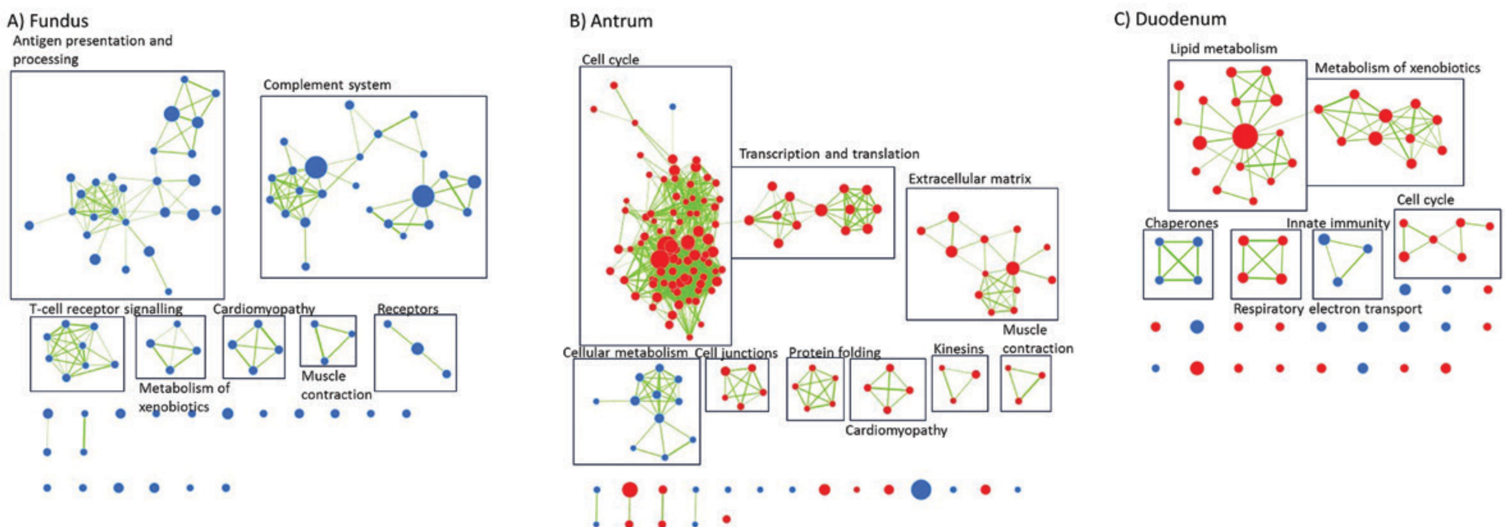


**Figure 3.2:** Gene set enrichment analysis of the fundus, antrum and duodenum. Each node indicates a conservative filtered gene set ($p<0.001$ and FDR $q<0.05$) and the connecting lines indicate overlapping genes between the nodes/gene sets. Red is enriched; blue are depleted gene sets.

## Changes in gastrointestinal hormone expression

Being one of the main gastric hormones, ghrelin is not only involved in appetite regulation but also in immunity (14). Two genes related to ghrelin were significantly changed in specific locations of the GI tract (figure 3.3). In the fundus, there was a downregulation of *MBOAT4* (FC=-1.49), the gene encoding the ghrelin-activating enzyme GOAT4. Furthermore, there was a trend for downregulation of ghrelin expression itself in the fundus (FC= -1.88, p= 0.19) and antrum (FC= -3.13, p= 0.11) and a significant downregulation in the duodenum (FC= -1.34).

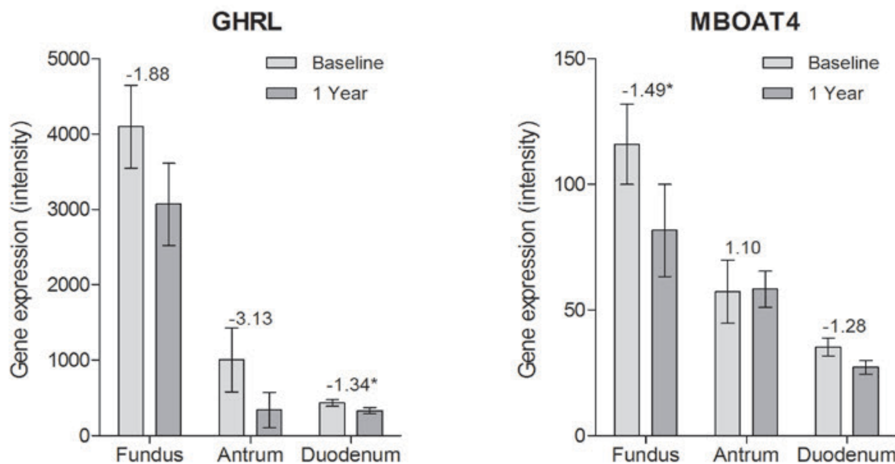


**Figure 3.3: Changes in gene expression of ghrelin and MBOAT4.** The graphs show RMA normalised intensities of microarray data at baseline and one year after gastroplication in biopsies of fundus, antrum and duodenum. Fold changes are indicated on top of the bars, significant changes are marked with * (p<0.05). All graphs show mean and SEM.

## Discussion

Results of the present study add important physiological background information to the clinical outcomes observed in patients after undergoing ACE stapler gastroplication. Our plasma analyses revealed beneficial effects on HbA1c and adiponectin levels one year after surgery, indicating an improvement of glycaemic control and a favourable shift in adipose tissue mass and (or) inflammatory status, respectively. This rise in plasma adiponectin and reduction of HbA1c is in line with previous studies in which a loss of excess body fat was achieved, including those involving other surgical and non-surgical weight loss interventions (15-18). We also found tendencies for reduced plasma IL-6 and MCP-1 levels, one year after intervention. Reduced levels of inflammatory markers are generally assumed to result from a reduction of visceral fat mass in particular, which plays a major role in the low grade inflammatory state associated with obesity (19). The total number of patients in our study population is quite small, and it is conceivable that statistical significance might have been reached with a larger patient group. Several other studies have found significant reductions in inflammatory markers like CRP, MCP-1 and IL-6 after bariatric surgery, while others did not find such an effect (20-22). To our knowledge, this study is the first to analyse long-term whole transcriptome changes in the upper gastrointestinal tract after a new transoral bariatric procedure. Gastroplication reduces gastric volume without altering intestinal anatomy, as is the case with Roux-en-Y gastric bypass (RYGB). In contrast to bypass surgery, exposure of the intestinal epithelium to nutrients and their metabolites is largely maintained after this endoscopic gastroplication. At the same time, small changes related to different GI transit characteristics or changes in the microbiome might still occur.

Analysis of the large amount of data using unbiased transcriptome analysis clearly pointed towards a reduction in inflammatory tone in the fundus and duodenum tissues as manifested by the downregulation of a wide variety of inflammation-related gene sets. The downregulated gene sets in the fundus were mostly related to innate immunity, and particularly associated with downregulation of the complement system, presentation and recognition of antigens (self or pathogenic), IFN-γ signalling and T-cell receptor signalling.

In the duodenum the main downregulated gene set was associated with the complement system. Moreover, the top 20 highly changed genes in these locations also suggest notable downregulation of many immune-related processes, of which several were related to chemokines, complement system, interferon signalling and immunoglobulins. These results coincided with the weight loss, improvement of HbA1c levels and decrease of whole-body inflammatory tone in these patients.

Based on the present study we cannot establish whether the apparent reduction of inflammatory tone in the upper GI tract has a predominantly local cause, *i.e.* due to a changed food-intake pattern or digestion process, or whether it is related to a reduction of low-grade systemic inflammation due to the reduction of body fat mass.

Increasing evidence points to a link between intestinal inflammation status in general, obesity and (or) diabetes. In obesity, increased innate cell densities, among which macrophages, natural killer cells and T-cells, especially the proportion of cytotoxic CD8 T-cells, have been observed in the jejunal epithelium. These epithelial T-cells were found to be associated with local and systemic comorbidities. Furthermore, isolated T-cells from obese patients decreased insulin sensitivity of epithelial cells *in vitro* (23). Another study reported that diet-induced weight loss resulted in a downregulation of inflammatory pathways and inflammatory cytokines IL-8, TNF-α, MCP-1 and IL-1β, in recto-sigmoid mucosal tissue (24). Moreover, increased intestinal inflammatory gene expression of TNF-α, IL-6, ICAM and PTGS-2 was found in insulin-resistant obese patients compared to non-insulin resistant obese patients, suggesting that intestinal inflammation is involved in diabetes during obesity (25, 26). A prominent feature of the immune system in the gastrointestinal tract is to provide adequate protection without stimulating excessive inflammation, thereby maintaining a fine balance (27, 28). A pro-inflammatory immune status of the gastrointestinal tract in obese patients might be protective against increased luminal challenges associated within obesity but deteriorating for insulin resistance (23). Furthermore, this pro-inflammatory status might be linked to the increased prevalence of inflammatory bowel disease and cancer in obese patients (29-31). In summary, we can only speculate whether the

reduced inflammatory microenvironment in gastric and duodenal tissue found after gastroplication can be considered as a positive or negative outcome.
In the antral tissue, gene sets related to cell cycle processes and extracellular matrix were increased. This might be explained by dilation of the stomach, a common observation after gastric volume reduction (32-35). While gastric volume was not quantified, we perceived the stomach as larger at one-year follow-up than immediately after gastroplication.
An interesting observation was that the gene expression of ghrelin and the enzyme GOAT, responsible for ghrelin acylation, decreased after gastroplication. At the same time, plasma fasted ghrelin was increased. Ghrelin is one of the most prominent hormones secreted from the upper gastrointestinal tract and does not only play a role in appetite regulation but also in inflammation (14). Consistent with our results, in RYGB patients significant lower levels of jejunal ghrelin gene expression have been reported after 10 months (6). Furthermore, GOAT mRNA expression and GOAT-positive cell numbers were lower in a non-obese group compared to morbidly obese patients, although no changes in jejunal ghrelin expression were detected (36). Moreover, more ghrelin positive cells were found in the stomach of morbidly obese and overweight patients compared to healthy normal weight controls (37, 38), which might indicate that with weight loss the number of ghrelin-releasing cells will decrease. The discrepancy with ghrelin expression in the gastrointestinal tract and plasma ghrelin values might be explained by a reduced secretory activity of (a higher number of) ghrelin producing cells in obesity, as suggested by Widmayer et al (37). The implications of these changes in ghrelin are not fully understood and need further investigation to crystallize the underlying mechanism and to explore the potential of these changes in obesity treatment.
There are some strengths and limitations to this study. The within person measurement of changes in gastrointestinal gene expression is unique as most studies in this field are observational. By applying a prospective design we were able to perform paired analysis and look specifically for changes induced by the gastroplication treatment instead of comparing obese subjects with lean controls. Whole transcriptome analysis enabled us to investigate changes in an unbiased manner. One of the limitations of this study is that it was not powered

to find differences in inflammatory markers. Therefore the inclusion of more patients could have strengthened the study. Furthermore, a control group on a lifestyle intervention program could help differentiate between weight loss effects and strictly procedural effects.

This study presents the long-term effects of a new transoral gastroplication treatment in morbidly obese patients. We show that this recently developed ACE stapler procedure was not only effective in reducing body weight as presented before (1), but also improved glycated haemoglobin levels and increased plasma adiponectin. Furthermore, whole transcriptome analysis suggested a marked downregulation of inflammatory gene sets in both the fundus and duodenum, coinciding with changes in plasma cytokines. Moreover, gene expression of ghrelin and its activating enzyme GOAT were reduced after gastroplication. The apparent reduction of inflammatory tone in the upper GI tract may be a consequence of an improved metabolic health status as associated with weight loss, or alternatively caused by the procedure itself. In conclusion, this new transoral gastroplication treatment which induced significant weight loss and improved plasma levels of adiponectin and glycated haemoglobin, coincides with a reduced inflammatory tone in the upper GI tract. The clinical relevance of our findings remains to be established, as there is still limited knowledge on the role of inflammatory pathways in the upper GI tract in obesity


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## Supplemental data

**Table S3.1: Full GSEA list of fundus, antrum and duodenum.** Gene sets within several processes using conservative filtering (p<0.001 and FDR q<0.05) with their normalised enrichment scores (NES).

| **Fundus** | | |
|---|---|---|
| **Process** | **Gene set** | **NES** |
| Antigen presentation and processing | INTERFERON.SIGNALING | -1.82327 |
| | KEGG_ALLOGRAFT.REJECTION | -2.51834 |
| | KEGG_VIRAL.MYOCARDITIS | -2.4598 |
| | WP2739.AMYLOIDS | -2.07757 |
| | KEGG_TUBERCULOSIS | -2.35037 |
| | KEGG_TYPE.I.DIABETES.MELLITUS | -2.6364 |
| | KEGG_TOXOPLASMOSIS | -1.86798 |
| | INTERFERON.GAMMA.SIGNALING | -2.2216 |
| | KEGG_SYSTEMIC.LUPUS.ERYTHEMATOSUS | -2.86087 |
| | KEGG_ASTHMA | -2.34147 |
| | CYTOKINE.SIGNALING.IN.IMMUNE.SYSTEM | -1.76089 |
| | KEGG_AUTOIMMUNE.THYROID.DISEASE | -2.49038 |
| | KEGG_STAPHYLOCOCCUS.AUREUS.INFECTION | -2.82522 |
| | WP1835.INTERFERON.ALPHA.BETA.SIGNALING | -1.83516 |
| | KEGG_CHAGAS.DISEASE.AMERICAN.TRYPANOSOMIASIS. | -2.24942 |
| | KEGG_INTESTINAL.IMMUNE.NETWORK.FOR.IGA.PRODUCTION | -2.33433 |
| | KEGG_CELL.ADHESION.MOLECULES.CAMS. | -2.59037 |
| | WP1836.INTERFERON.GAMMA.SIGNALING | -2.19636 |
| | KEGG_GRAFT.VERSUS.HOST.DISEASE | -2.67827 |
| | KEGG_ANTIGEN.PROCESSING.AND.PRESENTATION | -2.57427 |
| | WP2328.ALLOGRAFT.REJECTION | -2.33522 |
| | KEGG_LEISHMANIASIS | -2.43961 |
| | KEGG_INFLUENZA.A | -1.70275 |
| | KEGG_RHEUMATOID.ARTHRITIS | -1.97922 |
| Complement system | WP558.COMPLEMENT.AND.COAGULATION.CASCADES | -2.64551 |
| | WP2806.HUMAN.COMPLEMENT.SYSTEM | -2.14534 |
| | WP2775.TOLL.LIKE.RECEPTORS.CASCADES | -2.24403 |
| | WP1829.IMMUNOREGULATORY.INTERACTIONS.BETWEEN.A.LYMPHOID.AND.A.NON.LYMPHOID.CELL | -2.75048 |
| | WP1798.COMPLEMENT.CASCADE | -2.59545 |
| | WP1794.CELL.SURFACE.INTERACTIONS.AT.THE.VASCULAR.WALL | -2.22474 |
| | SCAVENGING.OF.HEME.FROM.PLASMA | -1.98708 |
| | RESPONSE.TO.ELEVATED.PLATELET.CYTOSOLIC.CA2. | -2.11246 |
| | PLATELET.DEGRANULATION | -2.13783 |
| | PLATELET.ACTIVATION.SIGNALING.AND.AGGREGATION | -1.87206 |
| | NITRIC.OXIDE.STIMULATES.GUANYLATE.CYCLASE | -2.28073 |
| | KEGG_COMPLEMENT.AND.COAGULATION.CASCADES | -2.67419 |
| | INNATE.IMMUNE.SYSTEM | -1.92568 |
| | INITIAL.TRIGGERING.OF.COMPLEMENT | -2.53372 |
| | IMMUNOREGULATORY.INTERACTIONS.BETWEEN.A.LYMPHOID.AND.A.NON.LYMPHOID.CELL | -2.73543 |
| | HEMOSTASIS | -2.03207 |
| | FORMATION.OF.FIBRIN.CLOT.CLOTTING.CASCADE. | -1.94238 |
| | FCGR.ACTIVATION | -2.31351 |
| | FCERI.MEDIATED.CA.2.MOBILIZATION | -2.07759 |
| | CREATION.OF.C4.AND.C2.ACTIVATORS | -2.33675 |
| | COMPLEMENT.CASCADE | -2.64418 |
| | CGMP.EFFECTS | -2.14792 |
| | CELL.SURFACE.INTERACTIONS.AT.THE.VASCULAR.WALL | -2.05582 |

| | | |
|---|---|---|
| T-cell receptor signaling | PD.1.SIGNALING | -2.40159 |
| | TCR.SIGNALING | -2.3198 |
| | DOWNSTREAM.TCR.SIGNALING | -2.18912 |
| | COSTIMULATION.BY.THE.CD28.FAMILY | -2.0165 |
| | WP1927.TCR.SIGNALING | -2.21863 |
| | PHOSPHORYLATION.OF.CD3.AND.TCR.ZETA.CHAINS | -2.30236 |
| | GENERATION.OF.SECOND.MESSENGER.MOLECULES | -2.67015 |
| | WP1799.COSTIMULATION.BY.THE.CD28.FAMILY | -2.11037 |
| Metabolism of xenobiotics | KEGG_CHEMICAL.CARCINOGENESIS | -2.10708 |
| | GLUTATHIONE.CONJUGATION | -1.77924 |
| | KEGG_METABOLISM.OF.XENOBIOTICS.BY.CYTOCHROME.P450 | -2.23614 |
| | KEGG_DRUG.METABOLISM.CYTOCHROME.P450 | -2.02946 |
| | KEGG_DILATED.CARDIOMYOPATHY | -1.97885 |
| Cardiomyopathy | WP2118.ARRHYTHMOGENIC.RIGHT.VENTRICULAR.CARDIOMYOPATHY | -1.96139 |
| | KEGG_ARRHYTHMOGENIC.RIGHT.VENTRICULAR.CARDIOMYOPATHY.ARVC. | -1.99686 |
| | KEGG_HYPERTROPHIC.CARDIOMYOPATHY.HCM. | -1.95811 |
| Muscle contraction | MUSCLE.CONTRACTION | -2.04807 |
| | WP1864.MUSCLE.CONTRACTION | -2.02105 |
| | SMOOTH.MUSCLE.CONTRACTION | -2.15383 |
| Receptors | PEPTIDE.LIGAND.BINDING.RECEPTORS | -1.73849 |
| | WP24.PEPTIDE.GPCRS | -1.82179 |
| | CHEMOKINE.RECEPTORS.BIND.CHEMOKINES | -2.16619 |
| Other | WP619.TYPE.II.INTERFERON.SIGNALING.IFNG. | -2.14764 |
| | BIOC_NKTPATHWAY | -2.18916 |
| | MHC.CLASS.II.ANTIGEN.PRESENTATION | -1.70662 |
| | AMYLOIDS | -1.81128 |
| | KEGG_HEMATOPOIETIC.CELL.LINEAGE | -1.92881 |
| | KEGG_LYSOSOME | -1.73537 |
| | WP560.TGF.BETA.SIGNALING.PATHWAY | -1.75296 |
| | KEGG_MALARIA | -1.94819 |
| | KEGG_LONG.TERM.DEPRESSION | -1.73225 |
| | MOLECULES.ASSOCIATED.WITH.ELASTIC.FIBRES | -2.00971 |
| | KEGG_PERTUSSIS | -2.42017 |
| | WP1833.INTEGRIN.CELL.SURFACE.INTERACTIONS | -1.98508 |
| | ARACHIDONIC.ACID.METABOLISM | -1.95268 |
| | KEGG_LEUKOCYTE.TRANSENDOTHELIAL.MIGRATION | -1.8182 |
| | G.PROTEIN.MEDIATED.EVENTS | -1.70815 |
| | WP2431.SPINAL.CORD.INJURY | -2.15311 |

| **Antrum** | | |
|---|---|---|
| **Process** | **Gene set** | **NES** |
| Cell cycle | KEGG_ALLOGRAFT.REJECTION | -1.85431 |
| | FORMATION.OF.THE.BETA.CATENIN.TCF.TRANSACTIVATING.COMPLEX | 1.970776 |
| | WP1775.CELL.CYCLE.CHECKPOINTS | 2.175551 |
| | RNA.POLYMERASE.I.PROMOTER.OPENING | 2.196208 |
| | CELL.CYCLE | 2.353103 |
| | TRANSCRIPTIONAL.REGULATION.BY.SMALL.RNAS | 1.887323 |
| | MITOTIC.METAPHASE.AND.ANAPHASE | 2.200172 |
| | ACTIVATION.OF.APC.C.AND.APC.C.CDC20.MEDIATED.DEGRADATION.OF.MITOTIC.PROTEINS | 1.747528 |
| | NORC.NEGATIVELY.REGULATES.RRNA.EXPRESSION | 1.875334 |
| | WP179.CELL.CYCLE | 2.256023 |
| | ORC1.REMOVAL.FROM.CHROMATIN | 1.964168 |
| | REGULATION.OF.DNA.REPLICATION | 2.05806 |
| | REGULATION.OF.APC.C.ACTIVATORS.BETWEEN.G1.S.AND.EARLY.ANAPHASE | 1.695511 |
| | CDC20.PHOSPHO.APC.C.MEDIATED.DEGRADATION.OF.CYCLIN.A | 1.642509 |
| | CYCLIN.A.CDK2.ASSOCIATED.EVENTS.AT.S.PHASE.ENTRY | 1.863858 |
| | ACTIVATION.OF.ATR.IN.RESPONSE.TO.REPLICATION.STRESS | 2.31258 |
| | WP1874.NUCLEOSOME.ASSEMBLY | 2.30575 |
| | WP1858.MITOTIC.G1.G1.S.PHASES | 2.262116 |
| | CYCLIN.E.ASSOCIATED.EVENTS.DURING.G1.S.TRANSITION | 1.828069 |
| | WP2369.HISTONE.MODIFICATIONS | 2.202452 |
| | WP2739.AMYLOIDS | 2.570812 |
| | TCF.DEPENDENT.SIGNALING.IN.RESPONSE.TO.WNT | 1.680383 |
| | REMOVAL.OF.LICENSING.FACTORS.FROM.ORIGINS | 1.97605 |
| | WP1928.TELOMERE.MAINTENANCE | 2.337343 |
| | KEGG_DNA.REPLICATION | 2.309638 |
| | CONDENSATION.OF.PROPHASE.CHROMOSOMES | 2.472027 |
| | PRC2.METHYLATES.HISTONES.AND.DNA | 2.179259 |
| | MITOTIC.ANAPHASE | 2.227793 |
| | SEPARATION.OF.SISTER.CHROMATIDS | 2.265669 |
| | WP45.G1.TO.S.CELL.CYCLE.CONTROL | 2.216835 |
| | MEIOTIC.RECOMBINATION | 1.907697 |
| | WP2757.MITOTIC.METAPHASE.AND.ANAPHASE | 2.385166 |
| | MITOTIC.G1.G1.S.PHASES | 2.388205 |
| | SIGNALING.BY.WNT | 1.675156 |
| | WP2698.MEIOTIC.RECOMBINATION | 1.839597 |
| | WP1898.REGULATION.OF.DNA.REPLICATION | 1.942631 |
| | NUCLEOSOME.ASSEMBLY | 2.48161 |
| | G0.AND.EARLY.G1 | 2.180428 |
| | WP2652.MITOTIC.PROMETAPHASE | 2.602052 |
| | SYNTHESIS.OF.DNA | 2.247251 |
| | MISSPLICED.LRP5.MUTANTS.HAVE.ENHANCED.BETA.CATENIN.DEPENDENT.SIGNALING | 1.682867 |
| | WP2446.RB.IN.CANCER | 2.758937 |
| | MEIOSIS | 1.730538 |
| | DNA.METHYLATION | 2.263346 |
| | MITOTIC.PROMETAPHASE | 2.418687 |
| | M.PHASE | 2.290548 |
| | NEGATIVE.EPIGENETIC.REGULATION.OF.RRNA.EXPRESSION | 1.848622 |
| | MITOTIC.G2.G2.M.PHASES | 1.640014 |
| | ACTIVATION.OF.THE.PRE.REPLICATIVE.COMPLEX | 2.57844 |

| | | |
|---|---|---|
| | S.PHASE | 2.294708 |
| | RNA.POLYMERASE.I.PROMOTER.CLEARANCE | 1.710418 |
| | DNA.REPLICATION | 2.317099 |
| | KEGG_SYSTEMIC.LUPUS.ERYTHEMATOSUS | 2.425107 |
| | XAV939.INHIBITS.TANKYRASE.STABILIZING.AXIN | 1.679086 |
| | TELOMERE.MAINTENANCE | 2.313958 |
| | CELL.CYCLE.CHECKPOINTS | 2.155747 |
| | MITOTIC.M.M.G1.PHASES | 2.536521 |
| | G1.S.SPECIFIC.TRANSCRIPTION | 2.569735 |
| | G2.M.CHECKPOINTS | 2.369734 |
| | WP1925.SYNTHESIS.OF.DNA | 2.370287 |
| | CELLULAR.SENESCENCE | 1.838437 |
| | WP2785.M.G1.TRANSITION | 2.181483 |
| | KEGG_CELL.CYCLE | 2.147555 |
| | WP2361.GASTRIC.CANCER.NETWORK.1 | 2.383789 |
| | PACKAGING.OF.TELOMERE.ENDS | 2.018427 |
| | CELL.CYCLE.MITOTIC | 2.420983 |
| | DEPOSITION.OF.NEW.CENPA.CONTAINING.NUCLEOSOMES.AT.THE.CENTROMERE | 2.464646 |
| | WP466.DNA.REPLICATION | 2.526486 |
| | CHROMOSOME.MAINTENANCE | 2.485708 |
| | RNF.MUTANTS.SHOW.ENHANCED.WNT.SIGNALING.AND.PROLIFERATION | 1.690068 |
| | REGULATORY.RNA.PATHWAYS | 1.751141 |
| | POLO.LIKE.KINASE.MEDIATED.EVENTS | 1.939158 |
| | SENESCENCE.ASSOCIATED.SECRETORY.PHENOTYPE.SASP. | 1.861184 |
| | APOPTOSIS | 1.650792 |
| | M.G1.TRANSITION | 2.204365 |
| | ASSEMBLY.OF.THE.PRE.REPLICATIVE.COMPLEX | 2.020908 |
| | E2F.MEDIATED.REGULATION.OF.DNA.REPLICATION | 2.469653 |
| | DNA.STRAND.ELONGATION | 2.291611 |
| | KEGG_ALCOHOLISM | 2.205282 |
| | EPIGENETIC.REGULATION.OF.GENE.EXPRESSION | 1.87112 |
| | EXTENSION.OF.TELOMERES | 2.140079 |
| | G1.S.TRANSITION | 2.515779 |
| | WP2772.S.PHASE | 2.367719 |
| | RESOLUTION.OF.SISTER.CHROMATID.COHESION | 2.435047 |
| | DNA.REPLICATION.PRE.INITIATION | 2.262319 |
| Transcription and translation | WP2710.NONSENSE.MEDIATED.DECAY | 1.956784 |
| | WP1889.PROCESSING.OF.CAPPED.INTRON.CONTAINING.PRE.MRNA | 1.987702 |
| | WP411.MRNA.PROCESSING | 2.125963 |
| | MRNA.SPLICING | 1.973784 |
| | WP1811.EUKARYOTIC.TRANSLATION.ELONGATION | 2.037809 |
| | KEGG_SPLICEOSOME | 1.874981 |
| | WP1812.EUKARYOTIC.TRANSLATION.INITIATION | 1.890107 |
| | WP2737.SRP.DEPENDENT.COTRANSLATIONAL.PROTEIN.TARGETING.TO.MEMBRANE | 1.809973 |
| | WP477.CYTOPLASMIC.RIBOSOMAL.PROTEINS | 2.03664 |
| | WP2683.INFLUENZA.LIFE.CYCLE | 2.231637 |
| | PROCESSING.OF.CAPPED.INTRON.CONTAINING.PRE.MRNA | 1.966031 |
| | MRNA.SPLICING.MAJOR.PATHWAY | 1.968078 |
| | WP1813.EUKARYOTIC.TRANSLATION.TERMINATION | 1.982535 |
| Extracellular matrix | EXTRACELLULAR.MATRIX.ORGANIZATION | 2.066144 |
| | WP2725.COLLAGEN.BIOSYNTHESIS.AND.MODIFYING.ENZYMES | 1.933647 |
| | KEGG_FOCAL.ADHESION | 1.739328 |
| | KEGG_ECM.RECEPTOR.INTERACTION | 2.178188 |

| | DEGRADATION.OF.THE.EXTRACELLULAR.MATRIX | 1.925916 |
|---|---|---|
| | WP306.FOCAL.ADHESION | 1.776627 |
| | COLLAGEN.BIOSYNTHESIS.AND.MODIFYING.ENZYMES | 2.084609 |
| | WP185.INTEGRIN.MEDIATED.CELL.ADHESION | 1.66432 |
| | INTEGRIN.CELL.SURFACE.INTERACTIONS | 1.809606 |
| | COLLAGEN.FORMATION | 2.233561 |
| | ELASTIC.FIBRE.FORMATION | 1.798224 |
| | WP2798.ASSEMBLY.OF.COLLAGEN.FIBRILS.AND.OTHER.MULTIMERIC.STRUCTURES | 2.186452 |
| | ASSEMBLY.OF.COLLAGEN.FIBRILS.AND.OTHER.MULTIMERIC.STRUCTURES | 2.156891 |
| Cellular metabolism | REGULATION.OF.PYRUVATE.DEHYDROGENASE.PDH.COMPLEX | -1.96206 |
| | WP1902.RESPIRATORY.ELECTRON.TRANSPORT.ATP.SYNTHESIS.BY.CHEMIOSMOTIC.COUPLING.AND.HEAT.PRODUCTION.BY.UNCOUPLING.PROTEINS. | -2.17445 |
| | WP111.ELECTRON.TRANSPORT.CHAIN | -2.04609 |
| | KEGG_COLLECTING.DUCT.ACID.SECRETION | -1.96777 |
| | KEGG_OXIDATIVE.PHOSPHORYLATION | -2.24102 |
| | THE.CITRIC.ACID.TCA.CYCLE.AND.RESPIRATORY.ELECTRON.TRANSPORT | -2.43706 |
| | PYRUVATE.METABOLISM.AND.CITRIC.ACID.TCA.CYCLE | -2.15426 |
| | RESPIRATORY.ELECTRON.TRANSPORT | -2.26064 |
| | WP623.OXIDATIVE.PHOSPHORYLATION | -2.21162 |
| | WP2766.THE.CITRIC.ACID.TCA.CYCLE.AND.RESPIRATORY.ELECTRON.TRANSPORT. | -2.00618 |
| | RESPIRATORY.ELECTRON.TRANSPORT.ATP.SYNTHESIS.BY.CHEMIOSMOTIC.COUPLING.AND.HEAT.PRODUCTION.BY.UNCOUPLING.PROTEINS. | -2.279 |
| Cell junctions | CELL.CELL.JUNCTION.ORGANIZATION | 1.957272 |
| | CELL.JUNCTION.ORGANIZATION | 2.081496 |
| | WP1793.CELL.JUNCTION.ORGANIZATION | 1.780006 |
| | CELL.CELL.COMMUNICATION | 1.77621 |
| | TIGHT.JUNCTION.INTERACTIONS | 2.166633 |
| Protein folding | CHAPERONIN.MEDIATED.PROTEIN.FOLDING | 1.957393 |
| | WP1892.PROTEIN.FOLDING | 2.131613 |
| | FORMATION.OF.TUBULIN.FOLDING.INTERMEDIATES.BY.CCT.TRIC | 2.236554 |
| | PREFOLDIN.MEDIATED.TRANSFER.OF.SUBSTRATE.TO.CCT.TRIC | 2.431509 |
| | COOPERATION.OF.PREFOLDIN.AND.TRIC.CCT.IN.ACTIN.AND.TUBULIN.FOLDING | 2.363184 |
| Cardiomyopathy | KEGG_ARRHYTHMOGENIC.RIGHT.VENTRICULAR.CARDIOMYOPATHY.ARVC. | 2.307521 |
| | KEGG_HYPERTROPHIC.CARDIOMYOPATHY.HCM. | 1.86531 |
| | KEGG_DILATED.CARDIOMYOPATHY | 2.017088 |
| | WP2118.ARRHYTHMOGENIC.RIGHT.VENTRICULAR.CARDIOMYOPATHY | 2.31039 |
| Kinesins (move along microtubule filaments) | KINESINS | 2.299073 |
| | WP1842.KINESINS | 2.420609 |
| | MHC.CLASS.II.ANTIGEN.PRESENTATION | 1.797628 |
| Muscle contraction | WP1864.MUSCLE.CONTRACTION | 2.033824 |
| | MUSCLE.CONTRACTION | 2.058598 |
| | SMOOTH.MUSCLE.CONTRACTION | 2.151354 |
| Other | WP1422.SPHINGOLIPID.METABOLISM | -2.01252 |
| | WP2377.INTEGRATED.PANCREATIC.CANCER.PATHWAY | 1.697525 |
| | KEGG_SALMONELLA.INFECTION | 1.824445 |
| | KEGG_PROPANOATE.METABOLISM | -1.91319 |
| | GAP.JUNCTION.TRAFFICKING | 1.829106 |
| | AXON.GUIDANCE | 1.769707 |
| | WP143.FATTY.ACID.BETA.OXIDATION | -2.18356 |
| | KEGG_VALINE.LEUCINE.AND.ISOLEUCINE.DEGRADATION | -2.03598 |

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|---|---|---|
| | BRANCHED.CHAIN.AMINO.ACID.CATABOLISM | -2.29066 |
| | KEGG_REGULATION.OF.AUTOPHAGY | -1.98463 |
| | WP1831.INTEGRATION.OF.ENERGY.METABOLISM | 1.920028 |
| | WP2664.GASTRIN.CREB.SIGNALLING.PATHWAY.VIA.PKC.AND.MAPK | 1.687694 |
| | WP368.MITOCHONDRIAL.LC.FATTY.ACID.BETA.OXIDATION | -1.97753 |
| | WP536.CALCIUM.REGULATION.IN.THE.CARDIAC.CELL | 1.945329 |
| | GLUTATHIONE.CONJUGATION | -1.8823 |
| | INTEGRATION.OF.ENERGY.METABOLISM | 1.788005 |
| | GENERATION.OF.SECOND.MESSENGER.MOLECULES | -1.8954 |
| | EPH.EPHRIN.SIGNALING | 1.729218 |
| | GENERIC.TRANSCRIPTION.PATHWAY | -2.103 |

| Duodenum | | |
|---|---|---|
| Process | Gene set | NES |
| Lipid metabolism | PHOSPHOLIPID.METABOLISM | 1.83 |
| | KEGG_FAT.DIGESTION.AND.ABSORPTION | 2.42 |
| | FATTY.ACID.TRIACYLGLYCEROL.AND.KETONE.BODY.METABOLISM | 1.77 |
| | REGULATION.OF.CHOLESTEROL.BIOSYNTHESIS.BY.SREBP.SREBF. | 2.14 |
| | WP197.CHOLESTEROL.BIOSYNTHESIS | 2.20 |
| | PEROXISOMAL.LIPID.METABOLISM | 1.99 |
| | ACTIVATION.OF.GENE.EXPRESSION.BY.SREBF.SREBP. | 2.11 |
| | KEGG_PEROXISOME | 1.93 |
| | SPHINGOLIPID.DE.NOVO.BIOSYNTHESIS | 1.81 |
| | KEGG_GLYCEROPHOSPHOLIPID.METABOLISM | 2.18 |
| | WP2740.GLYCEROPHOSPHOLIPID.BIOSYNTHESIS | 1.98 |
| | CHOLESTEROL.BIOSYNTHESIS | 2.07 |
| | METABOLISM.OF.LIPIDS.AND.LIPOPROTEINS | 2.07 |
| | GLYCEROPHOSPHOLIPID.BIOSYNTHESIS | 1.92 |
| | KEGG_SPHINGOLIPID.METABOLISM | 2.00 |
| | WP1817.FATTY.ACID.TRIACYLGLYCEROL.AND.KETONE.BODY.METABOLISM | 1.81 |
| Metabolism of xenobiotics | WP702.METAPATHWAY.BIOTRANSFORMATION | 2.09 |
| | KEGG_CHEMICAL.CARCINOGENESIS | 2.31 |
| | KEGG_METABOLISM.OF.XENOBIOTICS.BY.CYTOCHROME.P450 | 2.19 |
| | WP43.OXIDATION.BY.CYTOCHROME.P450 | 2.03 |
| | KEGG_DRUG.METABOLISM.CYTOCHROME.P450 | 1.93 |
| | CYTOCHROME.P450.ARRANGED.BY.SUBSTRATE.TYPE | 2.07 |
| | BIOLOGICAL.OXIDATIONS | 2.23 |
| | PHASE.1.FUNCTIONALIZATION.OF.COMPOUNDS | 2.11 |
| | PHASE.II.CONJUGATION | 1.73 |
| | KEGG_RETINOL.METABOLISM | 2.09 |
| Chaperones (protein) | WP2667.ACTIVATION.OF.CHAPERONE.GENES.BY.XBP1.S. | -1.97 |
| | UNFOLDED.PROTEIN.RESPONSE.UPR. | -2.33 |
| | XBP1.S.ACTIVATES.CHAPERONE.GENES | -2.00 |
| | IRE1ALPHA.ACTIVATES.CHAPERONES | -2.10 |
| Respiratory electron transport chain | WP1902.RESPIRATORY.ELECTRON.TRANSPORT.ATP.SYNTHESIS.BY.CHEMIOSMOTIC.COUPLING.AND.HEAT.PRODUCTION.BY.UNCOUPLING.PROTEINS. | 1.97 |
| | RESPIRATORY.ELECTRON.TRANSPORT | 1.91 |
| | WP111.ELECTRON.TRANSPORT.CHAIN | 1.93 |
| | RESPIRATORY.ELECTRON.TRANSPORT.ATP.SYNTHESIS.BY.CHEMIOSMOTIC.COUPLING.AND.HEAT.PRODUCTION.BY.UNCOUPLING.PROTEINS. | 1.86 |
| Innate immunity | INITIAL.TRIGGERING.OF.COMPLEMENT | -1.91 |
| | IMMUNOREGULATORY.INTERACTIONS.BETWEEN.A.LYMPHOID.AND.A.NON.LYMPHOID.CELL | -1.93 |
| | FCGR.ACTIVATION | -1.95 |
| Cell cycle | WP1928.TELOMERE.MAINTENANCE | 2.18 |
| | WP466.DNA.REPLICATION | 1.93 |
| | KEGG_DNA.REPLICATION | 2.05 |
| | TELOMERE.MAINTENANCE | 1.89 |
| | WP1925.SYNTHESIS.OF.DNA | 1.72 |
| | DNA.METHYLATION | 1.96 |
| | WP2739.AMYLOIDS | 1.98 |
| Other | PLATELET.DEGRANULATION | -1.94 |
| | KEGG_BILE.SECRETION | 2.10 |

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|---|---|---|
| | BIOSYNTHESIS.OF.THE.N.GLYCAN.PRECURSOR.DOLICHOL.LIPID.LINKED.OLIGOSACCHARIDE.LLO.AND.TRANSFER.TO.A.NASCENT.PROTEIN | -1.99 |
| | PPARA_TARGETS | 2.26 |
| | KEGG_PROTEIN.EXPORT | -2.04 |
| | KEGG_PROTEIN.PROCESSING.IN.ENDOPLASMIC.RETICULUM | -2.26 |
| | KEGG_DRUG.METABOLISM.OTHER.ENZYMES | 1.87 |
| | KEGG_PPAR.SIGNALING.PATHWAY | 2.05 |
| | APOPTOTIC.EXECUTION.PHASE | 1.83 |
| | KEGG_GLYCEROLIPID.METABOLISM | 1.93 |
| | WP2806.HUMAN.COMPLEMENT.SYSTEM | -2.01 |
| | WP716.VITAMIN.A.AND.CAROTENOID.METABOLISM | 2.17 |
| | KEGG_STAPHYLOCOCCUS.AUREUS.INFECTION | -2.30 |
| | KEGG_INTESTINAL.IMMUNE.NETWORK.FOR.IGA.PRODUCTION | -1.90 |
| | WP2840.HAIR.FOLLICLE.DEVELOPMENT.CYTODIFFERENTIATION.PART.3.OF.3. | -1.96 |
| | KEGG_PROTEIN.DIGESTION.AND.ABSORPTION | 1.92 |
| | KEGG_HOMOLOGOUS.RECOMBINATION | 2.05 |
| | WP2431.SPINAL.CORD.INJURY | -1.97 |
| | KEGG_FATTY.ACID.DEGRADATION | 1.99 |

# 4

# Steviol Glycoside Rebaudioside A Induces GLP-1 and PYY Release in a Porcine Ex Vivo Intestinal Model

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## Abstract

Glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) are hormones important for satiation and are involved in the process called “ileal brake”. The aim of this study was to investigate the GLP-1- and PYY-stimulating efficacy of rebaudioside A, casein, and sucrose. This was studied using tissue segments collected from various regions of the pig small intestine. GLP-1 release was strongest from the distal ileum. There, control release was 0.06 ± 0.01 (GLP-1) and 0.07 ± 0.01 (PYY) pmol/cm$^2$ of tissue. Rebaudioside A (2.5, 12.5, and 25 mM) stimulated GLP-1 release (0.14 ± 0.02, 0.16 ± 0.02, and 0.13 ± 0.02 pmol/cm$^2$ of tissue, $p < 0.001$) and PYY release (0.19 ± 0.02, 0.42 ± 0.06, and 0.27 ± 0.03 pmol/cm$^2$ of tissue, $p < 0.001$). Sucrose stimulated GLP-1 release (0.08 ± 0.01 pmol/cm$^2$ of tissue, $p < 0.05$) only at 10 mM. Casein (0.5%, 1%, and 2.5%, w/v) stimulated GLP-1 release (0.15 ± 0.03, 0.13 ± 0.02, and 0.14 ± 0.01 pmol/cm$^2$ of tissue, $p < 0.001$) and PYY release (0.13 ± 0.02, 0.20 ± 0.03, and 0.27 ± 0.03 pmol/cm$^2$ of tissue, $p < 0.01$). These findings may help in developing dietary approaches for weight management.

## Introduction

The presence of nutrients and other molecules in the small intestine stimulates the release of satiety hormones, such as Cholecystokinin (CCK), Glucagon-Like Peptide-1 (GLP-1), and Peptide YY (PYY)(1,2). The release of these hormones results in decreased gastric emptying and activation of the so-called intestinal brake. This poorly explored mechanism refers to a physiological feedback mechanism that is triggered by nutrients at a specific location in the intestine resulting in reduced appetite and food intake (3,4). Lipids have been widely researched and are known to activate this ileal brake mechanism resulting in the release of satiety hormones and reduced food intake (5-8). However, whether other macronutrients such as carbohydrates and proteins could also activate the ileal brake mechanism is still unknown (4). Knowledge of the satiety-hormone-inducing properties of macronutrients is relevant to better understanding the intestinal response to ingested food and may contribute to the development of products with satiating properties.

The efficacies of non-caloric sweeteners to induce the release of satiety hormones are also partly unknown. However, a study by Anton et al. (2010) showed that preloads containing the sweetener stevia reduced daily caloric intake by 301 kcal and reduced postprandial glucose and insulin levels compared to preloads containing sucrose (9). Such a reduced caloric intake as well as glucose and insulin responses may be caused by reduced caloric content of the preload but may also be caused by other mechanisms such as GLP-1 release. The glycoside rebaudioside A is the one of the most abundant sweet compound extracted from the leaves of Stevia rebaudiana bertoni. Rebaudioside A is reported to be the most potent sweetener among the steviol glycosides(10), with a relative sweetness of 200 – 400 times greater than that of sucrose (11). Rebaudioside A is increasingly used in food products as a natural noncaloric sweetener. It has been approved in the United States since 2008(12), and in the European Union since 2010 (13).

It is unknown if ileal delivery of macronutrients other than lipids and sweeteners could also stimulate the release of satiety hormones and consequently activate the ileal brake. Stimulation of GLP-1 and PYY release from isolated ileal tissue has been demonstrated for lipids including unsaturated fatty acids. This was

shown using a recently described *ex vivo* porcine intestinal model (14). For other macronutrients such as sucrose and casein such studies have not been performed yet, although their efficacy to induce gut hormone release has been shown in cell cultures (15-17). The pig was chosen as a model because the gastrointestinal tract of the pig shows a high degree of macroscopic and microscopic resemblance with that of humans (18-20). The present study primarily focused on the GLP-1 and PYY inducing efficacy of casein, sucrose and the non-caloric sweetener rebaudioside A at various locations of the small intestine. We hypothesize that rebaudioside A, the protein casein, and the carbohydrate sucrose are effective stimulators of GLP-1 and PYY release from various locations of the small intestine, and thus may be effective activators of the ileal brake *in vivo*.

## Materials and methods

### Chemicals

Casein protein (food grade, sodium caseinate purity >82% batch number 158156) was obtained from Dutch Protein Services BV, Tiel, The Netherlands. Rebaudioside A (figure 4.1, food grade, purity >97% batch number 20110301, ChemSpider ID: 5294031) was obtained from SteviJa Natuurlijk BV, Drachten, The Netherlands. Sucrose (food grade, purity >99.9%) was supplied by Suiker Unie, Oud Gastel, The Netherlands. Safflower oil (food grade, composed of 3-8% C16:0, 2-5% C18:0, 9-15% C18:1 and 75-85% of C18:2) was provided by De Wit Specialty oils, De Waal, The Netherlands. Xantham Gum and Guar gum were supplied by GF Supplies, Amsterdam, The Netherlands. Chemicals to make the Krebs Ringer bicarbonate (KRB) buffer (D-glucose 1.8 gram/L, Magnesium Chloride 0.0468 gram/L, Potassium chloride 0.34 gram/L, Sodium Chloride 7.0 gram/L, Sodium Phosphate Dibasic 0.1 gram/L, Sodium Phosphate Monobasic 0.18 gram/L, HEPES 5,579 gram/L) were obtained from Sigma, Zwijndrecht, The Netherlands. The pH of the KRB buffer was adjusted to pH 7.4.

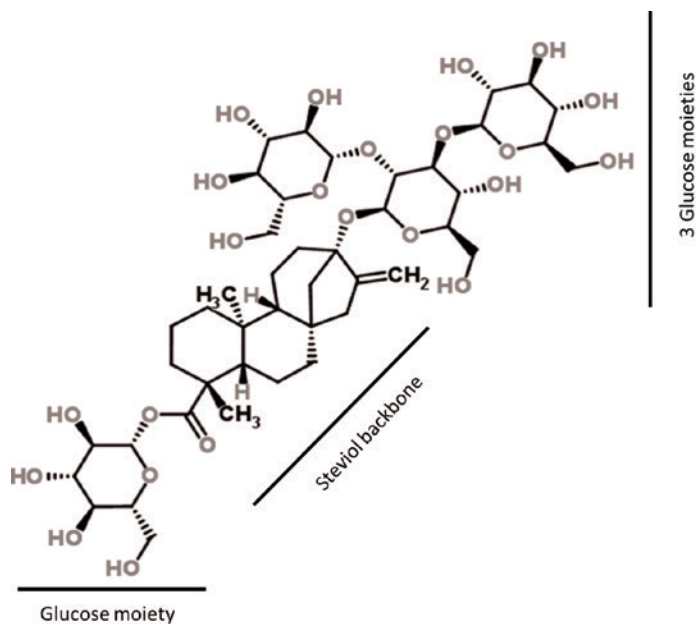


**Figure 4.1 Chemical structure of Rebaudioside A.**
Adapted from ChemSpider ID: 5294031

## Collection of porcine intestinal tissues

Intestinal tissues were obtained from ten healthy male pigs (*Sus scrofa domesticus*). The pigs were considered healthy after evaluation by a veterinarian. The protocol for this study was approved by the Animal Ethics Committee Utrecht (Ethics Committee permit number 2012.I.09.096, Utrecht, The Netherlands). Pigs had a mean (± SEM) body weight of 141 ± 2 kg, and mean (± SEM) age of 6 ± 0.1 months. They were housed in groups and were fed *ad libitum* with standard pig diet (vitaal P 003103, de Heus, Ede, The Netherlands) and free access to water. Within 10 minutes after the pigs were sacrificed, intestines were excised and segments of different anatomical regions were stored in ice-cold oxygenated (95% $O_2$, 5% $CO_2$) KRB buffer. Jejunum (4.5 meter proximal to the ileocecal junction) and proximal, mid and distal ileum (0.5 meter of intestine sampled from 1.5, 1 and 0.5 meter proximal to the ileocecal junction, respectively) were collected for the experiments. Tissues were transported to the laboratory and immediately used for *ex vivo* experiments. Time between excision and start of the experiments was approximately 30 minutes.

**Nutrient exposure of intestinal tissue and viability tests**

Incubation with casein (0.5, 1 and 2.5% w/v), rebaudioside A (2.5, 12.5 and 25 mM), sucrose (2.5, 5 and 10 mM), and safflower oil (3.35% w/v) were performed with porcine intestinal tissue as described previously(14) with some minor adaptations. In brief, upon arrival at the laboratory, the intestine was rinsed with KRB buffer and cut open in a longitudinal direction. The outer muscle layers were carefully stripped off with the basolateral side upwards. The mucosal tissue was placed on a gauze (pores = 250 μm, Sefar Nitex 03-250/50, Sefar Heiden Switzerland) with the apical side upward, and circles of tissue with a diameter of 11.8 mm (about 1.0 $cm^2$) were punched out using a biopsy punch. The intestinal segments were randomized, per region, in a beaker glass and transferred to a 24-wells plate, filled with 500 μl ice cold KRB buffer/well, and kept on ice until start of the experiment. Thereafter, tissues were brought to room temperature within 30 min, followed by an pre-incubation step at 37°C for 1 hour in a humidified incubator (5% v/v CO2). To study the release of GLP-1 and PYY, incubations were initiated by replacing the KRB buffer solution with 500 μl pre-warmed (37°C) KRB without D-glucose containing the test compounds. KRB buffer without D-glucose was used as a control since D-glucose might induce gastrointestinal hormone release. Then the tissue was put in a humidified incubator for 1 hour at 37°C at 5% v/v CO2. After incubation, solutions were collected, mixed with 100 μM phenylmethanesulfonyl floride (PMSF) (Sigma Aldrich, Zwijndrecht, The Netherlands) to inactivate dipeptidyl peptidase IV, aliquoted and stored at 4°C for further analysis of lactate dehydrogenase (LDH), or stored at -80°C for further analysis of GLP-1 and PYY.

Tissue viability was checked by measuring leakage of intracellular LDH in supernatant. LDH, a stable cytosolic enzyme, was analyzed using a LDH kit (Sigma Aldrich, Zwijndrecht, The Netherlands). LDH activity in supernatant was expressed as a percentage of the total LDH activity present in control tissue collected before incubation. Total tissue levels of LDH were determined per region in 1.0 $cm^2$ tissue samples, which were homogenized in ice-cold KRB buffer with a Potter-Elvehjem-type Teflon pestle tissue grinder (Braun, Melsungen, Germany) for 5 minutes at 200 rounds per minute. Samples were

only included for further analysis when LDH leakage did not exceed 10% of the total LDH tissue content (14,21). No samples had to be excluded applying this criterion.

**Analysis of satiety hormones GLP-1 and PYY**

GLP-1 active levels were analyzed using commercially available ELISA assay kits obtained from Millipore (Billerica, MA, USA) and used according to the manufacturer's instructions using a BioTek Synergy HT fluorescence microplate reader (BioTek Instruments Inc., Winooski Vermont, USA). Since the GLP-1 hormone gene sequence is highly preserved, this kit could be applied for analyzing porcine GLP-1. The range of this assay is approximately 2 – 100 pM. The inter-assay coefficient of variation is 11%, and the intra-assay variation is 6%. When necessary, samples were diluted in assay buffer from the kit to be in this range.

PYY release was measured from distal ileum only, since PYY is known to be co-localized with GLP-1 (22). PYY concentrations were measured using a PYY ELISA kit for total PYY (Bachem, Penisula Laboratories, San Carlos, CA, USA) according to the manufacturer's instructions using a BioTek Synergy HT microplate reader (BioTek Instruments Inc., Winooski Vermont, USA). This kit measures porcine PYY, which is identical to human PYY (23). The range of this assay is approximately 0.32 – 5 ng/ml (4.6 – 1150 pM). The inter assay coefficient of variation for this assay is 6% and the intra assay coefficient of variation is 3%. The lower detection limit of this kit was 20 pg/ml (4.6 pM) It was not necessary to dilute samples for this assay.

**Statistical analysis**

Statistical analysis was performed using the SAS statistical software package (SAS version 9; SAS institute, Cary, NC, USA). Both variables GLP-1 and PYY were compared with a mixed analysis of variance model (ANOVA). GLP-1 and PYY were both log transformed to meet the requirements for ANOVA. The ANOVA model included the fixed factors dose solution (control, sucrose 2.5, 5 and 10 mM, casein 0.5, 1 and 2.5% w/v, rebaudioside A 2.5, 12 and 25 mM and safflower oil 3.35% (w/v), intestinal location (jejunum, and proximal

mid and distal ileum) and the number of incubations per condition (incubations were performed in triplicate). The factor pig, which specifies the individual pig, was added to the model as random factor. The Tukey post hoc test was used to correct for multiple comparisons. Data are presented as the mean ± SEM and considered significant at $p < 0.05$.

## Results

### Effects of rebaudioside A and other compounds / mixtures on GLP-1 release

Table 4.1 shows the GLP-1 concentrations in the medium following incubation with the test compounds or mixtures using tissues taken from different locations. In all parts of the small intestine investigated, rebaudioside A caused a significant release of GLP-1 already at the lowest concentration tested (2.5 mM) ($p < 0.01$), except for the jejunum where the release was significant at a concentration of 12.5 mM ($p < 0.05$). The highest relative increase of GLP-1 after incubation with rebaudioside A was seen in the distal ileum ($p < 0.001$). The release at 25 mM was not higher than at 12.5 mM. As also shown in table 4.1, safflower oil (3.35% w/v) significantly increased GLP-1 release from the proximal ileum ($p < 0.05$) and even more from the distal ileum ($p < 0.001$). Safflower oil did not increase GLP-1 release from the mid-ileum and only marginally from the jejunum. At the concentrations tested sucrose did not stimulate GLP-1 release from jejunal and mid ileal tissue. The only significant stimulation was seen in tissues from the distal ileum at a sucrose concentration of 10 mM ($p < 0.05$).



Casein, at 1 and 2.5 % (w/v) increased GLP-1 release ($p < 0.001$) from tissues collected from the jejunum, whereas 0.5% (w/v) casein did not. Both 0.5 and 2.5% (w/v) casein stimulated GLP-1 release from the proximal ileum ($p < 0.001$). A concentration of 1% (w/v) casein stimulated GLP-1 release from proximal ileum ($p < 0.010$), but exposure to 0.5% and 2.5% (w/v) casein caused a higher GLP-1 release. None of the casein concentrations, 0.5, 1 and 2.5% (w/v), stimulated GLP-1 release from the mid ileum. All three casein

concentrations stimulated GLP-1 release from the distal ileum ($p < 0.001$). Similar to rebaudioside A, the most pronounced stimulation of GLP-1 by casein was observed in the distal ileum.

**Regional differences in GLP-1 release**

Exposing intestinal tissue to the control did not result in regional differences in control GLP-1 release. However, after exposure to the nutrients, regional differences were observed. For GLP-1 both an overall incubation and location effect ($p < 0.001$), and an interaction effect between incubation and location was found ($p < 0.05$). Overall GLP-1 release from all ileal locations (distal, mid and proximal ileum), was higher than GLP-1 release from the jejunal locations ($p < 0.001$). Furthermore, GLP-1 release from the distal ileum was higher than GLP-1 release from the mid ileum ($p < 0.001$). Neither hormone release from mid and proximal ileum differed, nor did release from proximal and distal ileum ($p < 0.08$).

**Effects of compounds and mixtures on PYY release**

The compounds and mixtures stimulating GLP-1 release were also tested for their PYY releasing capacity from the distal ileum (table 4.2). Safflower oil was found to stimulate PYY release, whereas sucrose at the concentrations tested did not. Casein stimulated PYY release from the distal ileum at all three concentrations tested. Release after exposure to a concentration of 1% (w/v) was higher than with 0.5% (w/v) casein ($p < 0.05$). Rebaudioside A stimulated PYY release from distal ileum ($p < 0.001$). The effect at 12.5 mM rebaudioside A was higher than that at 2.5 mM ($p < 0.05$).

**Table 4.1: GLP-1 concentrations released from jejunal, proximal, mid and distal ileum intestinal tissue segments after exposure to various compounds.**

a $p < 0.05$, b $p < 0.01$, c $p < 0.001$; p-values are based on comparison to control (ANOVA + Tukey correction). The incubations were performed in triplicate (n=6 for jejunum, n=10 for proximal, mid and distal ileum)

| Solution | Concentration | GLP-1 release (pmol/cm² tissue) | | | |
|---|---|---|---|---|---|
| | | *Jejunum* | *Proximal ileum* | *Mid ileum* | *Distal ileum* |
| Control | | 0.04± 0.01 | 0.07 ± 0.02 | 0.07 ± 0.01 | 0.06 ± 0.01 |
| Safflower oil (%) | 0,0335 | 0.07± 0.02 | 0.11±0.03 a | 0.09±0.02 | 0.13 ±0.02 c |
| Sucrose (mM) | 2,5 | 0.05±0.01 | 0.10±0.02 | 0.07±0.01 | 0.06±0.01 |
| | 5 | 0.05±0.01 | 0.17±0.05 | 0.08±0.01 | 0.07±0.01 |
| | 10 | 0.06±0.01 | 0.07±0.01 | 0.08±0.01 | 0.08±0.01 a |
| Casein (%) | 0,5 | 0.06±0.01 | 0.12±0.02 c | 0.09±0.01 | 0.15±0.03 c |
| | 1 | 0.09±0.01 c | 0.09±0.02 b | 0.08±0.01 | 0.13±0.02 c |
| | 2,5 | 0.08±0.02 c | 0.13±0.03 c | 0.08±0.01 | 0.14±0.01 c |
| Rebaudioside A (mM) | 2,5 | 0.07±0.01 | 0.14±0.02 c | 0.13±0.02 b | 0.14±0.02 c |
| | 12,5 | 0.07±0.01 b | 0.11±0.02 c | 0.18±0.04 c | 0.16±0.02 c |
| | 25 | 0.07±0.01 b | 0.14±0.02 c | 0.13±0.02 b | 0.13±0.02 c |

**Table 4.2 PYY concentrations released distal ileum intestinal tissue segments after exposure to various compounds.**

a $p < 0.05$, b $p < 0.01$, c $p < 0.001$; p-values are based on comparison to control (ANOVA + Tukey correction). The incubations were performed in triplicate (n=10).

| Solution | Concentration | PYY (pmol/cm² tissue) |
|---|---|---|
| | | *Distal ileum* |
| Control | | 0.07±0.01 |
| Safflower oil (%) | 3,35 | 0.17±0.01 c |
| Sucrose (mM) | 2,5 | 0.14±0.03 |
| | 5 | 0.12±0.02 |
| | 10 | 0.08±0.01 |
| Casein (%) | 0,5 | 0.13±0.02 b |
| | 1 | 0.20±0.03 c |
| | 2,5 | 0.27±0.03 c |
| Rebaudioside A (mM) | 2,5 | 0.19±0.02 c |
| | 12,5 | 0.42±0.06 c |
| | 25 | 0.27±0.03 c |

## Discussion

Our study confirmed our hypothesis that rebaudioside A and casein induce GLP-1 and PYY release from porcine intestinal tissue segments. Remarkably, rebaudioside A was found to stimulate GLP-1 release from all ileal and jejunal regions investigated, whereas sucrose (10 mM) only caused GLP-1 release from the distal ileum. Rebaudioside was effective in stimulating hormone release from more locations of the small intestine than casein. Therefore, taking into account that the model and the tested concentrations make a direct comparison difficult, our results suggest that rebaudioside A and the protein casein are candidate substances that may activate the ileal brake *in vivo* via the release of GLP-1 and PYY.

Some sweeteners including sucralose were found not to affect satiety hormone release *in vivo* (24,25). For *Stevia* Anton *et al*. (2010) demonstrated satiating inducing properties which may be due to increased satiety hormone release. However, since satiety hormones were not measured in that study, this association cannot be made from these findings. To our knowledge no reports have shown the satiety hormone-inducing capacity of the non-caloric sweetener rebaudioside A as was done in our study.

Nutrients, such as casein and sucrose, could stimulate satiety hormone release following interaction with G-protein coupled receptors and some solute carrier transporters located on enteroendocrine cells in the intestinal epithelium (26). A potential mechanism by which rebaudioside A could stimulate GLP-1 and PYY release is via the sweet taste receptor. Recently, it was found that rebaudioside A has affinity for the sweet taste receptor TAS1R2/TASR3 (10). This receptor is involved in glucose dependent release of GLP-1 and PYY *in vivo* (27). Furthermore, TAS1R2/TASR3 is present on brush cells and enteroendocrine cells (28). However, other mechanisms than via the taste receptors by which rebaudioside A and casein could induce satiety hormone release cannot be excluded and should be further studied.

Already more than 100 compounds have been identified in *Stevia rebaudiana*. However, the steviol glycosides stevioside and rebaudioside A have been found to be the most abundant. Steviol glycosides are metabolized into steviol upon their arrival in the colon and are absorbed thereafter (29). Incubation

of the murine enteroendocrine STC-1 cells with *Stevia* preparations resulted in higher GLP-1 and CCK release compared to incubation with sucrose (17), which is in line with our findings. The same study also demonstrated GLP-1 and CCK release after incubation with sucralose. However, only relatively high concentrations of 150 and 300 mM sucralose and of 600 mM sucrose were able to stimulate GLP-1 and CCK release from STC-1 cells. In our study 2.5 mM rebaudioside A resulted in higher GLP-1 release compared to 2.5 mM sucrose ($0.14 \pm 0.02$ *vs.* $0.06 \pm 0.01$ $p < .001$, distal ileum). Concentrations of sucrose and rebaudioside A in this study were in the same range (0-25 mM). Further *in vitro* studies should explore dose-effect relationships of these compounds.
Safflower oil was used as a positive control in our study. Safflower oil (3.35%) was found to activate the ileal brake *in vivo* and to increase satiety and gastrointestinal hormone concentrations (5,7). As expected safflower oil effectively stimulated GLP-1 release from the ileum mainly from the distal part whereas no such stimulation occurred from the mid ileum. This difference may relate to the enteroendocrine cell distribution. Enteroendocrine L-cells are most abundant in the distal part of the small intestine (30) corresponding to the intestinal tissue content of GLP-1 (14). The protein casein increases satiety *in vivo* (31) and *in vitro* it stimulates GLP-1 release from enteroendocrine murine STC-1 and human NCI-H716 cells (15,16,32). In our study casein induced GLP-1 and PYY release which confirms these previous findings and suggests that casein is a candidate to induce the ileal brake *in vivo*.
Apart from the differences in efficacy of various nutrients to stimulate GLP-1 and PYY some other questions remain. An important question is to what extent the effects on satiety hormone release found in our model can be reproduced in *in vivo* models and specifically in humans. Another relevant question is whether the capacity to induce satiety hormone release will lead into satiation *in vivo*. In one study of Veldhorst *et al.*(2009) it was shown that breakfast with higher casein content (25% *vs.* 10%) increased VAS scores in fullness and satiety whereas subsequent energy intake was not reduced and GLP-1 plasma concentrations did not differ per condition (33). Food intake is regulated by a whole cascade of responses which have to be taken into account if studying satiation *in vivo*. Therefore, knowledge of the satiety hormone releasing

capacity of macronutrients and non-caloric sweeteners could be useful to understand this so called 'satiety cascade' (34).

The current *in vivo* data are based on lipid infusions mainly. Those data could be extended into infusion studies using rebaudioside A and casein since the results from this study suggest that the satiety hormone releasing effect of sweeteners and proteins might be at least as effective as fat. These human interventions will then further substantiate the relevance of this *ex vivo* intestinal model.

One could question whether the concentrations of the selected compounds are relevant in view of those reached in the gut after dietary intake. Nutrient concentrations in the small intestine depend on the rate of gastric emptying of a meal, the rate of absorption, and (or) digestion of the nutrients the meal contained (35-37). Data on actual concentrations of the selected products are very scarce. There is only one animal study showing that luminal glucose concentrations after a meal are varying between 0.2 and 48 mM depending on postprandial time and the small intestinal region (38). Although the data are limited one could estimate intestinal concentrations on the following assumptions; 1 cup of coffee or tea (200 ml) with 0.3 gram of rebaudioside A (based on ADI of 0.4 mg/kg BW/day), or 1 cube of sugar (1 gram), in a total volume of 300 ml (100 ml gastric juice is added in the stomach)(17). These assumptions would yield an estimate of approximate concentrations of 10 mM sucrose and 1 mM rebaudioside A for the small intestine. The estimate for casein is based on the assumption that 1 glass of milk is consumed (total volume including gastric juice 300 ml) with a casein concentration of 80% of total milk protein content which is approximately 35 g/L. This means that a concentration of approximately 3% may initially be reached in the small intestine. Although these values are estimates only they suggest that the concentrations used in this study may be relevant from a dietary perspective.

The hormone secreting capacity of nutrients and other compounds is often studied *in vitro* using enteroendocrine cell lines including murine STC-1 (15,39-41), GLUTag cells (42) and the human NCI-H716 cell line (43). The advantage of the current *ex vivo* model compared to cell lines is that this model consist of all different cell types present in the gut. Another model to study the GLP-

1 and PYY inducing capacity of nutrients is the Ussing chamber technology using animal or human biopsies (44). It is however, challenging to obtain human biopsies especially from the ileum which makes the *ex vivo* intestinal porcine segment system a valuable alternative. However, there are still some technical limitations such as tissue viability and intra-subject variation. In this study tissue viability was evaluated by LDH leakage as before (14) and LDH leakage did not exceed 10%. Also, hormone release in the control condition was low. Both conditions indicate a good tissue viability. However, we cannot exclude that hormone release may be due to aspecific processes like cell lysis. Also, a high number of replicates was needed because of a high intra-subject variation. This variation may be caused by the relatively low number of enteroendocrine cells (reported to account for only 1% of the epithelial cell population in the small intestine) and these cells may be unevenly distributed (2,28).

In conclusion, the present study showed that rebaudioside A and casein, but not sucrose, induced satiety hormone release at various locations of the small intestine using an *ex vivo* model. These findings may contribute to the development of dietary approaches improving weight management and glucose control. However, *in vivo* studies are necessary to confirm the potential satiety inducing properties of rebaudioside A and casein *in vivo*.



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# 5

# Rebaudioside A-induced GLP-1 secretion from enteroendocrine cells is independent of the sweet taste receptor

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In preparation.

## Abstract

In food products, the molecular mechanisms of action of steviol glycosides is only partly understood. Given their intestinal stability, it is likely that interaction takes place with nutrient sensing receptors present on the apical membrane of enteroendocrine cells. However, these interactions and their possible post-receptor effects have not been reported in much detail. Here, we have used enteroendocrine STC-1 cells and two dimensional organoids prepared from mouse ileal crypt cells to investigate the effects of steviol glycosides on GLP-1 and CCK release. The role of the sweet taste receptor and the involvement of cAMP- and calcium signaling in the effects of rebaudioside A were investigated using specific inhibitors and live cell imaging. It was established that rebaudioside A stimulated GLP-1 and CCK release from STC-1 cells and organoids. In STC-1 cells, Rebaudioside A-induced GLP-1 release was reversed by MDL-12330A, an adenylate cyclase inhibitor, while gurmarin, a murine-specific inhibitor of the sweet taste receptor, did not affect GLP-1 release. Our findings led us to conclude that the induction of GLP-1 release by rebaudioside A occurs independently of the sweet taste receptor (Tas1R2/Tas1R3) and that the underlying pathway involves cAMP signaling.

## Introduction

Since the approval of steviol glycosides by EFSA and FDA, stevia products are increasingly used as non-caloric sweeteners in food products (1). Steviol glycosides are components of the plant *Stevia rebaudiana* Bertoni, of which the leaves are traditionally used in South America (2). Extracts of the plant contain numerous steviol glycosides, among which the most abundant and best characterized constituents are stevioside and rebaudioside A (3). The different steviol glycosides vary in their potencies to induce sweet taste sensations. Stevioside, rebaudioside A and rebaudioside D are reported to have the lowest sweet taste threshold compared to other common steviol glycosides, when tested by a sensory panel. Comparable relative potencies were found in cell-based assays functionally expressing the sweet taste receptor (4).

Despite their increasing use in food products, the effects of steviol glycosides on food intake and glucose homeostasis are still far from clear with several studies reporting contradictory outcomes. Stevia preloads in humans were reported to reduce daily food intake (5). In addition, intragastric infusion of rebaudioside A in combination with umami and bitter tastants reduced subsequent food intake (6). Furthermore, stevia and stevioside reduced post-prandial blood glucose in healthy-, obese- and type II diabetic subjects (5, 7). However, these results diverge from other studies where no effect on fasted glucose or energy intake was observed (8-10). Also animal studies show contradictory results. Fujita et al. found no effects of stevia on glucose levels in a glucose tolerance test or on plasma GLP-1 and GIP incretin secretion in rats (11), while others did find effects of stevioside and rebaudioside A on glucose and/or insulin in healthy and diabetic rodents (12, 13).

Underlying mechanisms mediating effects of steviol glycosides on food intake and metabolic parameters are still poorly understood. After their ingestion steviol glycosides remain intact in the small intestine. Only upon contact with bacteria in the colon these compounds are degraded and their degradation product, steviol, is subsequently absorbed (2). Therefore, stevia compounds may interact with chemosensory receptors expressed by the small intestinal epithelium (14). Most of these receptors are located on enteroendocrine cells. These cells comprise about 1% of the epithelial cells and are well-known for

the secretion of satiety hormones like GLP-1, PYY and CCK (15). Recently, we showed that rebaudioside A can induce both GLP-1 and PYY release in a porcine *ex vivo* intestinal model (16).

Here, we aimed to further elucidate the underlying mechanism of GLP-1 release induced by steviol glycosides, using both STC-1 enteroendocrine cells and intestinal organoid cultures. We focused on the role of the sweet taste receptor in GLP-1 release and explored intracellular signaling pathways.

## Materials and methods

### Cell culture

The STC-1 cell line (ATCC, LGC Standards, Teddington, UK) is a pluripotent enteroendocrine cell line derived from a double-transgenic mouse. STC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS) (Lonza, Verviers SPRL, Belgium). The cells were passaged when reaching 80% confluence; passage numbers between 25-50 were used.

### Secretion studies

STC-1 cells were grown in 24-well plates. When reaching 80% confluence, the medium was removed and the cells were rinsed with HBSS (containing 1.26 mM $Ca^{2+}$, 10 mM HEPES with pH 7.4) (Life technologies, Carlsbad, USA). Subsequently, the cells were incubated in HBSS or HBSS supplemented with rebaudioside A, stevioside or sucralose (Sigma-Aldrich, St. Louis, USA), at 37°C. After 2 hours of incubation, the buffer was removed, mixed with phenylmethanesulfonyl fluoride (Sigma-Aldrich, St. Louis, USA) to a final concentration of 100 µM. Subsequently, this mixture was centrifuged to remove cell debris and stored for analysis. The involvement of cAMP in gut hormone secretion was investigated by addition of 10 µM MDL-12330A (Sigma-Aldrich, St. Louis, USA) at the start of the 2 hour incubation period. The role of the sweet taste receptor was studied using gurmarin (kindly provided by Loic Briand, INRA Dijon Centre, France). This purified recombinant gurmarin was shown to have a half-maximal inhibitory concentration of 0.030 µg/ml for the sweet taste receptor (Tas1R2/Tas1R3) (17). For experiments using gurmarin,

STC-1 cells were pre-incubated with various concentrations of gurmarin and subsequently incubated with rebaudioside A in combination with gurmarin or with HBSS, for 2 hours.

**Cytotoxicity**

Effects of the test compounds on the viability of the STC-1 cells were assessed using an LDH Cytotoxicity Detection Kit and an XTT Cell Proliferation Kit II (Roche Applied Science, Almere, The Netherlands) according to the manufacturer's instructions. Briefly, cells were first incubated with the test compounds for two hours. Thereafter, the activity of lactate dehydrogenase, an enzyme released from damaged cells into the supernatant, was measured. Simultaneously, the cells' ability to metabolize tetrazolium salt (XTT) to formazan was assessed as a measure for cell viability. As a control, Triton X-100 was added to the cells, resulting in total cell lysis.

**Intestinal organoid secretion study**

The use of mouse intestine had been approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University (Permit number: 2014036.b). Intestinal tissue was collected from six-month old C57Bl/6J mice. The isolation and culturing of intestinal organoids will be described in chapter 6 of this thesis. Briefly, intestinal crypts were isolated from the ileum and cultured in Matrigel$^{R}$ basal membrane matrix, growth factor reduced (Corning Incorporated, Corning, USA). For the organoids DMEM/F12 medium (containing GlutaMAX supplement, 1mM sodium pyruvate, MEM Non-Essential Amino Acids, 100U/ml Penicillin-Streptomycin, R-spondin 1 (homebrew), WNT (homebrew), 0.5 μg/ml DMH1, 10% FCS) was used. For the GLP-1 secretion study, organoids were cultured in 24 wells plates as described in chapter 6. After four days, the 2D organoids were pre-incubated for one hour with glucose-free DMEM. Subsequently, the organoids were incubated with glucose-free DMEM supplemented with the test compounds at 37°C and 5% $CO_2$. After one hour, supernatants were collected and mixed with phenylmethanesulfonyl fluoride (Sigma-Aldrich, St. Louis, USA) to a final concentration of 100 μM for subsequent hormone measurements.

**Gut hormone measurements**

GLP-1 (active) levels were determined using ELISA (EGLP-35K, Merck Millipore, Darmstadt, Germany), according to the manufacturer's instructions. Standard curves were constructed using Prism 5 (GraphPad Software, La Jolla, USA). Concentrations of CCK-8 (CCK 26-33) were analyzed with an optimized and validated commercial RIA kit (EURIA-CCK, RB302, Euro-diagnostica, Malmö, Sweden). Radioactivity of [125I]-CCK-8 was measured by liquid scintillation counting using a gamma counter (EC&G, Breda, The Netherlands).

**Intracellular calcium ion measurements**

Intracellular [$Ca^{2+}$] ([$Ca^{2+}$]$_i$) fluctuations were measured using a Fura-2 calcium indicator or using STC-1 cells stably expressing the Cameleon YC3.6 calcium reporter protein (18). STC-1 cells were plated onto clear bottom black 96 wells plates (BD Biosciences, Erembodegem, Belgium). After one day, the cells were washed with HBSS and loaded with the acetoxymethyl(AM)-ester of Fura-2 (5μM, Life technologies, Carlsbad, USA) for 30 minutes at 37°C. After a subsequent wash with HBSS the plate was transferred to the incubation chamber of the BD pathway™ 855 (BD Biosciences, Erembodegem, Belgium), which was kept a 37°C and 5% $CO_2$. Measurements were performed with excitation at 340 and 380 nm for ratiometric analysis and images were acquired using 20x magnification. Cells were automatically identified as regions of interest and changes in 340/380 fluorescence emission ratio for these regions of interest were analyzed using BD Attovision software (BD Biosciences, Erembodegem, Belgium). Stimuli were applied in a fully automated manner, adding 20 μl of 10 times concentrated solution.

STC-1 cells stably expressing the Cameleon YC3.6 calcium reporter protein were seeded on a disposable 15×45 $mm^2$ borosilicate cover slides, coated with 100 μg/ml poly-L-Lysine (P6282, Sigma-Aldrich, St. Louis, USA) at a density of $3\times10^5$ cells/ml in DMEM supplemented with 5.5 mM glucose and grown in a $CO_2$ incubator at 37°C for 24h. Subsequently, the slides were mounted in a flow cell (Micronit Microfluidics B.V., Enschede, The Netherlands) (18). The cells were imaged with a Leica M205 FA Fluorescence Stereo Microscope, equipped with CFP and FRET emission filters and images were analyzed using ImageJ

software. Slides were given a constant flow of 100 µl/min and samples were injected into the flow cell by a 50 µL sample loop.

### RNA isolation and microarray hybridization and analysis

RNA was isolated by using TRIzol reagent (Life technologies, Bleiswijk, Netherlands) and further purified using the RNeasy mini kit (Qiagen Venlo, Netherlands) with on column DNase treatment (Qiagen, Venlo, Netherlands). RNA yield was measured with the Nanodrop ND-1000 spectrophotometer and the quality of RNA samples was verified with an Agilent 2100 Bio analyzer (Agilent Technologies, Amstelveen, Netherlands). One hundred nanogram of RNA was used for whole transcript cDNA synthesis (Affymetrix, inc., Santa Clara, USA). Hybridization, washing and scanning of Affymetrix GeneChip Mouse Gene 1.1 ST arrays was carried out according to standard Affymetrix protocols. Arrays were normalized using the Robust Multi-array Average method (19, 20). Probe sets were assigned to unique gene identifiers, in this case Entrez IDs. The probes on the Mouse Gene 1.1 ST arrays represent 21,213 Entrez IDs (21). Array data were analyzed using an in-house, on-line system (22). All data were filtered, and probe sets with expression values above 20 in at least 3 arrays were included in further analysis. In addition, a cut-off of $IQR>0.1$ was used to filter out genes that showed no variation between the samples. Intensity-Based Moderated T-statistics (IBMT) were used to assess significant differences with $p\text{-value} < 0.05$.

### Statistical analysis

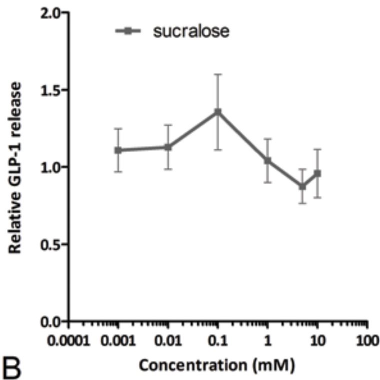
Results were plotted in Prism 5 (GraphPad Software, La Jolla, USA), and statistical analyses were conducted using a student's t-test or ANOVA with Dunnet's post hoc test to compare treatment against control or Tukey post hoc test to compare multiple treatments. Significant differences are indicated as * $p<0.05$; ** $p<0.01$; and *** $p<0.001$.

## Results

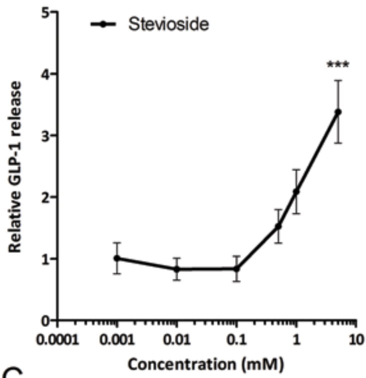
### Rebaudioside A and stevioside induce GLP-1 and CCK secretion from STC-1 cells

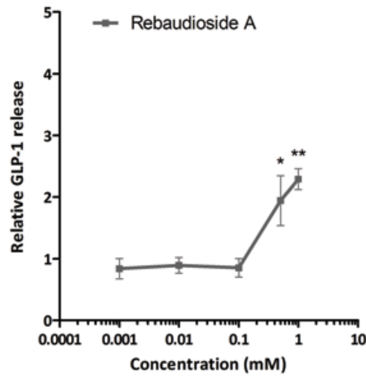
To investigate whether the low-caloric sweeteners sucralose, rebaudioside A and stevioside can induce gut hormone release from enteroendocrine cells, STC-1 cells were stimulated for 2 hours with these compounds. Sucralose did not induce any significant GLP-1 secretion upon stimulation within a concentration range of 0.001 mM to 10 mM (figure 5.1A). Stimulation with rebaudioside A as well as stevioside elicited a significant GLP-1 release from enteroendocrine STC-1 cells. Concentrations of 0.5 and 1 mM rebaudioside A induced a 1.9 and 2.3-fold increase in GLP-1 release, respectively. Stevioside (5mM) significantly induced a 3.38-fold increase in GLP-1 release (figure 5.1B). However, only rebaudioside A was able to induce CCK secretion in the STC-1 cells (figure 5.1). Interestingly, a significant increase for rebaudioside A-induced CCK release was already observed at a 5-fold lower concentration compared to rebaudioside-induced GLP-1 release, namely at 0.1 mM. Also, CCK release decreased when cells were stimulated with rebaudioside A at a concentration of 1mM while GLP-1 secretion still showed an increase at 1 mM. Stevioside did not induce any significant increase in CCK release. The compounds at their tested concentrations did not induce any cytotoxicity to the cells as determined by an LDH assay (figure S5.1).

A

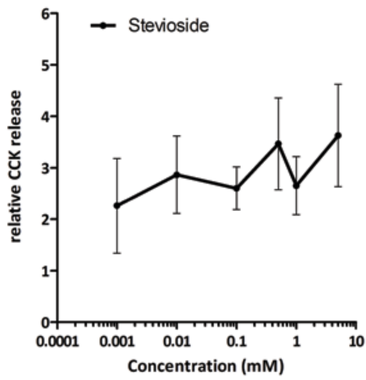


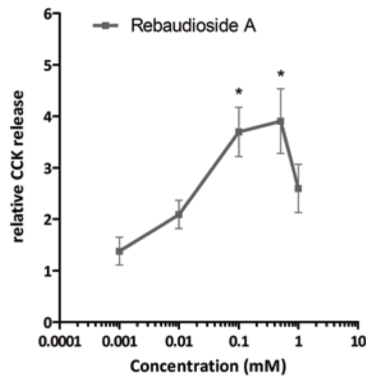

B




C



< **Figure 5.1: Gut hormone secretion by STC-1 cells in response to sweeteners.** A) GLP-1 release after 2 hours of incubation with HBSS supplemented with different concentrations of sucralose. Values indicate relative GLP-1 secretion compared to basal GLP-1 secretion following incubation with HBSS alone. B) GLP-1 release after 2 hours of incubation with HBSS supplemented with different concentrations of rebaudioside A and stevioside, relative to the basal GLP-1 secretion following incubation with HBSS alone. C) CCK release after 2 hours of incubation with HBSS supplemented with different concentrations of rebaudioside A and stevioside, relative to the basal GLP-1 secretion following incubation with HBSS alone. Values are means ± SEM, n=7 for sucralose, n=4 for Rebaudioside A and n=3 for stevioside. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

## Rebaudioside A induces GLP-1 release in ileal organoids

The effect of steviol glycosides on GLP-1 release was also investigated in ileal murine organoids. Using a two-dimensional organoid model, we showed that rebaudioside A induced a 3.0-fold and a 7.0-fold GLP-1 release at a concentration of 5 and 10mM, respectively.

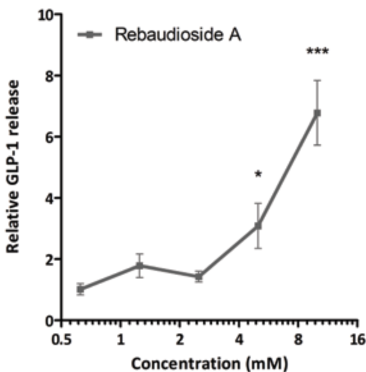



**Figure 5.2: Relative GLP-1 secretion by ileal organoids in response to rebaudioside A.** GLP-1 release after one hour of incubation with DMEM supplemented with different concentrations of Rebaudioside A, relative to the basal GLP-1 secretion following incubation with DMEM alone. Values are means ± SEM, 7 individual stimulations were performed, * $p<0.05$, *** $p<0.001$

5

### GLP-1 release induced by rebaudioside A involves cAMP signaling and not intracellular calcium signaling

To investigate intracellular mechanisms that underlie rebaudioside A-induced gut hormone release, the role of possible second messengers was investigated. Cytosolic [$Ca^{2+}$]fluctuations in STC-1 cells were measured using the [$Ca^{2+}$]-sensitive Fura-2 dye. Ionomycin as a control elicited an increase in $[Ca^{2+}]_i$ levels. However, upon stimulation with rebaudioside A (2.5 mM) no rise in $[Ca^{2+}]_i$ levels was observed. Subsequently, we investigated the role of cAMP in the rebaudioside A-induced GLP-1 release using MDL-12330A, an inhibitor of adenylate cyclase. Adding MDL-12300A to incubations with rebaudioside A resulted in a 77% reduction of GLP-1 release compared to rebaudioside A treatment alone, suggesting a cAMP-dependent signaling pathway in rebaudioside A-induced GLP-1 release in STC-1 cells.

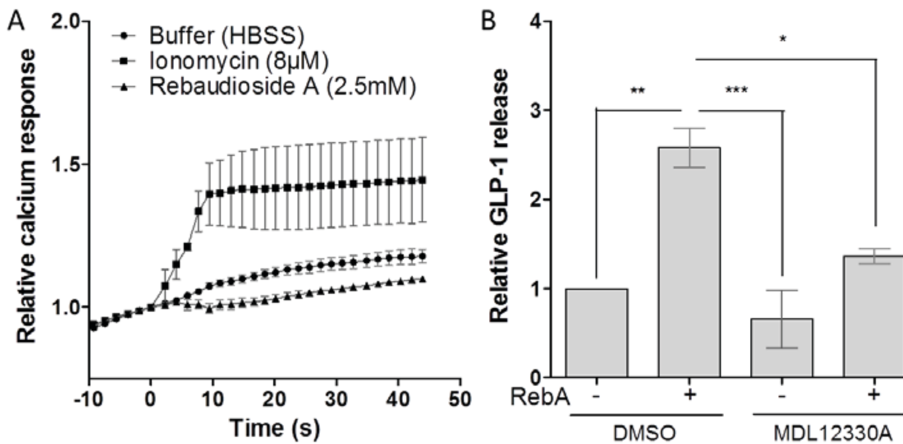
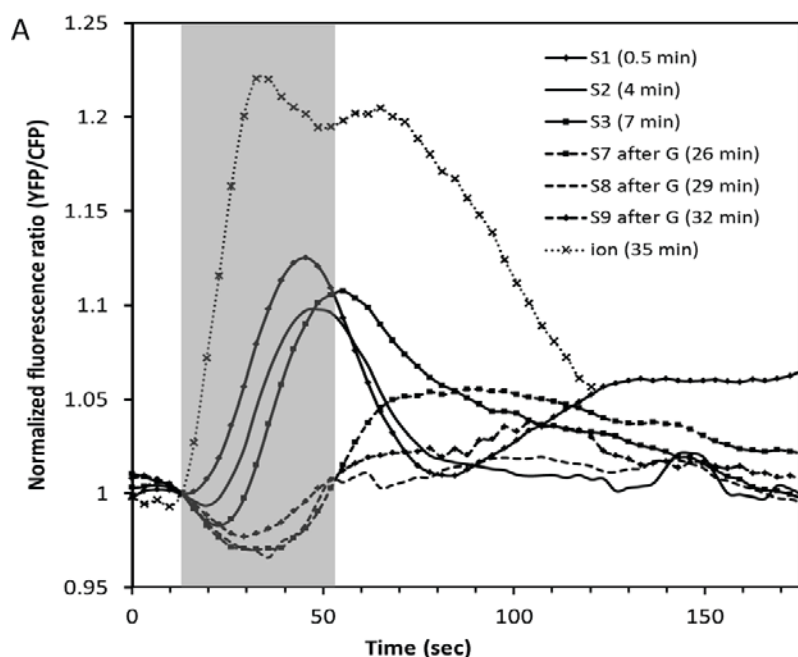
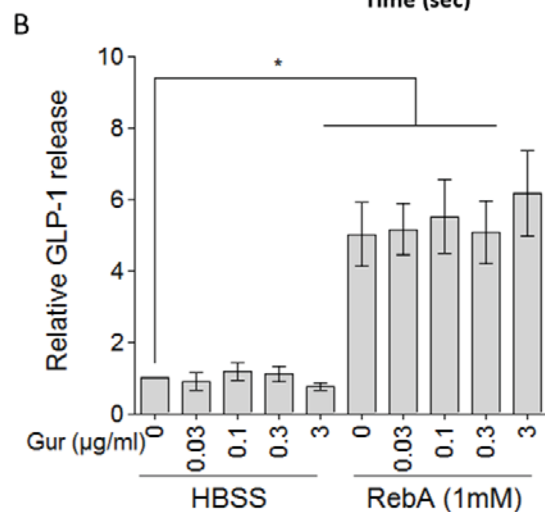


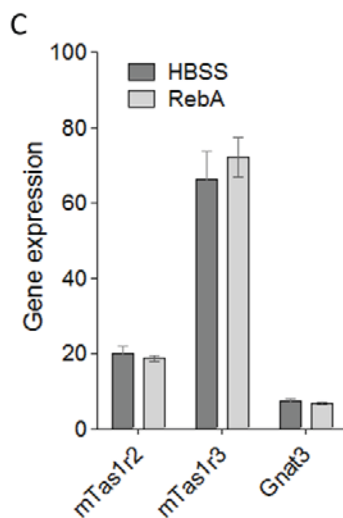

**Figure 5.3: Role of intracellular signaling in GLP-1 response to Rebaudioside A.**
A) Intracellular [Ca2+]changes in response to buffer, ionomycin and rebaudioside A were measured with Fura-2. Values represent the average 340/380nm ratio of about 40-60 regions of interest normalized to the time of injection. Values are expressed as means ± SEM, n=3. B) GLP-1 release after a 2 hour incubation with HBSS supplemented with Rebaudioside A and/or adenylate cyclase blocker MDL12330A. Values are means ± SEM, n=3. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Tukey multiple comparison).

**The sweet taste receptor is not involved in Rebaudioside A- induced GLP-1 release**

The murine sweet taste receptor antagonist gurmarin was used to investigate the role of the sweet taste receptor (*Tas1r2/Tas1r3*) in the rebaudioside A-induced GLP-1 release in STC-1 cells. Firstly, effects of gurmarin on sucrose-induced $[Ca^{2+}]_i$ levels were assessed in STC-1 cells stably expressing the Cameleon YC3.6 calcium reporter protein. Figure 5.4A shows that STC-1 cells respond to sucrose with a primary immediate $[Ca^{2+}]_i$ response and a delayed secondary response, around 20-50 seconds later. After the gurmarin injections only the secondary response is seen in response to sucrose, indicating that gurmarin blocks the primary $[Ca^{2+}]_i$ response to sucrose. Subsequently, a concentration series ranging from 0.03 to 3 mM gurmarin was used in combination with 1 mM rebaudioside A in order to investigate the involvement of the sweet taste receptor in rebaudioside A-induced GLP-1 secretion. However, at none of the concentrations tested gurmarin could reverse the rebaudioside A-induced GLP-1 release (figure 5.4B), suggesting that rebaudioside A does not mediate its effects via the sweet taste receptor. Furthermore, gene expression levels of *mTas1r2, mTas1r3* and *Gnat3* (α-gustducin) were assessed by microarray analysis in STC-1 cells. It was found that *mTas1r3* was expressed, while *mTas1r2* and particularly *Gnat3* were expressed around or below threshold levels (figure 5.4C).


A
Normalized fluorescence ratio (YFP/CFP)
Time (sec)
S1 (0.5 min)
S2 (4 min)
S3 (7 min)
S7 after G (26 min)
S8 after G (29 min)
S9 after G (32 min)
ion (35 min)


B
Relative GLP-1 release
Gur (µg/ml)
HBSS
RebA (1mM)


C
Gene expression
HBSS
RebA
mTas1r2
mTas1r3
Gnat3

**< Figure 5.4: Role of sweet taste receptor in Rebaudioside A-induced GLP-1 response.** A) Effect of gurmarin on sucrose-induced cytoplasmic calcium ion levels in STC-1 cells stably expressing the Cameleon YC3.6 calcium reporter protein. Shown are superimposed sucrose responses of STC-1 cells before and after application of the sweet receptor blocker gurmarin. Cells expressing Cameleon YC3.6 were exposed to 100 mM sucrose (S1-S9) for 42 seconds every 3 minutes before and after a series of gurmarin injections (2 times 0.3 μM gurmarin, followed by 3 times 0.3 μM gurmarin plus 100 mM sucrose, followed by 3 μM gurmarin all in a period of 18 minutes, figure S5.2). The ionomycin (ion) injection (10 μM) at the end demonstrates cells were not at their [$Ca^{2+}$] maximum yet. Injections at t=0 reached cells ca 16.25 seconds later (normalization point =1, greyed time zone). Shown are the global averages of all imaged cells (1315 ROIs). A moving average (15 seconds, 5 values) was used to suppress effects of high frequency calcium oscillations (~3-6 sec, ~0.1 amplitude) and cell synchronization. B) GLP-1 release after 30 minutes pre-incubation with HBSS supplemented with gurmarin and subsequent addition of rebaudioside A (1mM) for 2 hours. Values represent relative GLP-1 release compared to the basal GLP-1 secretion following incubation with HBSS alone. Values are means ± SEM, n=3, * $p<0.05$ (Tukey multiple comparison). C) Gene expression levels (Robust Multi-array Average normalized intensity) assessed by microarray analysis of the sweet taste receptor (Tas1R2/Tas1R3) and α-Gustducin (Gnat3) after incubation with HBSS or rebaudioside A (1mM) for 2 hours.

5

## Discussion

Currently, steviol glycosides are increasingly used in food products. However, knowledge on molecular mechanisms underlying steviol glycoside-associated effects is still limited. Recently, we reported that the steviol glycoside rebaudioside A can elicit GLP-1 and PYY secretion in porcine *ex vivo* intestinal segments (16). Here, we show that steviol glycosides, in particular rebaudioside A, are able to enhance the release of GLP-1 and CCK in murine enteroendocrine STC-1 cells. The GLP-1 response evoked by rebaudioside A was also present in two dimensional ileum-derived organoids. Furthermore, with this study we aimed to gain more insight in the sensing mechanisms responsible for the effects of rebaudioside A on intestinal GLP-1 release. Data indicate that rebaudioside A-induced GLP-1 release occurs independently of the sweet taste receptor (*Tas1r2/Tas1r3*) and suggest that the underlying pathways involve signaling by the second messenger cAMP.

Steviol glycosides were shown to be potent ligands for the human sweet taste receptor (4), and in mice the sweet taste receptor (Tas1R3) was found to be essential for the detection of stevia taste (23). It can be estimated that the effective concentrations of steviol glycosides in the two murine *in vitro* models used in our study are in the same order of magnitude as those reached by intake of 0.3 gram of rebaudioside A in a cup of coffee (16). The EFSA has established an acceptable daily intake level of 4 mg/kg BW/day. The concentrations used in our study seem therefore to lie within the range of normal consumption. Interestingly, rebaudioside A also evoked a significant CCK release in STC-1 cells, and the effective concentration was slightly lower for CCK than for GLP-1. Furthermore, we did not find a significant effect of stevioside on CCK release, whereas in the same experiments a clear effect on GLP-1 was shown. CCK has been found to affect the release of digestive enzymes and bile acids, it plays a role in the control of food intake, and inhibits gastric emptying (24, 25). Contrary to GLP-1, CCK is known to be most prominently expressed in the proximal small intestine which was also confirmed in our earlier studies (14).
In view of the still disputed roles played by the sweet taste receptor in intestinal nutrient sensing, we aimed to unravel the involvement of this receptor in steviol

glycoside-induced hormone secretion. The sweet taste receptor is known to be expressed in many extra-oral tissues like the pancreas and the gastrointestinal tract. In the intestinal tract the receptor was found to be co-expressed with GLP-1 and GIP positive cells (26, 27). Several cell-based studies provide evidence for a role of the sweet taste receptor in mediating gut hormone release. For example, Jang et al. showed that GLP-1 secretion in response to sucralose in colon-derived NCI-H716 cells was reduced by the sweet taste receptor inhibitor lactisole and these authors also found that this process was dependent on signaling via α-gustducin (26). Also, in GLUTag cells sucralose-stimulated GLP-1- and GIP release were inhibited by gurmarin (28). However, others found no evidence for the involvement of the sweet taste receptor in GLP-1 release, and showed that glucose-induced GLP-1 release was mediated via SGLT-1-dependent transport and inhibition of ATP dependent potassium channels. Additionally, in small intestinal primary cells or perfused tissue no effect of sucralose on GLP-1 release was observed (29-32).
The data presented here suggest that the sweet taste receptor is not involved in rebaudioside A-induced GLP-1 release. Gurmarin, a specific sweet taste receptor inhibitor in mice (17) was able to inhibit the primary rise in intracellular [$Ca^{2+}$] evoked by sucrose but did not reverse GLP-1 release induced by rebaudioside. Moreover, gene expression of both *Tas1r2*, one of the sweet taste receptor heterodimers, and α-gustducin, the sweet taste receptor G-protein were very low in the STC-1 cells used. In the present study also no effects of sucralose on GLP-1 release were observed, which is in line with the above mentioned reports performed in small intestinal primary cells or perfused tissue (29-32). Altogether, this points to the conclusion that other pathways are mediating rebaudioside A-induced GLP-1 responses in STC-1 cells.

We provide evidence that the GLP-1 release induced by rebaudioside A is dependent on cAMP signaling. The compound MDL-12330A in STC-1 cells reduces cAMP production and has been shown to block cAMP-dependent GLP-1 release (33-35). Also in our hands it reduced GLP-1 secretion elicited by rebaudioside A. Cyclic AMP has been shown to trigger GLP-1 release (36),

suggesting that rebaudioside A by changing cellular cAMP levels might also induce GLP-1 release. We did not observe any changes in intracellular $[Ca^{2+}]$ levels after addition of rebaudioside A to the cells using the $[Ca^{2+}]$sensitive Fura-2 dye (figure 5.3b), nor in the STC-1 cells stably expressing the Cameleon YC3.6 calcium reporter protein (not shown). The absence of a rise in $[Ca^{2+}]_i$ in combination with an increase in cAMP in intestinal cells was reported previously (37). A potential explanation for the absence of increased $[Ca^{2+}]$ levels might be the oscillatory behavior of the cells. Cyclic AMP induces the opening of ion channels, subsequently leading to increased $[Ca^{2+}]_i$. However, this cAMP dependent increase in $[Ca^{2+}]_i$ is regulated by a local increase in $[Ca^{2+}]_i$ through a feedback mechanism (38, 39). This process might lead to high frequency calcium oscillations which may cause signals to remain undetected when measuring large ensembles of cells. We have observed continuous oscillatory behavior in single STC-1 cells (data not shown).

Apart from their sweetness, steviol glycosides are also known for their bitter taste. Steviol glycosides bind and activate two distinct human bitter taste receptors, hTas2R4 and hTas2r14 (4). Many bitter receptors are expressed along the gastrointestinal tract (40) and their activation has been related to GLP-1 and CCK secretion (41-44). Our preliminary data obtained in collaboration with the laboratory of Meyerhof (DIfE, Potsdam, Germany) indeed showed that rebaudioside A can activate the mouse homologue of hTas2R4, namely mTas2R108. Furthermore, agonists of this receptor could induce GLP-1 release in STC-1 cells, and mTas2R108 was found to be expressed in STC-1 cells (data not shown). These preliminary data indicate that the Tas2R108 receptor might be involved in rebaudioside A-induced gut hormone release. However, additional studies are needed to definitely prove the involvement of Tas2R108 or other receptors in steviol glycoside-mediated incretin release.

In conclusion, rebaudioside A significantly enhanced the release of GLP-1 and CCK *in vitro*. The stimulation of GLP-1 secretion seems to involve signaling by the second messenger cAMP via a sensing mechanism independent of the sweet taste receptor. The bitter receptor Tas2R108 might be a likely candidate receptor involved in steviol glycosides-related gut hormone release. However, future studies are needed to elucidate depict the responsible receptor(s) and

underlying mechanism(s) mediating rebaudioside A-induced gut hormone release. Given the fact that GLP-1 has satiety and incretin effects, these findings are of interest for the development of novel strategies in the treatment of obesity and type II diabetes.

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## Supplemental data

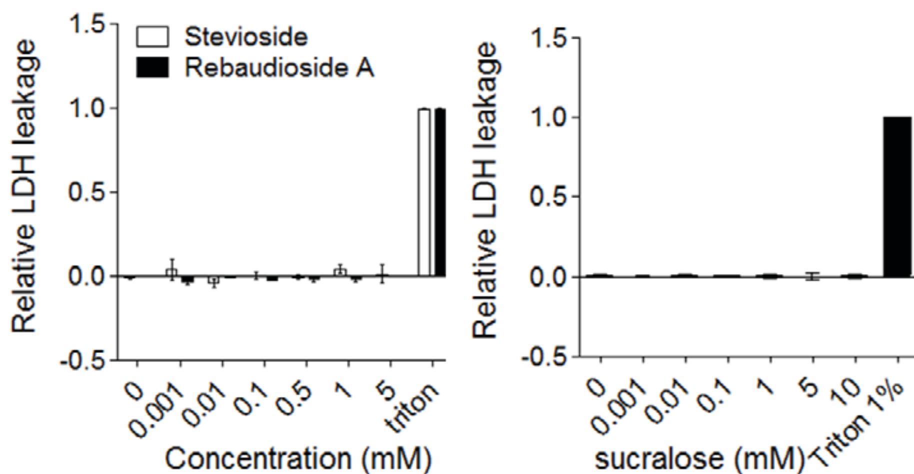


**Figure S5.1: LDH leakage by STC-1 cells.** Relative LDH leakage is calculated with the OD value of HBSS treated (negative control) cells and triton treated cells (positive control). Values are means ± SEM, n=2 for Rebaudioside A and n=3 for stevioside, n=4 for sucralose.

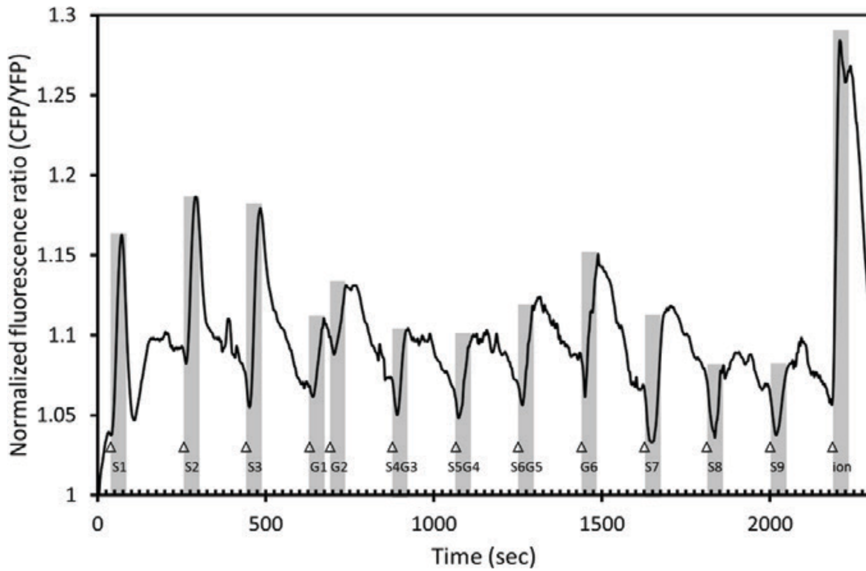


**Figure S5.2: Intracellular [Ca2+] changes of the series of injections of sucrose (S), gurmarin (G) and the combinations of both.** Effect of gurmarin on sucrose-induced calcium responses in STC-1 cells stably expressing the Cameleon YC3.6 calcium reporter protein. Shown are the global averages of all imaged cells (1315 ROIs). A moving average (16.25 seconds, 5 values) was used to suppress effects of high frequency calcium oscillations (~3-6 sec, ~0.1 amplitude) and cell synchronization.

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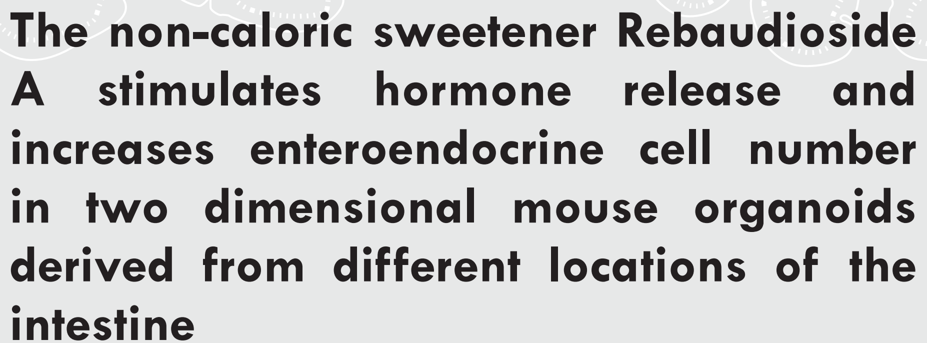

# The non-caloric sweetener Rebaudioside A stimulates hormone release and increases enteroendocrine cell number in two dimensional mouse organoids derived from different locations of the intestine

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Submitted.

## Abstract

The gut hormone GLP-1 contributes to satiety and has a pivotal role in insulin secretion and glucose homeostasis. Modulation of GLP-1 release has been implicated as a potential strategy for the treatment of obesity and type II diabetes. Here, we investigated the effects of stevia-derived rebaudioside A on the development of enteroendocrine cells and their secretion of GLP-1 and other gut hormones. To this end, we developed a two dimensional (2D) organoid model derived from mouse duodenal, jejunal and ileal tissues. The 2D organoids contained crypt cells and differentiated villus cells such as enterocytes, goblet- and enteroendocrine cells and maintained their location-specific gene expression patterns. The non-caloric sweetener rebaudioside A induced the secretion of GLP-1 and PYY in a location-specific manner and chronic stimulation with this sweetener induced the expression of enteroendocrine-specific markers. These results might give rise to potentially interesting dietary application of rebaudioside A for type 2 diabetic patients.

## Introduction

Cells along the intestinal tract secrete different peptide hormones with autocrine, paracrine and endocrine functions. Their release is time- and location specific and amongst others modulated by the presence of food and bacterial activity. Given their role in food-intake regulation and incretin effects, several of these peptides, including cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), are considered potential targets for weight management or (and) type II diabetes (1, 2).

The incretin GLP-1 is released by enteroendocrine cells (EECs), and signals in an endocrine manner via the circulation, and in a paracrine manner via receptors located on sensory nerve endings. To study its secretion and interactions with other hormones and intestinal cells in more detail, organoids provide useful models. These self-sustained mini guts, grown from adult Lgr5 positive stem cells, show good resemblance with the *in vivo* architecture and contain all different epithelial cell types (3, 4). This includes EECs, which generally comprise about 1% of the intestinal epithelium (5). Previous studies by Petersen et al. showed that the relative number and secretory activity of GLP-1 positive EECs in organoids is enhanced by short chain fatty acids and pharmacologic agents (6, 7).

So-called non- or low-caloric sweeteners are increasingly used in foods and beverages, however their role in gut hormone release remains controversial. Recently, stevia-derived glycosides have been approved in the European Union and the United States. One of the most commonly used steviol glycosides is rebaudioside A, which is known to activate the sweet taste receptor, a heterodimer of Tas1R2 and Tas1R3, and two bitter receptors, Tas2R4 and Tas2R14 (8). Upon ingestion, rebaudioside A remains intact throughout the small intestinal tract (9), enabling interactions with receptors on EECs (10, 11). Recently, we have demonstrated that rebaudioside A augments GLP-1 and PYY release from isolated porcine intestinal segments (12).

Here, we aimed to further investigate the effects of rebaudioside A on the number of EECs and their secretion of GLP-1 and other gut hormones, using intestinal organoids derived from different locations of the mouse small

intestine. For that purpose, we developed and used a novel two dimensional organoid model which contains crypt cells and the differentiated villus cells such as enterocytes, goblet cells and EECs.

## Materials and methods

### Organoid isolation and culturing

The use of mouse intestine had been approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University (Permit number: 2014036.b). Intestinal tissue was collected from six-month old C57BL/6J mice. The small intestine, from pylorus to ileocecal junction, was divided into three equal parts, further referred to as duodenum, jejunum and ileum. These intestinal segments were opened and thoroughly rinsed with PBS containing calcium and magnesium. The tissue was digested twice for 15 minutes with 0.35mg/ml Collagenase XI (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) in Dulbecco's Modified Eagle Medium (DMEM)(Lonza, Verviers SPRL, Belgium). The digests were centrifuged at 100RCF for 3 minutes and resuspended in DMEM. Subsequently, the crypts were plated in growth factor reduced Matrigel basal membrane matrix (Corning Incorporated, Corning, USA) with organoid medium (DMEM/F12 medium containing GlutaMAX supplement, 1mM sodium pyruvate, MEM Non-Essential Amino Acids, 100U/ml Penicillin-Streptomycin, R-spondin 1 (homebrew), WNT (homebrew), 0.5μg/ml DMH1) to stimulate organoid formation. These organoids were passaged once a week in a 1:4 ratio.

For the secretion assays, immunofluorescent stainings and qPCR analysis, these 3D organoids were plated to obtain 2D structures by growing them onto Matrigel pre-coated wells. For this 24 wells plates were first coated with a thin layer of two times diluted Matrigel. After polymerization of the Matrigel, a suspension of about 40 crypts in organoid medium supplemented with 10% FCS was added to the wells. These organoids were grown for four days at 37°C and 5% $CO_2$.

**Organoid secretion assay**

Four days after 2D organoid plating, the wells were washed twice with DMEM without glucose, and subsequently incubated for 1 hour with DMEM without glucose. Subsequently, the organoids were incubated with DMEM without glucose, supplemented with rebaudioside A (Sigma-Aldrich Chemie ,Zwijndrecht, The Netherlands) at 37°C and 5% $CO_2$. After one hour, supernatants were collected and mixed with phenylmethanesulfonyl fluoride (Sigma-Aldrich Chemie ,Zwijndrecht, The Netherlands) to a final concentration of 100 μM.

**Gut hormone measurement**

Concentrations of active amylin, total gastric inhibitory polypeptide (GIP), active ghrelin, active GLP-1, insulin, leptin, pancreatic polypeptide and total PYY were simultaneously quantified using the Milliplex MAP mouse gut hormone magnetic bead panel (Millipore, MGTMAG-78K) on a Luminex MAGPIX with xPonent software. Concentrations of CCK-8 (CCK 26-33) were analyzed with an optimized and validated commercial RIA kit (Euro-diagnostica, Malmö, Sweden). Due to the volume needed for the analysis, supernatants of three experiments were pooled. Radioactivity of [125I]-CCK-8 was measured by liquid scintillation counting using a gamma counter (EC&G, Breda, The Netherlands).

**Immunofluorescent staining**

Cultured organoids were fixed with 4% formaldehyde and permeabilized with 0.5% Triton-X100. These organoids were incubated with anti-chromogranin A antibody (Abcam, Cambridge, UK), anti-GLP-1 (7-36) antibody (Phoenix Pharmaceuticals, Karlsruhe, Germany), anti-PYY antibody (Abcam, Cambridge, UK), anti-serotonin antibody (Merck Millipore, Darmstadt, Germany), anti-MUC2 antibody (Abcam, Cambridge, UK), Anti-TROY antibody (abcam, Cambridge, UK) or FITC labeled anti-CD24 antibody (BD Biosciences, Erembodegem, Belgium) for 2 hours at 37°C. Alexa Fluor 488 goat anti-rabbit (Life Technologies, Bleiswijk, The Netherlands) and Alexa Fluor 488 donkey anti-rat (Life Technologies, Bleiswijk, The Netherlands) were used as secondary antibodies. Actin and nuclei were stained using rhodamine

phalloidin (Life Technologies, Bleiswijk, The Netherlands) and 4',6-diamidino-2-fenylindool (DAPI) (Life Technologies, Bleiswijk, The Netherlands) or Hoechst 33342 (Sigma-Aldrich, Zwijndrecht, The Netherlands). Images were acquired on a fluorescence Olympus IX71microscope using cellSens software (Olympus Corporation, Tokyo, Japan).

### Quantitative PCR and Microarray analysis

RNA was isolated and purified with Quick-RNA MiniPrep kits (Zymoresearch, Irvine, USA). cDNA was synthesized using an iScript cDNA synthesis kit (Bio-rad Laboratories, Hercules, USA). The qPCR reactions were performed on the CFX384™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, USA) using SensiMix SYBR No-ROX kits (Bioline, London, UK). The primer sequences are specified in table 6.1. To compare the location-specific expressions in mice and the organoids, an extensive dataset of ten regions of the small intestine sampled in vivo was used (13).

**Table 6.1: Primer sequences used for qPCR analysis.**

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *Chga* | CAGGGACACTATGGAGAAGAGA | GGTGATTGGGTATTGGTGGCT |
| *Gcg* | AGGGACCTTTACCAGTGATGT | AATGGCGACTTCTTCTGGGAA |
| *Pyy* | ACGGTCGCAATGCTGCTAAT | GACATCTCTTTTTCCATACCGCT |
| *Cck* | AGCGCGATACATCCAGCAG | ACGATGGGTATTCGTAGTCCTC |
| *Hprt* | GAGAGCFTTGGGCTTACCTC | ATCGCTAATCACGACGCTGG |
| *Gpr120* | AGGCGGCACTGCTGGCTTTC | GCGGGACCACGCGGAACAAG |
| *Slc2a2* | CATCAGCCAGCCTGTGTATGC | CATGCCAATCATCCCGGTTAGG |
| *Slc30a2* | AACTGCCAGGCGTGCCAGGG | CCGTGGAGTGGTCCAGGCTGTG |
| *Apoa4* | CAACAGGCTGAAGGCTACGAT | CGATTTTTGCGGAGACCTTGG |
| *Lyz2* | ATGGAATGGCTGGCTACTATGG | ACCAGTATCGGCTATTGATCTGA |
| *Lgr5* | CCTACTCGAAGACTTACCCAGT | GCATTGGGGTGAATGATAGCA |
| *Vil1* | TACCTCAAGACCCACCCTGGAA | AAAGCCCTTGAAGGCAGGGTAG |
| *Muc2* | AGGGCTCGGAACTCCAGAAA | CCAGGGAATCGGTAGACATCG |

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### Statistical analysis

All qPCR gene expression data was first normalized to reference gene HPRT, subsequently plotted relative to control. The effect of rebaudioside A treatment on hormone release and gene expression was tested using Mann-Whitney test, significant differences are indicated as * $p<0.05$; ** $p<0.01$; *** $p<0.001$. All graphs show means with SEM.

## Results

### Two dimensional organoids harbor different epithelial cell types

Two-dimensional organoid structures were obtained by plating 3D organoids derived from isolated small intestinal crypts of the duodenum, jejunum and ileum in wells coated with two times diluted Matrigel. Matrigel appeared to function as basal lamina for the epithelial organoids. Upon contact with the Matrigel layer and addition of FCS, different epithelial cell types started to grow from the crypts. Intact crypts and associated cells were sustained in the presence of R-spondin 1 and BMP4 inhibitor noggin. This way, the luminal side of the 2D cultured organoids is easily accessible for test compounds such as nutrients and sweeteners.

The 2D differentiated organoids were characterized using immunofluorescent staining and gene expression analysis. From each crypt, a large villus domain developed (figure 6.1, 2D overview). The villus domain mostly consisted of absorptive and secretory cells. Next to these the presence of enterocytes and goblet cells was demonstrated using villin and muc2 antibodies (figure 6.1, Muc2 and Villin). The expression of *Lgr5* and *Lyz2* in the 2D organoids was compared with that in the 3D model and demonstrated the presence of intact crypts (figure S6.1). The 2D grown organoids also contained enteroendocrine cells (EECs), which were identified by the use of several EEC markers among which chromogranin A (14). Different types of EEC were identified by staining for GLP-1, PYY and serotonin positive cells. Figure 2 shows 2D cultured organoids which express the typical L-cell hormones GLP-1 and PYY, and enterochromaffin cells which contain serotonin.

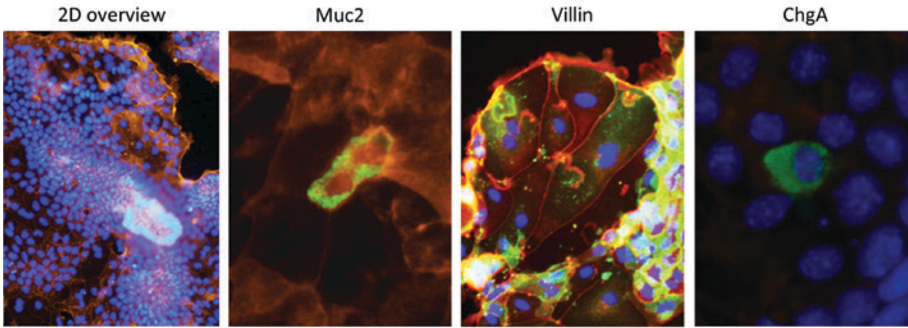


**Figure 6.1: 2D cultured organoids from the small intestine.** A) Overview showing actin and nuclei staining of whole 2D cultured organoids. Identification of enterocytes, goblet cells and enteroendocrine cell using villin, muc2 and chromogranin A staining, respectively. Magnification used were 60x,60x, 40x and 10x, from right to left.

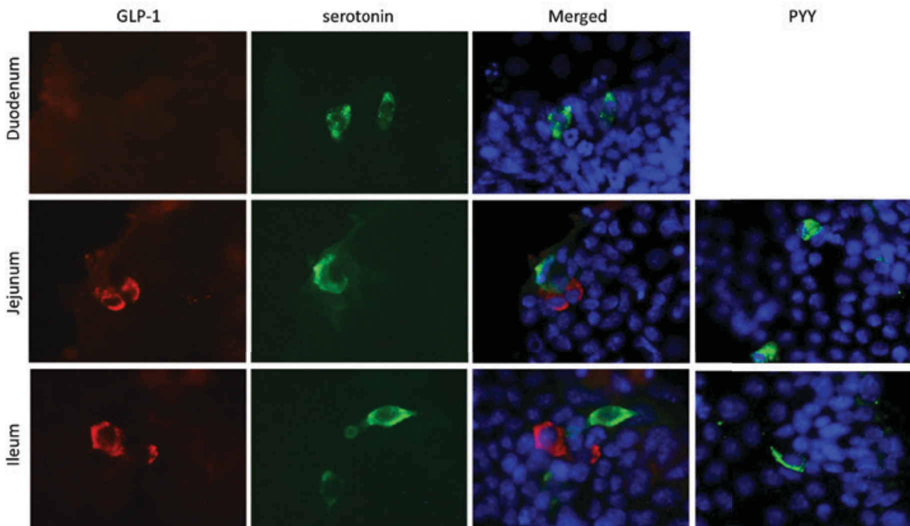


**Figure 6.2: Immunostaining of 2D cultured organoids derived from duodenum, jejunum and ileum.** Identification of GLP-1, PYY and serotonin positive cells using specific antibodies. Merged picture showed co-staining of GLP-1 and serotonin.

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**Intestinal location-specific expression of PYY and GLP-1 is conserved *in vitro* and *in vivo***

The three small intestinal locations, duodenum, jejunum and ileum, are characterized by their specific gene expression profiles. To establish whether organoids originating from different locations along the intestine retained their location-specific gene expression profiles, these were assessed and compared with those *in vivo*.

In line with our immunofluorescent staining data, expression of *Gcg*, encoding GLP-1, was very low in the duodenum and increased along the intestinal tract. This was in concordance with the Gcg expression in murine intestine (figure 6.3). *Pyy* and *Gpr120* were highly expressed in the ileum, which was also found in the mouse intestinal samples. *Slc2a2*, a duodenum specific villus gene which encodes for the glucose transporter GLUT2 (15), showed a similar expression profile along the small intestinal axis in both mice as our 2D organoids. A comparable parallelism was seen for *Slc30a2*, a zinc transporter, and *Apoa4*, an apolipoprotein, although variation in the organoids was relatively large.

**Rebaudioside A augments gut hormone secretion in a location-specific release pattern**

To investigate the effects of Rebaudioside A on gut hormone release in organoids from different intestinal locations, we determined the secretion of GLP-1, PYY and CCK after a 1 hour rebaudioside A exposure in serum- and glucose-free medium. We established that region-specific 2D cultured organoids remained functional with respect to hormone secretion. We measured high levels of basal GLP-1 release in ileum of 350.5±109.0 pg/ml (mean ± SEM), whereas GLP-1 release was relatively low in duodenum- (132.2±18.2 pg/ml) and jejunum-derived (173.3±9.6 pg/ml) organoids. The relative induction of GLP-1 release by rebaudioside A was also highest in ileum, being a factor 4 higher compared to medium (figure 6.4). Ileum-derived organoids released the highest amounts of PYY (9.98±1.90 pg/ml). Jejunal basal secretion of PYY was 8.70±1.29 pg/ml and undetectable in the medium of duodenum-derived organoids. For PYY, the relative induction by rebaudioside A was highest in ileum showing a 3-fold increase. The basal release of CCK was relatively low in the duodenum-

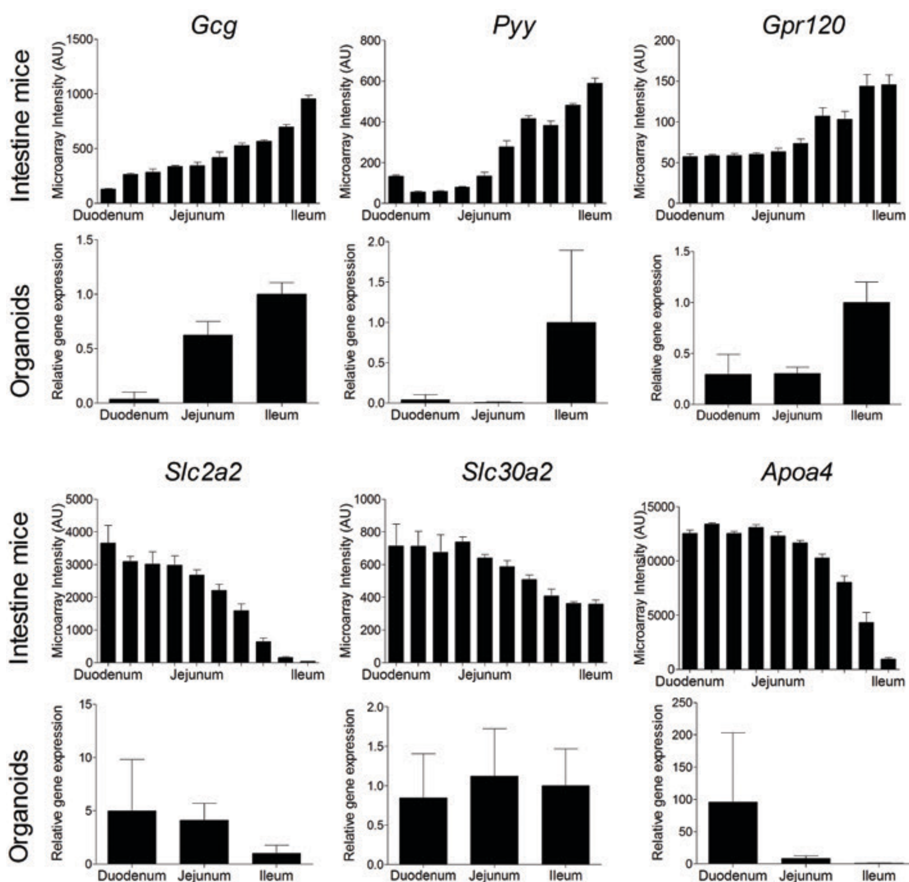


**Figure 6.3: Gene expression of location-specific genes in the murine intestine and 2D organoid cultures.** Murine intestinal gene expression data were obtained from a deposited data set previously generated in our lab (13). Microarray intensity was shown for the intestinal expression of 4 mice fed a chow diet. Organoid data show relative expression corrected for the reference gene HPRT and were determined using qPCR analysis. RNA was isolated from 2D cultured organoids (N=6 wells), it was shown relative to the ileal gene expression. Specific locations were depicted on the x-axis of the graphs. All graphs show mean and SEM.

derived organoids (0.73±0.36 pM) and higher in jejunum- (1.37±0.60 pM) and ileum-derived organoids (2.15±1.35 pM). The release of CCK was not significantly changed by rebaudioside A exposure, although a trend for increased CCK release could be observed.

**Rebaudioside A increases enteroendocrine cell number in ileal-derived 2D organoids**

To examine the effect of prolonged rebaudioside A stimulation, ileal organoids were incubated for 18h with this compound at 10mM. Next, qPCR analyses were performed for *Chga*, *Gcg*, *Pyy* and *Cck*. Increased mRNA expression was observed for all these EEC markers (figure 6.5A). The increase induced by rebaudioside A compared to medium for *Chga*, *Gcg*, *Pyy* and *Cck* was 3.5, 3.5, 5.2 and 6.2 fold, respectively. Furthermore, the number of chromogranin A positively stained cells increased by prolonged rebaudioside A treatment (figure 6.5B).

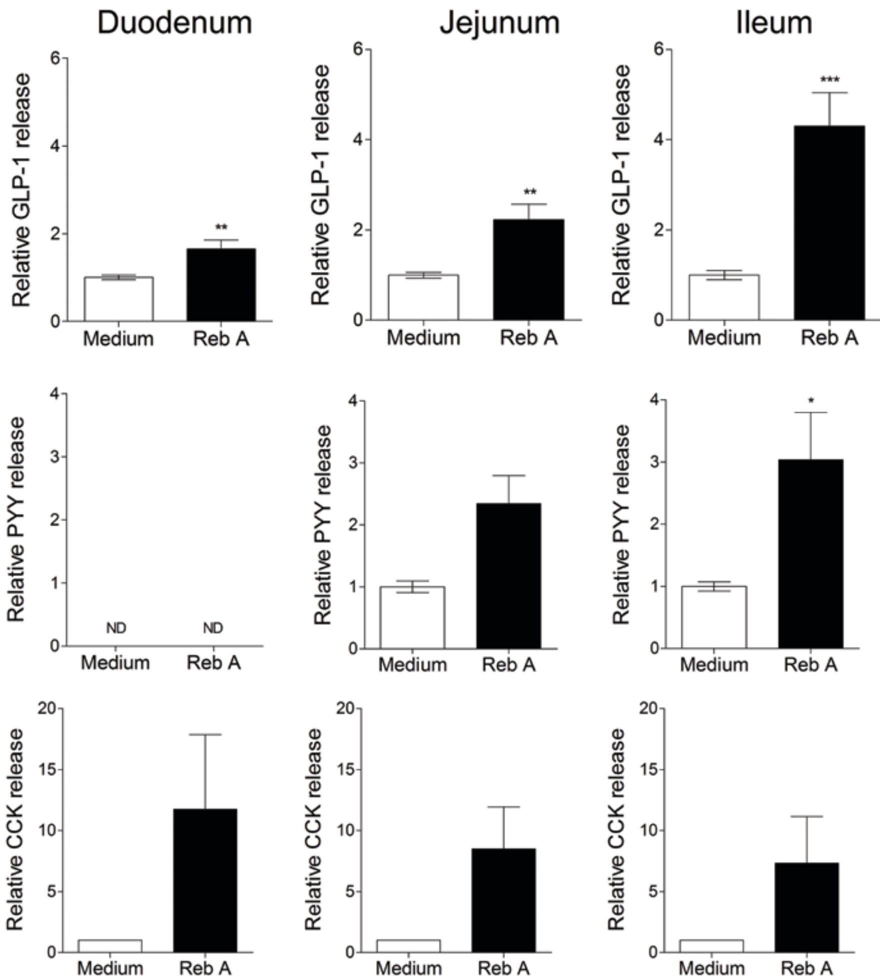


**Figure 6.4: Rebaudioside A-induced gut hormone (GLP-1, PYY and CCK) release in organoids originating from three different locations.** Organoids were exposed to Rebaudioside A (10mM) or control medium for 1 hour. From top to bottom: GLP-1 release, PYY release and CCK release. For GLP-1 and PYY 9 individual stimulations were performed, N=9. For CCK release 9 individual stimulations were performed and pooled per 3 experiments, N=3. Graphs show mean and SEM, statistical differences are tested with Mann-Whitney test, * $p<0.05$; ** $p<0.01$, *** $p<0.001$

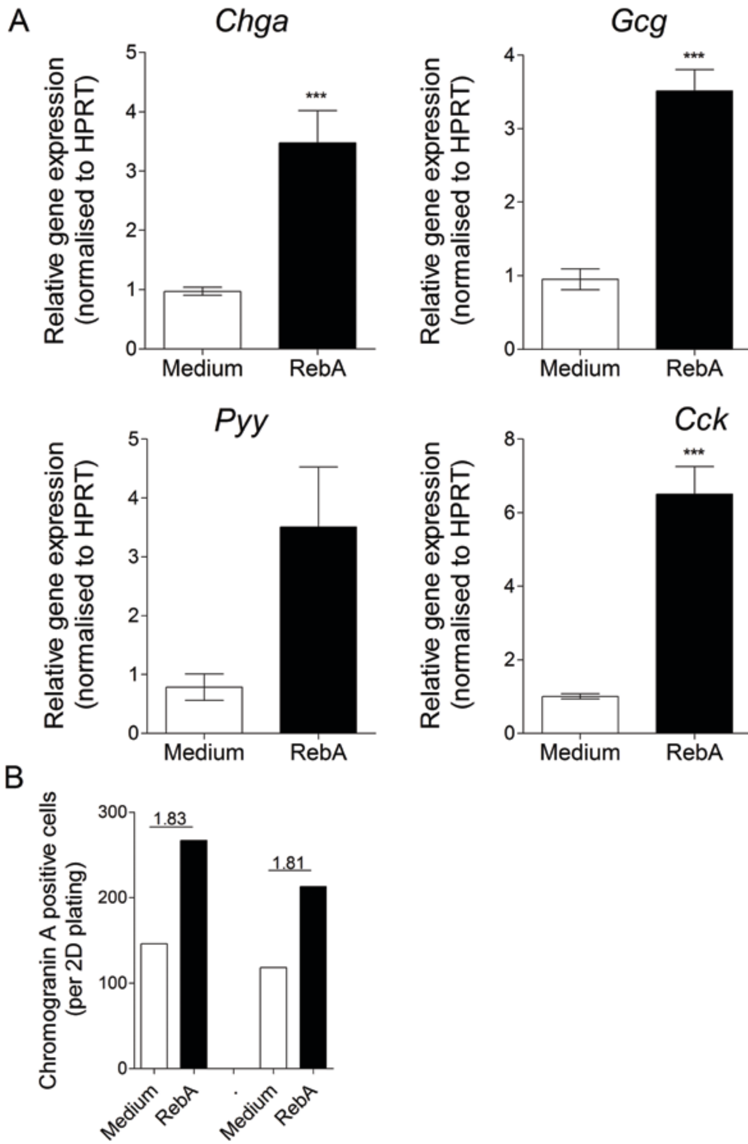


**Figure 6.5: Increased expression of specific enteroendocrine markers in ileal organoids after overnight culture with rebaudioside A (10mM).** A) Graphs show relative gene expression determined using qPCR analysis . Organoid RNA was isolated from three independent experiments, it was normalized to reference gene HPRT and data was shown relative to medium incubation. All graphs show mean and SEM, significant differences of $p<0.001$ were indicated with ***. B) Amount of chromogranin A positive cells per well in 2D plated ileal organoids. Graphs show induction in two individual experiments, the relative change compared to medium was indicated at the top of each experiment.

## Discussion

Results of the present study add to our knowledge on the effects of the steviol glycoside Rebaudioside A in the intestinal tract, focusing on GLP-1 secretion and EEC differentiation. Rebaudioside A and other preparations from the S*tevia rebaudiana* plant are increasingly used in foods and beverages as non-caloric sweetener. For our study we established a new two-dimensional (2D) organoid model from mouse intestinal stem cells, highly suitable to explore effects of (dietary-) compounds on gut hormone release. We characterized this 2D organoid model by visualizing the presence of the different epithelial cell types. Furthermore, we showed that the organoids derived from different segments maintained their regional-specific gene expression patterns. When exposed to rebaudioside A the secretion of GLP-1 and PYY were stimulated in a location-specific manner and chronic stimulation with this sweetener increased the number of enteroendocrine cells.

Our 2D system has been optimized for direct apical stimulation of intestinal organoids without disturbance of the organoids by pipetting. In the 3D culturing system as it is commonly used thus far, the villus space is shared among multiple crypts (3). In contrast, our 2D system showed large villus-like areas, which grew from each individual crypt. As isolated villus cells from mice were shown to contain higher glucagon expression compared to the crypt cells (6), a larger villus area could be beneficial for determining GLP-1 release. Previously, other 2D intestinal models have been developed for the study of host-pathogen interaction. However, these are likely to show more complete differentiation of the crypt cells as these organoids were cultured without R-spondin 1 and Noggin, typical stem cell niche factors, for three consecutive days (16). Furthermore, a human 2D intestinal cell model was shown to have only rare Paneth cell expression (17). As these two models do not give rise to all epithelial lineages typically found in the intestine, they are less representative for studying intestinal physiology compared to our 2D model, in which all typical intestinal epithelial cell types are present. Another model similar to ours, but more laborious is the 2D culture of air-liquid interface differentiated intestinal stem cells (18). Altogether, our 2D model contains all intestinal cell types, and

because of the presence of stem and progenitor cells differentiation can be studied. Moreover, the mucus membrane is present which is an essential barrier in the intestine. Additionally, our 2D model provides direct apical stimulation. For the development of intestinal organoids we have explicitly discriminated between different intestinal regions by culturing duodenal, jejunal and ileal organoids. Furthermore, we have shown that the location specific expression of *Gcg, Pyy, Gpr120, Slc2a2, Slc30a2* and *ApoA4 in vivo* remains present in cultured organoids, indicating that these organoids keep their regional characteristics. Our results are in agreement with those of Middendorp et al., who showed the similarities between intestinal gene expression and organoid gene expression on whole transcriptome level (15). Furthermore, stem cells from different regions showed region specific signatures on gene expression and their air-liquid interface differentiated cells show even difference in morphology (18). These results indicated that stem cells are intrinsically programmed with location-specific properties. However, this applies not for all genes, for example the expression of *Cck* in our organoids did not overlap with *in vivo* expression. This was in concordance with raw gene expression data of Middendorp et al (15).

Our data showed that the sweetener rebaudioside A augments the release of the incretin GLP-1 in all three organoid-derived intestinal locations. Ileum-derived organoids generated the highest relative release of both GLP-1 and PYY by rebaudioside A. Furthermore, these organoids were also capable of secreting basal levels of GLP-1, PYY and CCK. These data confirm our previous studies in pig intestinal segment explants, which showed GLP-1 and PYY release induced by rebaudioside A (12). Nutrient-sensing receptors and nutrient transporters on gut hormone secreting enteroendocrine cells (EECs) like the sweet taste receptor and the SGLT-1 transporter have been shown to induce gut hormone secretion upon stimulation by glucose and other dietary compounds. Recently, KO-organoid models showed that SGLT-1 mediates GLP-1 release upon glucose stimulation (19). Rebaudioside A has also been shown to be able to activate the sweet taste receptor Tas1R2/Tas1R3 and human bitter receptors Tas2R4 and Tas2R14 in a transfected HEK cell reporter assay

(8). Activation of these sweet receptors, bitter receptors and transporters in the intestine can generate gut hormone release (11, 19-21), making it highly plausible that rebaudioside A might mediate its effects via one of these nutrient sensors. Satiating effects of nutrients have been shown to be region-specific as gut hormone secretion is dependent on the location of exposure. Van Avesaat et al. showed that ileal infusion of protein resulted in higher plasma GLP-1 levels than jejunal or duodenal infusions (22). Interestingly, infusion of a mixture of rebaudioside A, quinine and monosodium glutamate in the human duodenum resulted in a significant decrease in food intake of the participants (23). These findings indicate that the effects induced by rebaudioside A may be relevant for food intake regulation.

Interestingly, long term incubation with rebaudioside A induced the expression of enteroendocrine markers. Not only chromogranin A, an EEC marker, but also *Gcg*, encoding GLP-1, *Pyy* and *Cck* were increased by overnight stimulation with rebaudioside A. Furthermore, the number of chromogranin A positive cells was increased by the treatment. Several other dietary compounds or their metabolites have been shown to influence the number of EECs. In intestinal organoids, as well as in EECs, the supplementation with short chain fatty acids resulted in increased expression of glucagon and L-cell progenitor Foxa1/2 (6, 24, 25). Furthermore, dietary intake of fibres resulted in enhanced expression of glucagon *in vivo* as well (26, 27). In contrast, a reduction in EEC numbers was observed with a high fat diet. Here, a decrease in enteroendocrine progenitors Math1 (Atoh1), NeuroD1 and Pax6 was shown (28). The question remains what underlies our observed enteroendocrine enrichment. Could it be modulation of EEC number by rebaudioside A or does persistent stimulation of hormone secretion by rebaudioside A stimulate enteroendocrine development in its surrounding cells? Stimulating enteroendocrine L-cell differentiation in a pharmacological manner *in vivo* can restore glucose tolerance (7). We conclude that long term rebaudioside A exposure results increased enteroendocrine cell numbers in our 2D organoid model.

We have highlighted the usefulness of the two dimensional organoid model for studying gastrointestinal hormone release. However, the model may be less suitable for studying CCK release. The location-specific gene expression of CCK did not reflect the in vivo expression (data not shown). Moreover, we have observed highest release of CCK in the ileum contrary to the duodenum, which is the main site of CCK release in vivo.

In conclusion, we showed here that rebaudioside A induced hormone release in a location-specific manner and increased EEC number in a developed 2D intestinal organoid model. Our results might give rise to potential dietary strategies for type II diabetic patients. However, the latter merits further investigations.

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## Supplemental data:

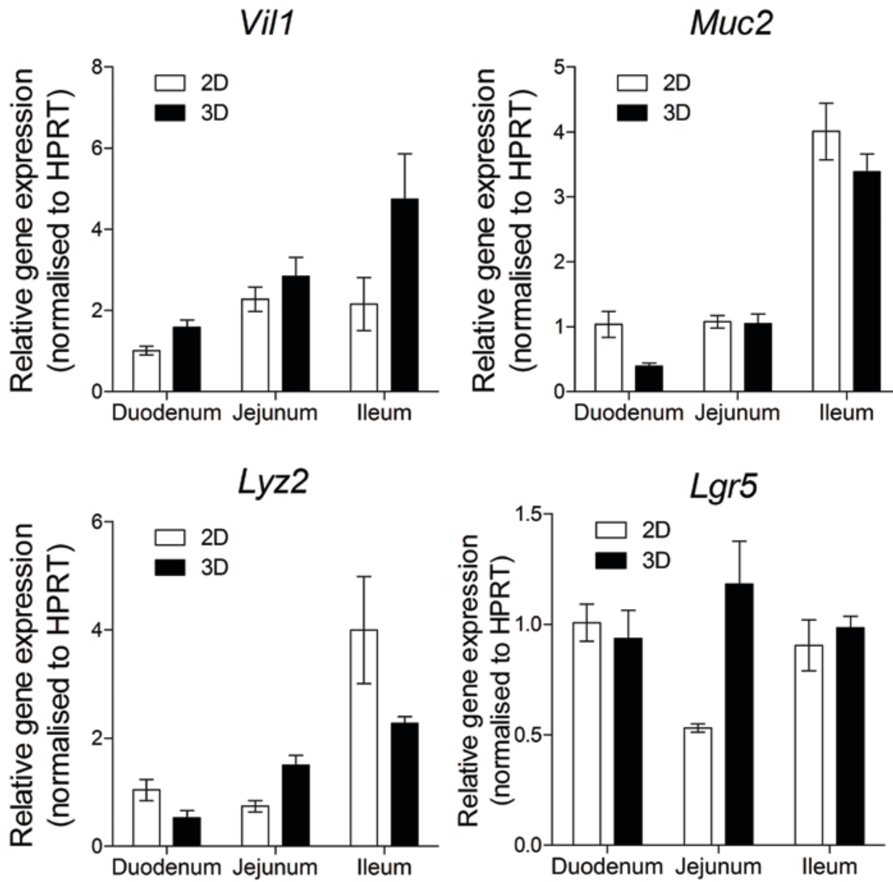


**Figure S6.1: Comparison of gene expression of Vil1, Muc2, Lyz2, Lgr5 in 2D and 3D cultured organoids derived from the duodenum, jejunum and ileum.**

6

# 7

# General discussion

The studies presented in this thesis add to our understanding of the role of receptors and other molecular structures assumed to be involved in nutrient sensing and gut hormone release. The research presented here was part of a larger research project investigating the possibilities to apply nutrient sensing for the modulation of food intake. A better understanding of the regulation of satiety and satiation remains crucial for developing strategies and new products that contribute to effective weight management. In most cases, such knowledge will be applied to body weight reduction and management. However, there are also groups and individuals for whom involuntary weight loss can pose a serious health risk. This general discussion will start with the main findings of the thesis. Next, some important issues in intestinal nutrient sensing are discussed, followed by general conclusions and future directions.

## Main outcomes of this thesis

In summary, the main outcomes of this thesis were:

- ☐ Gene expression of receptors, transporters and peptides involved in nutrient sensing showed a distinctive distribution pattern along the small intestine, which is in the distal small intestine highly similar between mouse, pig and man.
- ☐ One year after bariatric surgery using a recently developed gastroplication technique, tissue expression of genes associated with nutrient sensing was hardly changed, while a considerable reduction in inflammatory pathways was observed.
- ☐ The steviol glycoside rebaudioside A was found to induce GLP-1 and PYY release *ex vivo* from porcine intestinal tissue and two dimensional mouse organoids. This induction of the release was specific for intestinal location, with the ileum being the most potently stimulated by rebaudioside A. Moreover, this steviol glycoside increased enteroendocrine cell numbers in two dimensional mouse organoids.
- ☐ Rebaudioside A induced GLP-1 and CCK release in STC-1 cells, which was not mediated by the sweet taste receptor.

## The role of GLP-1 in the regulation of food intake

It is clear that food intake regulation is a very complex process, involving many environmental, behavioral, physiological and other factors. This thesis focuses on gut hormones, in particular GLP-1, as intermediate markers. There is little doubt that GLP-1 is a physiological signal related to satiety, a process that inhibits hunger and further eating which arises as a consequence of food ingestion (1). This is amongst others underpinned by the observation that exogenous infusion inhibits food intake in both man and rodents (2, 3). Furthermore, GLP-1 has been related to fullness as the postprandial plasma levels of this hormone ran parallel with feelings of fullness measured by visual analogue scores (4). However, it should be kept in mind that several factors are involved in food intake regulation, which likely explains the fact that GLP-1 alone does not represent a proper biomarker for satiety (4).

As has been described in chapter 1 of this thesis, GLP-1 is not only involved in food intake regulation but also has incretin effects. Its potency in maintaining glucose homeostasis is underpinned by the fact that GLP-1 analogues are currently used for the treatment of type II diabetic patients. Although this thesis focused on the role of gut hormones in food intake, strategies influencing endogenous GLP-1 release via nutrient sensing receptors might also contribute to type II diabetes management (5).

## Methodological considerations: Local GLP-1 release

Effects of nutrients on gastrointestinal hormone release are often studied with the ultimate goal to achieve effective weight management. Therefore, understanding intestinal hormone release *in vivo* is important. However, measuring GLP-1 in plasma does not give a good representation of local intestinal hormone release because of its short half-life. This short half-life might explain why effects of intestinal nutrient infusion on food intake were found without significant changes in plasma GLP-1 levels (6). After secretion from enteroendocrine cells, active GLP-1 will diffuse to the lamina propria and enter the blood stream, where it is rapidly degraded by DPP IV, with less than 15% reaching the circulation (7). With the short half-life of GLP-1 in mind, it

is likely that the activation of GLP-1 receptors by GLP-1 released in the gut is predominantly local. In fact the presence of GLP-1 receptors on the nerve fibers in the mucosa, in some cases relatively close to the GLP-1 releasing L-cells, confirms the idea of a local signaling effect (8). These nerves are mainly vagal nerves, which signal to the brain stem, but the GLP-1R was also found to be present on the enteric nervous system, meyenteric ganglia, and dorsal root ganglia (8, 9). Considering the recent discovery that enteroendocrine cells have extensions that often reach beneath 10 to 15 epithelial cells, it is even less surprising that there are local signaling effects of GLP-1 and perhaps other gut hormones and possibly epithelial cell interactions (10).
To account for these apparently local roles of GLP-1, effects of nutrient stimulation on GLP-1 release were mainly studied in *ex vivo* and *in vitro* models in which local intestinal endocrine release could be determined. The used models enabled us to study the effect of nutrients and other compounds on hormone release and the underlying mechanisms. The enteroendocrine STC-1 cell line used in this thesis was ideal as a quick screening tool and allowed us to investigate intracellular signaling. However, with this model no interactions can be studied between different types of epithelial cells. Therefore, *ex vivo* porcine intestinal tissue and organoids were used, as in these models the enteroendocrine cells are situated in their normal epithelial environment. To determine the release of GLP-1 and other gut hormones, a two dimensional organoid model was developed. The additional advantage of intestinal organoids over the *ex vivo* model is their viability and flexibility, which enabled us to investigate long-term effects of rebaudioside A stimulation as described in chapter 6.
However, some limitations of the used models warrant consideration. A major pitfall is their lack of discrimination between apical and basolateral sides. Furthermore, these models do not properly reflect signaling and regulation that is normally taking place in an intact organism. For example, insulin can stimulate GLP-1 secretion (11) and vascular exposure to intestinal compounds was in some cases more efficient than luminal exposure (12, 13). Therefore, care should be taken when translating the results of our models to the *in vivo* situation.

## Molecular targets for nutrients that influence hormone release in the intestinal tract

There are several gut nutrient sensing receptors and transporters which are found to be involved in gut hormone release and food intake regulation. Table 7.1 shows a short overview of nutrient sensing receptors and transporters for (digested) macronutrients and sweet compounds with their effect on gut hormone release, food intake regulation and body weight. In chapter 2 we have explored the expression pattern of these receptors and transporters in the intestinal tract.

### Effects of diet on the expression of these nutrient sensing related targets

In life, the ability to sense and respond to fluctuations in environmental nutrient levels is vital. The same holds true for the response to the body's nutrient or energy status. Gastrointestinal nutrient sensing is an important feature related to these mechanisms. In view of this, it is of interest to investigate to what extent the processes involved in nutrient sensing themselves are affected by dietary patterns. In chapter 2, we have tested the effect of a high fat diet on the expression of several nutrient sensing related genes in mice. In the statistical model, combining the expression pattern of several genes along the intestine, we saw only slight changes induced by a high fat diet in the distal small intestine of the mice. However, when looking at individual genes there were some changes in the expression. However the sizes of these changes were minor, especially compared to those reported in the literature. From literature it is known that the expression of several receptors and transporters can be increased by a diet rich in their ligands. For example, the expression of peptide transporter, Pept1, was found to be increased by a high-protein diet (38-41). However, inconsistent results were found with some diets and molecular targets. For example, GPR120 expression in the corpus of the stomach and proximal intestine was increased in mice having received a high fat diet, whereas it was decreased in the antrum of the stomach (42, 43). Taken together, there appear to be several knowledge gaps regarding the long term effects of diet and other lifestyle factors on the mechanisms involved in intestinal nutrient sensing.

**Table 7.1: Receptors and transporters involved in nutrient sensing**

| Receptor | Effect on gut hormone release | Effect on food intake regulation |
|---|---|---|
| **GPR120 (FFAR4)** for detection of long-chain fatty acids | GLP-1 release ↑ (14, 15), CCK ↑ (16), GIP ↑ (17) | Body weight in mice↔ (15, 18) |
| **GPR119** for detection of lipid compounds like oleoylethanolamide | GLP-1 release ↑ (19, 20) | Food intake and body weight in rats↓ (21), OEA suppression of food intake also in GPR119-/- mice (22) |
| **Pept1 (SLC15A1)** for transport of di/tri peptides | GLP-1 ↑ (23) | N/A |
| **SGLT-1 (SLC5A1)** for transport of glucose | GLP-1 ↑ (24-26), GIP ↑ (26-28) | N/A |
| **GPR93 (LPAR5)** for detection of peptones | GLP-1 ↔ (23), CCK ↑ (29) | N/A |
| **CaSR** for detection of L-amino acids | GLP-1 ↑ (23, 30, 31), CCK ↑ (31-33), PYY ↑ (30), GIP ↑ (30) | N/A |
| **GPRC6A** for detection of L-amino acids | GLP-1 ↑ (34, 35) | N/A |
| **Tas1R1/Tas1R2** for detection of umami tastants | CCK ↑ (36), GLP-1 ↑ (37) | N/A |
| **Tas1R2/Tas1R3** for detection of sweet tastants | Specified in a specific section below | |

**Effects of bariatric surgery on the expression of nutrient sensing related targets**

To date, the most effective medical treatment for obesity remains bariatric surgery. Surgical procedures used for weight loss influence fasted gut hormone levels, and following surgery many gut hormones are increased postprandially (44). The observed changes in GLP-1 following Roux-en-Y gastric bypass (RYGB) were found to be associated with decreased appetite and weight loss, although a causal relationship is not clear (45). The gene expression of several of nutrient sensing receptors was found to be changed in obesity and after weight loss treatment (42, 46-48). Furthermore, the density of enteroendocrine cells and the expression levels of gastrointestinal hormones alters after changes in body weight (43, 48, 49). This thesis included a study on the effects of a minimally invasive bariatric procedure, endoscopic gastroplication. This procedure has shown to induce weight loss in morbidly obese patients and to alter gut hormone levels, both in fasted and postprandial states (50). Therefore, we sought to investigate the effects of this procedure on the expression levels of nutrient sensing related genes. However, whole transcriptome analysis showed more pronounced effects in relation to inflammatory pathways, as highlighted in chapter 3. Relatively few effects on the expression of nutrient sensing receptors and transporters were observed one year after the procedure. Interestingly however, we found the expression of the calcium sensing receptor, CaSR, to be increased in the antrum (FC= 1.78, p=0.01). This receptor for both calcium ions as well as L-amino acids was shown to be enriched in ghrelin-positive cells and its activation was able to inhibit ghrelin secretion *ex vivo* (51). In these patients the postprandial decrease in ghrelin levels was found to be restored after weight loss (50). Surprisingly, no significant changes in GPR120 expression were detected after one year of weight loss resulting from gastroplication. This was in contrast to other studies which found increased levels of gastric GPR120 expression in morbidly obese subjects compared to normal weight subjects (46), and an observed positive correlation between duodenal GPR120 expression and BMI (48). A plausible explanation for this could lie in the degree of weight loss. Even though these patients lost on average 20kg, their BMI was still relatively high (33.4kg/m$^2$) one year after intervention. Furthermore,

there might be some other changes in the gastrointestinal physiology or on the whole body level induced by gastroplication that influences the expression of these receptors. Young et al. showed that not only luminal nutrients but also plasma glucose levels can influence the expression of the sweet taste receptor (52). Lastly, it could be that the nutrient sensing mechanisms are not always regulated at the gene expression level of receptors and transporters, but by post-translational modifications or via their cellular location, for example regulation through brush border membrane mobilization.

### Other nutrient sensing receptors and targets of potential interest

This thesis focused on the receptors and transporters for macronutrients and non-caloric sweet compounds. As a consequence, a number of other potentially relevant molecular targets received less attention. These include receptors for short chain fatty acids and bile acids (53-56). Next to these well-known chemosensory receptors, intestinal olfactory receptors may become of more interest as targets for the regulation of food intake. These receptors represent a large family of GPCRs that is primarily known for its capacity to detect odors. However, increasing evidence suggests a role in the intestines as well. Several olfactants have been shown to influence serotonin release from enterochromaffin cells (57, 58). As the olfactory receptors are a very large family of more than 500 genes or pseudogenes, specialized in the detection of many chemical compounds (59), it would be interesting to see if the expression of these receptors is enriched in enteroendocrine cells compared to other epithelial cells and to further investigate their role in gut hormone release.

## The importance of location-specific nutrient sensing

### Location-specific hormone release

Considering the effectiveness of especially RYGB in reducing weight, it has been suggested that nutrient delivery to the more distal parts of the small intestines is of particular importance. In human infusion studies, location-specific differences in food intake and plasma hormone levels in response to nutrients were observed. Ileal exposure to casein resulted in reduced food

intake, compared to duodenal or placebo infusion, which had no effect. Furthermore, plasma GLP-1 levels were highest after casein stimulation of the ileum compared to jejunum and duodenum (60). We were able to confirm location-specific effects on gut hormones as presented in chapters 4 and 6. In the intestinal organoids derived from different locations, we found the highest GLP-1 and PYY release in response to rebaudioside A in the ileum. In chapter 4 we showed that rebaudioside A was able to stimulate GLP-1 release from all regions, whereas sucrose only induced GLP-1 release from the distal ileum, confirming region-specific stimulation of GLP-1. These region-specific releases of hormones may result from location-specific receptor or transporter expression and location-specific hormone expression, which was shown in chapter 2. The direct link between the level of expression and the release of hormones would be interesting for further investigation. Obviously, when studying these location-specific effects in species other than man, differences between species should be taken into account.

**Cross-species comparison**

In chapter 2, we sought to investigate location-specific differences between species. The first difficulty we encountered were differences in anatomy and nomenclature of the intestinal locations in different species. For example the anatomy of the pig large intestine, which has a spiral form, is different compared to that in humans (61). There even appears to be some degree of inconsistence between definitions of anatomical locations within a certain species (van der Meulen, personal communication). Therefore, we chose to compare levels of expression between the species based on location relative to the full length of the intestine. Our key finding was that there appears to be a strong parallelism between the expression patterns of genes related to nutrient sensing in the distal ileum of the three species, but larger differences in the proximal intestine. The main differences in nutrient sensing related genes we observed in the proximal intestine might be related to general differences in the digestive system. On top of this, dietary effects are also likely to play a role for a given species.

## Rebaudioside A

### Effects of non-caloric sweeteners on food intake regulation and weight management

Changing food composition using specific components plays an important role in current weight management strategies. Since a few decades low-caloric sweeteners of different chemical structure are in use. Their use is to replace sugar and thus reduce caloric intake. Their application is not without debate, one of the arguments being the suggestion that consumption of low-caloric sweeteners might lead to compensation as these compounds are thought not to satiate and even to increase hunger (62). However, systematic reviews and meta-analyses of studies have shown that, in general the use of low-caloric sweeteners is associated with a reduced energy intake and body weight (63). In this thesis we have specifically focused on the low-caloric sweetener Rebaudioside A from *Stevia rebaudiana* Bertoni. The leaves of this plant were traditionally used in South America (64). Preparations from *Stevia rebaudiana* are receiving considerable interest as it is marketed as a 'natural' sweetener. When using stevia as a preload, it reduced total daily energy intake compared to a sugar-sweetened preload (65). Also, intraduodenal infusion of rebaudioside A in combination with other tastants reduced subsequent energy intake (6). However, studies on long-term intake of capsulated rebaudioside A showed no effects on body weight or food intake compared to control (66, 67). A recent systemic review of the randomized clinical trials concluded that based on the currently available studies, rebaudioside A may influence blood glucose although effects are small and more studies are needed (68).

### Involvement of the sweet taste receptor in GLP-1 release

The role of the sweet taste receptor in GLP-1 secretion is still very controversial. Different models have generated different outcomes. Moreover, the use of different agonists and antagonists has led to inconsistent conclusions. These differences are discussed in the next paragraph.

In cell studies, results show convincingly that GLP-1 is released in response to sweet tasting compounds. Jang et al. showed the induction of GLP-1 release by different sweet taste receptor ligands; glucose (165-555mM), sucrose (40-

145mM) and sucralose (1-5mM) in human colon NCI-H716 cells. The effect of sucralose was inhibited by the hTas1R3 inhibitor lactisole. Furthermore, sweet taste receptor related G-protein α-gustducin knock-down resulted in a reduction of glucose-stimulated GLP-1 release (69). Similar to these results, sucralose, saccharin, acesulfame K and glycyrrhizin (50mM) were shown to induce GLP-1 release in human duodenum-derived Hutu-80 cells, which was inhibited by lactisole (70). Moreover, GLP-1 inducible effects of sucralose (50mM) could be inhibited by the mTas1R3 inhibitor gurmarin in GLUTag cells (71). Also, in isolated primary murine colon cells, glucose (0.1-100mM) and sucralose (20mM) induced GLP-1 release while acesulfame K (2mM) did not. In primary murine cells of the upper small intestine no effects of sucralose (1-20mM) were found, although glucose (1-100mM) did induce GLP-1 release (72). This observation was confirmed by extensive studies using isolated perfused rat small intestine, where neither sucralose (0.25% w/v) nor acesulfame K (0.78% w/v) were found to induce GLP-1 release (24).
In all these *in vitro* studies there is a discrepancy between the concentrations of ligands used, and besides that the relative sweetness of the tested solutions was not always taken into account. In general, most of the used concentrations of non-caloric sweeteners were high, considering the relative sweetness compared to sucrose (73, 74). Another complicating aspect is the fact that some sweet compounds might induce GLP-1 release via other mechanisms not involving the sweet taste receptor. Glucose for instance has been shown to also mediate its effects on GLP-1 independently of the sweet taste receptor and to use other mechanisms including those involved in absorption and metabolism of glucose (24, 25, 72, 75).
*In vivo* studies, measuring GLP-1 in plasma, have not clarified the role of the sweet taste receptor in intestinal sensing either. A study in rats showed no effects on GLP-1 plasma levels following oral delivery of low-caloric sweeteners, whereas glucose (all 1g/kg) elevated plasma GLP-1 in this study (76). In line with these animal studies there are several human studies that could also not detect a relation between low-caloric sweeteners and GLP-1 release. Intragastric sucralose (0.4-4mM) infusion caused no significant increase in plasma GLP-1 compared to saline, whereas a sucrose load, equisweet to

the lowest sucralose solution, did (77). However, low-caloric sweeteners, given as a pre-load before glucose ingestion showed contradicting effects on GLP-1 plasma levels (78-81). Studies in mice showed that glucose can increase plasma GLP-1 levels and support the involvement of the sweet taste receptor in the observed GLP-1 release as α-gustducin$^{-/-}$ mice showed reduced plasma GLP-1 levels after an oral glucose load (69). Also, human studies, using the inhibitor lactisole to block the glucose-induced rise in plasma GLP-1 levels by intragastric or intraduodenal infusion, suggested the involvement of the sweet taste receptor (82, 83).

Altogether, available *in vivo* studies where sweet taste receptor Tas1R3 blockers and G-protein α-gustducin knock-out models were used, clearly point towards the involvement of the sweet taste receptor in glucose-induced GLP-1 release. However, although low-caloric sweeteners are potent agonists of the sweet taste receptor in both gustation and cell-based assays, a lack of effects of these sweeteners on plasma GLP-1 is observed. In our hands, the low-caloric sweetener sucralose had no effect on GLP-1 release, whereas the sweetener rebaudioside A showed consistent GLP-1 induction in the models used.

**Rebaudioside A induces gut hormone release**

As described in chapters 4, 5 and 6 we showed that GLP-1 (and PYY, CCK) release was potently stimulated by rebaudioside A in multiple models. Rebaudioside A is a steviol glycoside that is known for its sweet taste and it was shown to activate the human sweet taste receptor (88). In chapter 5, we have shown that blocking the murine Tas1R3 with gurmarin did not attenuate GLP-1 release in STC-1 cells. These results have led us to believe that the sweet taste receptor is not involved in the release of GLP-1 by rebaudioside A. Furthermore, we provided evidence that rebaudioside A-elicited GLP-1 secretion involves cAMP signaling.

## The role of nutrient sensing in relation to food intake in man

Targeting gut nutrient sensing receptors and transporters by specific stimulation of the enteroendocrine system might contribute to a solution for the obesity pandemic. This view is also supported by results coming from pharmacological interventions involving liraglutide, a GLP-1 analogue, which was recently approved for the treatment of obesity. Two years of treatment with this GLP-1 analogue resulted in an average weight loss of about 10 kg (BMI reduction of 3.4). However, this degree of weight loss was mainly achieved by emotional, restrained or indifferent eaters and not by external eaters (84). Liraglutide was also reported to have side-effects, which consisted mostly of gastrointestinal-related complaints like nausea and vomiting (85). An endogenously generated stimulation of GLP-1 release by nutrients in the intestine might in theory be attractive when it would result in a similar loss of body weight, without producing these side-effects. As most of the consumed food is digested and absorbed before it reaches the distal intestine, encapsulation of specific secretagogues might provide interesting options. Recently, the effect of 6 gram encapsulated glutamine was tested in healthy and diabetic volunteers. The intervention resulted in increased peak GLP-1 levels, but surprisingly led to a rise instead of a decrease in subsequent food intake in healthy volunteers (86). It was suggested that other GLP-1 secretagogues or higher doses of the compound might provide other results. Two human studies have shown that a combination of tastants infused in the duodenum and 6 gram safflower oil or 15 gram casein, or 13 gram sucrose infused in ileum resulted both in a reduction of food intake. However, in both studies no effects on plasma AUC GLP-1 levels were observed (6, 87). Altogether, to influence food intake using gut nutrient sensing mechanisms, multiple factors should be taken into account among which dose, compound(s), intestinal location of delivery and timing. Finally, a proof of principle study is needed, exploring the effects outside the laboratory environment and including the effects on long-term weight management.

## General conclusions and future directions

The world-wide rising obesity rates continue to fuel research on the modulation of body weight. Achieving sustained weight loss in obese patients by dietary or lifestyle intervention has proven to be very challenging. At the same time, pharmacological modulation of body weight has not been very successful as well, and the few compounds available do not reach a similar extent of weight loss as bariatric surgery, which is commonly regarded as the most effective treatment for obesity.

Based on the results presented in this thesis and data from other studies it would be too optimistic to conclude that promising strategies to modulate body weight based on targeting gastrointestinal nutrient sensing mechanisms are on the horizon. What we do know now is that several factors play a role in gut hormone release. This includes not only the nature and dose of the active compound(s), but also the location and timing of its (their) interactions with receptors and other targets along the gastrointestinal tract. As previously described, metabolic effects of RYGB appear to go beyond creating a situation of malabsorption only. Changes in location and timing of nutrient-receptor interactions are probably also playing a major role. In that respect these effects could be used a guiding principle to develop less invasive strategies. We have shown that rebaudioside A may be a potential compound to induce gut hormone release *in vivo*, especially when applied to the distal small intestine. Therefore, rebaudioside A could be of interest to influence food intake and it is likely to have the most potent effects when delivered in the ileum. Based on the studies presented in this thesis, some recommendations for future research on rebaudioside A can be formulated.

Considering the fact that the sweet taste receptor does not seem to play a role in rebaudioside A-induced hormone release, future studies should include further investigations on the mechanism(s) underlying rebaudioside A-induced hormone release. Steviol glycosides can also activate the human bitter receptors Tas2R4 and Tas2R14 (88). Their mouse homologues are Tas2R140 and Tas2R108, and these receptors are putative candidates to explain rebaudioside A-induced GLP-1 release found in our murine models. Preliminary results generated as a result of a recently started collaboration with the laboratory of Prof W.

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Meyerhof (DIfE, Potsdam, Germany) showed that rebaudioside A is able to activate (murine) Tas2R108. Furthermore, our experiments showed that agonists of this receptor induced GLP-1 release in STC-1 cells. These preliminary data suggest that the Tas2R108 receptor is involved in rebaudioside A-induced gut hormone release, which will be investigated in future studies by knocking out this receptor.

Another topic that merits further investigation are possible effects of prolonged exposure to rebaudioside A, as the compound seems to increase the number of enteroendocrine cells. It would be interesting to see if this effect is due to persistent GLP-1 release or induced by rebaudioside A stimulation itself. Moreover, it should be studied whether the increased number of enteroendocrine cells also results in a substantially enhanced GLP-1 release upon stimulation. Knowing the effects of rebaudioside A on GLP-1 release, in vivo effects of rebaudioside A on food intake should be studied more extensively. As an increase in enteroendocrine L-cell number by pharmacologic treatment could restore glucose tolerance in mice (89), the effects of rebaudioside A should also be tested in relation to glucose homeostasis. Several factors, like timing, dosage form, dosing concentration and intestinal location, seem to play a role.

Intestinal organoids provide a perfect model to study the cell interactions between different epithelial cell types. The possibility of this model to study intercellular signals and crosstalk in the intestinal epithelial tissue gives rise to a new interesting field of research in intestinal nutrient sensing. Future studies using murine and human organoids might add to the development of novel pharmacological or nutritional strategies in order to modulate food intake.

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# SUMMARY

The alarming increase in obesity rates creates an urgent need for effective prevention and treatment strategies. The most effective treatment for obesity today is bariatric surgery. Bariatric surgery comprises a number of different procedures having in common that they induce weight loss and alter gut hormone release. Gut hormones are well known for their effects on food intake behavior and their role in weight loss after bariatric surgery is undeniable. In addition, the therapeutic use of GLP-1 (Glucagon-Like Peptide-1) analogues including liraglutide in type II diabetes and obesity is on the rise. This underlines why gut hormones are considered promising targets for the development of new treatment strategies against obesity and its comorbidities.

The secretion of gut hormones, among which GLP-1, is influenced by nutrient ingestion. The interactions of dietary components or their breakdown products with receptors and transporters located on the enteroendocrine cells of the intestinal tract can induce their release, a process called intestinal nutrient sensing. In this thesis, we aimed to further elucidate intestinal nutrient sensing mechanisms on a cellular level. First, the regional expression of several gut nutrient sensing related genes along the intestinal tract was assessed in three commonly studied species, namely mouse, pig and man. Gene expression of receptors, transporters and peptides involved in nutrient sensing shows a distinctive distribution pattern along the small intestine, which is in the distal small intestine highly similar between the species. Subsequently, we sought to investigate if this expression was changed after a weight loss inducing bariatric procedure. By whole transcriptome analysis, we showed that upper gastrointestinal tissue expression of genes associated with nutrient sensing was hardly changed. In contrast, a considerable reduction in inflammatory pathways was observed.

Next, we sought to investigate the effects of the non-caloric sweetener rebaudioside A. This *Stevia rebaudiana*-derived compound was approved on the European market in 2011. As there is still some controversy about the effects of sweeteners in general on GLP-1 release, we investigated the effects of this specific sweetener. Because of the short half-life of GLP-1, the effect

of nutrient stimulation was mainly studied in *ex vivo* and *in vitro* models in which local intestinal hormone release could be determined. A two dimensional gut model using intestinal organoids derived from murine intestinal crypts was developed to study location-specific hormone secretion. Rebaudioside A was found to induce GLP-1 and PYY release *ex vivo* from porcine intestinal tissue and in two dimensional organoids. This induction of the release was specific for the intestinal location, with the ileum being most potently stimulated by rebaudioside A. Moreover, prolonged exposure to rebaudioside A increased enteroendocrine cell numbers in two dimensional organoids. When studying the underlying mechanism in enteroendocrine STC-1 cells, we concluded that rebaudioside A-induced GLP-1 release was independent of the sweet taste receptor.

The studies presented in this thesis add to our understanding the role of receptors and other molecular structures that are likely to be involved in nutrient sensing and the modulation of gut hormone release. What we know now is that several factors play a role in gut hormone release. This includes not only the nature and dose of the active compound(s), but also the location and timing of its (their) interactions with receptors and other targets along the gastrointestinal tract. We have shown that rebaudioside A may be a potential compound to induce gut hormone release *in vivo*, especially when applied to the distal small intestine. Therefore, rebaudioside A may be a promising compound to influence food intake, possibly most potent when delivered in the ileum.

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# ABOUT THE AUTHOR

## Curriculum vitae

Nikkie van der Wielen was born on the 29th of July, 1988 in Eindhoven, the Netherlands. After completing secondary school at the 'Scholengroep Cambium' in Zaltbommel, she started the BSc program 'Nutrition and Health' at Wageningen University in 2006. During her BSc studies she was board member of the study association 'Di-et-Tri'. She proceeded with a MSc program in 'Nutrition and Health', specializing in molecular nutrition. In 2010, she conducted her MSc thesis at FrieslandCampina, developing an *in vitro* assay. For her internship she went to Orebro University in Sweden, where she investigated the role of microbiota in irritable bowel syndrome. In September 2011, she started her PhD project at the Nutrition and Pharmacology group of the Division of Human Nutrition of Wageningen University. She executed her PhD research under guidance of Prof. Renger Witkamp, Dr. Jocelijn Meijerink and Dr. Henk Hendriks. Her research was part of the project entitled 'Gut nutrient sensing in relation to appetite control', funded by the Top Institute of Food and Nutrition. During her PhD, she closely collaborated with researchers from Maastricht University, TNO (The Netherlands Organization of Applied Scientific Research), Plant Research International Wageningen, and University of Applied Sciences Utrecht. The results from her PhD project are described in this thesis entitled 'Intestinal Nutrient Sensing: a gut feeling for food'. In addition to her research activities, she organized a study tour to Australia for 25 PhD students of the division of Human Nutrition. Nikkie was also responsible for the innovation of the BSc course 'Nutrition and Pharmacology' in 2015. Furthermore, she was involved in teaching and she supervised several students during their BSc and MSc thesis. In 2016 she was selected to participate in the 22$^{nd}$ seminar of the European Nutrition Leadership Platform. Currently, Nikkie works as a post-doctoral researcher on a protein quality project funded by the Dutch Dairy Association. Her project aims to develop a dual stable isotope method to measure protein digestibility, amino acid bioavailability and protein nutritional quality for humans.

## List of publications

**van der Wielen, N.**, J. P. ten Klooster, H. F. Hendriks, S. Rouschop, M. Jongsma, J. van Arkel, R. Pieters, R. F. Witkamp and J. Meijerink. "Rebaudioside A-induced GLP-1 secretion from enteroendocrine cells is independent of the sweet taste receptor." In preparation.

**van der Wielen, N.***, J.P. ten Klooster*, S. Muckenschnabl, R. Pieters, H. F. Hendriks, R. F. Witkamp and J. Meijerink. "The non-caloric sweetener Rebaudioside A stimulates hormone release and increases enteroendocrine cell number in two dimensional mouse organoids derived from different locations of the intestine." Submitted.

**van der Wielen, N.***, G. Paulus*, M. van Avesaat, A. Masclee, J. Meijerink and N. Bouvy. "Effect of endoscopic gastroplication on the genome-wide transcriptome in the upper gastrointestinal tract." Submitted.

Ripken, D., **N. van der Wielen**, H. M. Wortelboer, J. Meijerink, R. Witkamp and H. Hendriks. "Glucagon like peptide-1 release is modulated by serotonin." Submitted.

Ripken, D., **N. van der Wielen**, J. van der Meulen, T. Schuurman, R. F. Witkamp, H. F. Hendriks and S. J. Koopmans (2015). "Cholecystokinin regulates satiation independently of the abdominal vagal nerve in a pig model of total subdiaphragmatic vagotomy." Physiology & Behaviour.

Ripken, D., **N. van der Wielen**, H. M. Wortelboer, J. Meijerink, R. F. Witkamp and H. F. Hendriks (2014). "Steviol Glycoside Rebaudioside A Induces Glucagon-like Peptide-1 and Peptide YY Release in a Porcine ex Vivo Intestinal Model." Journal of Agricultural and Food Chemistry.

**van der Wielen, N.**, M. van Avesaat, N. J. de Wit, J. T. Vogels, F. Troost, A. Masclee, S. J. Koopmans, J. van der Meulen, M. V. Boekschoten, M. Muller, H. F. Hendriks, R. F. Witkamp and J. Meijerink (2014). "Cross-species comparison of genes related to nutrient sensing mechanisms expressed along the intestine." PLoS One.

* both authors contributed equally

## Overview of completed training activities

**Discipline specific activities**

22nd European Congress on Obesity (Prague, 2015)

Dutch Nutritional Science days (Deurne, Heeze, 2012, 2013, 2014, 2015)

Advanced visualization, integration and biological interpretation of -omics data (Wageningen, 2014)

Stevia tasteful conference (Berlin, 2014)

TI Food and Nutrition Conference (Arnhem, Utrecht, 2012, 2014)

33th Blankenese conference 'Nutrient sensing from brain to gut' (Hamburg, 2013)

Science camp GRK1482 Technical University Munich (Seeon, 2013)

Gut mini symposium Danone Research (Utrecht, 2013)

Joint international neurogastroenterology and motility meeting (NGM) (Bologna, 2012)

Regulation of energy intake: the role of product properties (Wageningen, 2012)

Workshop 'Measuring Gut Hormones' (Copenhagen, 2012)

Fifth National Nutrition Conference (Ede, 2012)

8th NUGO week (Wageningen, 2011)

**General courses**

Alison Douglas Summer School (Fraueninsel am Chiemsee, 2013)

Teaching and supervising thesis students (Wageningen, 2012)

Presentation skills (Wageningen, 2012)

Basic Intellectual property for researchers of TI food and nutrition (Wageningen, 2012)

VLAG PhD week (Baarlo, 2012)

Techniques for Writing and Presenting Scientific Papers (Wageningen, 2012)

Speed reading (Wageningen, 2011)

Project realisation programme of TI Food and Nutrition 'from ambition to results' (Wageningen, 2011)

**Optional activities**

Project meetings TI Food and Nutrition (2011-2015)

Nutrition and Pharmacology scientific meetings (weekly, 2011–2015)

NMG-Pharma scientific meetings (weekly, 2011–2015)

PhD study tour and organisation committee (Australia, 2013)

Preparation research proposal (2011)

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