**School of Pharmacy and Biomedical Sciences**

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# Lysophosphatidylinositol-glucagon like peptide 1 crosstalk in metabolic diseases

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Declaration

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# Abstract

Metabolic abnormalities, affecting a third of the world population, have reached pandemic proportions. Obesity, defined as a pathologically elevated body weight, is known to predispose to a vast gamut of chronic diseases that underpin its soaring socio-economical morbidity. Obesity often evolves into a more complex condition referred to as Type 2 diabetes (T2D), whereby glucose metabolism is disrupted in a multi-faceted way. In the last few decades Glucagon-like peptide-1 (GLP-1) mimicking drugs, a novel class of therapeutics, has shown promising anti-diabetic, yet non curative properties. GLP-1 is a beneficial hormone secreted into the bloodstream by a sub-family of cells, part of the broader Enteroendocrine cells system (EECs) lying along the gastrointestinal tract.

In recent years, it has become clear that the biology of GLP-1 is more nuanced than once thought, offering a much broader therapeutical potential. Indeed, despite the cardio-vascular benefits reported with the use of GLP-1 mimicking drugs, stimulating the endogenous secretion from the gut is in principle safer, and more physiological, widening the therapeutic potential of this approach.

In the first chapter of this thesis, 3 first author review articles provide an in-depth discussion of the complex biology of enteroendocrine cell hormones, and particularly the physiology of GLP-1 in the context of metabolic diseases and related morbidities such as pancreatic cancer. We discuss the physiology and pathology of this peptide in frame with novel evidence, reinstating the superiority of GLP-1 secreting drugs in contrast to GLP-1 mimetics currently on the market for the management of type 2 diabetes, and obesity.

In the study presented in chapter §2, a published first-author manuscript, the endogenous lipid Oleoyl-lysophosphatidylinositol (O-LPI) is shown to be an effective GLP-1 secreting agent signalling via the activation of GPR119. Different *in vitro*, and *ex-vivo* EECs models, indicate that O-LPI is a potent and efficient GLP-1 releasing drug. In this study we also characterized the downstream signalling pathways that this lipid elicits in L-cell lines, highlighting how particularly the MAPK-ERK1/2 and CREB activity are necessary to elicit GLP-1 secretion.

O-LPI is a labile molecule and its negligible concentration in any dietary food source, such as olive oil, complicates its direct therapeutical use. Multiple attempts have been

made at synthesizing small molecules targeting different EECs-specific receptors which activation is known to induce the release of GLP-1. In the last decade this therapeutical approach has become the priority of multiple commercial enterprises, which have generated following classic top-down methodologies, vast libraries of molecules targeting primarily the GLP-1-secreting G-protein coupled receptor GPR119; nonetheless no compound has yet reached the market, primarily due to off target toxicity. O-LPI herein identified as a potent and safe GLP-1 secreting agent, offers a valuable starting point for the study of GLP-1 secretagogues. In the study detailed in chapter §3, we present a receptor-agnostic bottom-up approach, with a small-scale Structural Activity Relationship (SAR) study of different drugs based on the structure of O-LPI. A library of 18 different compounds was screened *in vitro*, and 3 lead compounds were identified for their robust GLP-1 secreting properties. We identified compound ps297b as the O-LPI analogues capable to successfully ameliorate the glucose-stimulated plasmatic levels of GLP-1 in the diabetic db/db mouse model.

We have also generated an *in silico* 3D-model of the receptor GPR119 interacting with either O-LPI or ps297b, highlighting the interaction of the compounds with their Oleoyl-chain within a deep orthosteric lipophilic site, rationalizing the tropism for this receptor, and providing groundwork for future explorative studies aimed at experimentally validating this model.

In the final chapter of this thesis, we consider the pharmacological impact that the endocannabinoid system has on metabolic diseases. Recent evidence suggests that some cannabis-drugs can signal through the endocannabinoid system and modulate the enteroendocrine cell system. In chapter §4 of this thesis, we show that enteroendocrine cell lines express cannabinoid receptors, providing further evidence that they are elements of the endocannabinoid system. In addition, we report that the phytocannabinoids Tetrahydrocannabinol (THC), Tetrahydrocannabivarin (THCV), and Cannabidiol (CBD) can induce the release of GLP-1 *in vitro*. We contrast the pharmacology of these drugs demonstrating that only THC signals through CB1 and CB2 receptors in the STC-1 cell system. We conclude by discussing the implications of this pharmacology, providing the rationale for future investigations aimed at developing safer, and more efficient GLP-1 secretagogues in metabolic diseases.

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# List of publications

## Primary publications

**Paternoster S**, Falasca M. The intricate relationship between diabetes, obesity and pancreatic cancer. *Biochim Biophys Acta Rev Cancer*. 2020;1873(1):188326. doi:10.1016/j.bbcan.2019.188326

**Paternoster S**, Keating D, Falasca M. Editorial: Gastrointestinal Hormones. *Front Endocrinol (Lausanne).* 2019;10:498. Published 2019 Jul 25. doi:10.3389/fendo.2019.00498

**Paternoster S**, Falasca M. Dissecting the Physiology and Pathophysiology of Glucagon-Like Peptide-1. *Front Endocrinol (Lausanne).* 2018;9:584. Published 2018 Oct 11. doi:10.3389/fendo.2018.00584

In the following publication I share with Arifin Ahmad Syamsul the first-name authorship for equal contribution

Arifin SA, **Paternoster S**, Carlessi R, et al. Oleoyl-lysophosphatidylinositol enhances glucagon-like peptide-1 secretion from enteroendocrine L-cells through GPR119. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2018;1863(9):1132–1141. doi:10.1016/j.bbalip.2018.06.007

## Secondary publications

The following manuscripts do not directly relate to this PhD research thesis; nonetheless they are highlighted here to show the scientific productivity during this PhD project

Caporale, C.; Ranieri, A.M.; **Paternoster, S.**; Bader, C.A.; Falasca, M.; Plush, S.E.; Brooks, D.A.; Stagni, S.; Massi, M. Photophysical and Biological Properties of Iridium Tetrazolato Complexes Functionalised with Fatty Acid Chains. Inorganics 2020, 8, 23

**Paternoster S**, Falasca M. Targeting the adipose tissue to fight prostate cancer. Transl Androl Urol. 2019;8(Suppl 3):S229–S231. doi:10.21037/tau.2019.01.13

Domenichini A, Edmands JS, Adamska A, Begicevic RR, **Paternoster S**, Falasca M. Pancreatic cancer tumorspheres are cancer stem-like cells with increased

chemoresistance and reduced metabolic potential. Adv Biol Regul. 2019;72:63–77. doi:10.1016/j.jbior.2019.02.001

Simpson PV, Casari I, **Paternoster S**, Skelton BW, Falasca M, Massi M. Defining the Anti-Cancer Activity of Tricarbonyl Rhenium Complexes: Induction of G2/M Cell Cycle Arrest and Blockade of Aurora-A Kinase Phosphorylation. Chemistry. 2017;23(27):6518–6521. doi:10.1002/chem.201701208

## Abbreviations

| | |
|---|---|
| 2-AG | 2-Arachidonoyl-glycerol |
| 2-OG | 2-Oleoyl-glycerol |
| A-LPI | Arachidonoyl-LPI |
| AEA | Arachidonoylethanolamide / Anandamide |
| CBD | Cannabidiol |
| CBDV | Cannabidivarin |
| CBG | Cannabigerol |
| CCDS | Consensus coding sequence |
| CCK | Cholecystokinin |
| CD | Chron's disease |
| CNS | Central Nervous System |
| CRC | Colorectal cancer |
| db/db | $LepRD^{+/+}$ - leptin receptor deficient - Diabetic mouse |
| Diabesity | Obese type 2 diabetics |
| DPP-4 | Dipeptidyl peptidase-4 |
| EECs | Enteroendocrine cell system |
| GIP | Gastric Inhibitory Polypeptide |
| GLP-1 | Glucagon-like Peptide-1 |
| GLP-1R | Glucagon-like Peptide-1 Receptor |
| GLP-1RA | Glucagon-like Peptide-1 Receptor Agonist |
| GPCR | G-protein Coupled Receptors |
| IBD | Inflammatory bowel disease |

| | |
|---|---|
| INSL-5 | Insulin-like peptide-5 |
| LPI | Lyso-phosphatidylinositol |
| mAEA | methanandamide |
| $NaN_3$ | Sodium Azide |
| NOD | Novel Onset Diabetes |
| Noladin ether | 2-Arachidonoyl glyceryl ether |
| OAE | O-arachidonoyl ethanolamine / Virodhamine |
| OEA | Oleoyl-ethanolamide |
| O-LPI | Oleoyl-lysophosphatidylinositol |
| PAM | Positive allosteric modulator |
| PDAC | Pancreatic Ductal Adenocarcinoma |
| PET | PEG400 80%, Ethanol 10%, Tween 10% vehicle |
| PG | Proglucagon |
| PK/PD | Pharmacokinetic / Pharmacodynamic |
| PYY | Peptide YY |
| RYGB | Roux-en-y gastric bypass |
| SAR | Structure Activity Relationship |
| S-LPI | Stearoyl-LPI |
| T2D | Type 2 Diabetes |
| TBS-T | Tris-Buffered Solution – Tween 80 |
| THC | $\Delta^9$-Tetrahydrocannabinol |
| THCV | Tetrahydrocannabivarin |
| UC | Ulcerative cholitis |

## Index

# Chapter 1

# Chapter 1: Introduction to the pathophysiology of Glucagon-like peptide-1

**The content of this chapter is presented under the form of two peer-reviewed literature reviews and a commentary manuscript**

**Review 1**

**Paternoster S**, Falasca M. Dissecting the Physiology and Pathophysiology of Glucagon-Like Peptide-1. *Front Endocrinol (Lausanne)*. 2018;9:584. Published 2018 Oct 11. doi:10.3389/fendo.2018.00584

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**Editorial 1**

**Paternoster S**, Keating D, Falasca M. Editorial: Gastrointestinal Hormones. *Front Endocrinol (Lausanne)*. 2019;10:498. Published 2019 Jul 25. doi:10.3389/fendo.2019.00498

## 1.1 Synopsis of Review 1

The gastrointestinal tract, beyond its mechanistic role in digesting food, embodies the biggest endocrine organ of the human body, the enteroendocrine cell system (EECs). This organ, composed of distinct endocrine cells lying across the mucosa, secretes tens of different types of hormones into the systemic circulation. Surprisingly, the ability of the intestines to influence the biology of other endocrine organs, such as the pancreas, was described at the beginning of last century (Moore, 1906). Nonetheless only in the 1990s the complex physiology of gut-derived insulin stimulating molecules, or incretins, has started to be characterized at the molecular level in health and disease (Drucker, 2006). In particular, the powerful anti-diabetic properties of Glucagon-like peptide-1 (GLP-1), have started to be appreciated after the discovery of a natural mimetic with increased resistance to the ubiquitous protease DPP-4 (Eng, 1992). GLP-1 is indeed the genetic product of the same gene codifying for the Glucagon peptide, although it is post-translationally processed in a tissue specific

manner by the proteases Pcsk1/3 and Pcsk2. Proprotein convertase subtilisin/kexin type 1, is known as Pcsk1/3, PC1/3, PC1, PC3 NEC1, SPC3, BMIQ12 [NCBI Human gene ID 5122], while the proprotein convertase subtilisin/kexin type 2, is known as Pcsk2, PC2, NEC2, SPC2, [NCBI Human gene ID 5126]. After authoring this review nonetheless, we noticed that we wrongly referred to these enzymes with the acronyms Psck1/3 and Psck2, as some other authors did, such as (Good & Braun, 2013; Sandoval & D'Alessio, 2015). Indeed a third unrelated enzyme of the same kexin type family, known as Pcsk3, or Furin, also generates confusion [NCBI Human gene ID 5045], with at least an author referring to it as Psck1-3 (Vrang, Hansen, Larsen, & Tang-Christensen, 2007). For better clarity, the proteases involved in the processing of the preproglucagon peptide are most often known as PC1/3 and PC2. Type 2 diabetes is a chronic disease treated symptomatically with different drugs, mainly by controlling blood glucose levels. A remarkable case-study is represented by morbidly obese patients undergoing reconstructive bariatric surgery, such as Roux-en-y gastric bypass (RYGB). These patients are known to experience an almost instantaneous remittance from diabetes, a spectacular phenomenon which has been shown to be paralleled by an ten-fold increase in plasmatic GLP-1, (Davis et al., 2018; Salehi, Prigeon, & D'Alessio, 2011; E. P. Smith et al., 2014) raising the question whether this hormone might indeed be the key to replicate these benefits. Nonetheless, the degree by which the increase in GLP-1 is mechanistically causative, or merely secondary to the remittance is yet unclear, highlighting the gap in the knowledge that we have for this hormone.

Drugs mimicking the action of GLP-1 have swiftly been adopted as powerful glucose-lowering drugs to tackle the surge of metabolic diseases such as obesity, metabolic syndrome, and type 2 diabetes (T2D), which are currently plaguing an unprecedented fraction of the world population. Akin to Insulin analogues, GLP-1 mimetics are also peptides mirroring the physiological counterpart, and therefore require to be injected subcutaneously ultimately acting systemically. These drugs are known to possess multiple gastrointestinal side-effects, which are likely to be ascribed to the de-localized, systemic and sustained activation of the GLP-1 Receptor (GLP-1R).

In September 2019, the first orally available GLP-1 analogue, Semaglutide, received FDA approval for its efficacious glucose and weight lowering properties in type 2 diabetics. Nonetheless, independently from its route of administration gastrointestinal

upset is still reported by most patients. In particular as reported in the PIONEER 4 study, 14 mg of oral Semaglutide per day (R. Pratley et al., 2019), as well as 1 mg of Semaglutide injected subcutaneously once a week in the SUSTAIN 7 trial (R. E. Pratley et al., 2018), both elicit widespread gastrointestinal distress, primarily nausea, diarrhoea, vomiting and reduced appetite, in most patients, with 5% of events leading to a premature termination of the study. These reports further indicate that a supra-physiological localized, or systemic GLP-1R activation, equally impinges on diabetics' quality of life

Recent studies have implicated pancreatic α-cells as a secondary source of multiple EECs-derived peptides including GLP-1 (Kilimnik, Kim, Steiner, Friedman, & Hara, 2010), questioning its hormone-like nature given its physical vicinity to insulin secreting β-cells.

Indeed, key evidence presented by Chambers et al (Chambers et al., 2017) bolstered this new model of GLP-1 acting primarily as a paracrine agent. This study relied on transgenic mice genetically deficient for the Gcg gene, which physiologically codifies for a long proglucagon (PG) protein post-translationally modified, in a tissue specific manner, in at least six bio-active peptides: GLP-1, Oxyntomodulin, Glicentin, Glucagon, miniglucagon, and GLP-2 (Bataille & Dalle, 2014; Holst, 2007).

Mice systemically deficient for Gcg, or GLP-1R, did not respond to systemic administration of the GLP-1 receptor antagonist Exendin 9-39 (Ex-9), confirming that the physiological response to Ex-9 specifically requires an intact GLP-1 to GLP-1R axis. Unexpectedly animals with a restored intestinal-only Gcg production did not respond to Ex-9 either.

On the contrary, restoration of Pancreatic-only Gcg confers sensitivity to Ex-9, leading to an impaired oral and intraperitoneal glucose tolerance, supporting the key. Indeed these findings are phenocopied by animals deficient for GLP-1R specifically in β-cells only (E. P. Smith et al., 2014), adding further doubts onto the endocrinal nature of GLP-1 being secreted from the gut, and acting on the pancreas. In the paracrine-only model, GLP-1 is seen as being capable to influence the whole-body metabolism in a tissue specific, localized manner, yet engaging in a gut-brain-islet axis as depicted in Figure 2 of the presented Review 1.

Another key tenet surrounding the physiology of GLP-1 is that this peptide has only two active forms, named GLP-1 $(7\text{-}36)_{NH2}$ and GLP-1 (7-37) that are capable to activate the eponymous receptors. Novel evidence suggests that its once-thought inactive metabolites obtained after processing by the proteases DPP-4 and Neprilysin, do indeed possess biological activity. Multiple studies have implicated the small DPP-4 product GLP-1$(9\text{-}36)_{NH2}$ as well as the Neprylisin-processed GLP-1 $(29\text{-}36)_{NH2}$ and GLP-1$(32\text{-}36)_{NH2}$ in beneficial metabolic properties, such as increased glucose disposal and cardiovascular resilience (Ban et al., 2008; Elahi et al., 2014; Guglielmi & Sbraccia, 2017; Nikolaidis, Elahi, Shen, & Shannon, 2005; Ossum, van Deurs, Engstrøm, Jensen, & Treiman, 2009; Sun et al., 2015; Tomas et al., 2015).

These biologically active metabolites, acting independently of GLP-1R, are not formed as by-products of DPP-4-resistant GLP-1 mimetics, nor when using DPP-4 inhibitors, possibly explaining not only the reported side effects seen with these drugs, but also the supraphysiological activation of GLP-1R that is required in order to elicit metabolic benefits.

### 1.1.1 Significance

In this review, we have collated the most recent evidence, critically examining and expanding our understanding of the complex paracrine nature of GLP-1, and all its metabolites, paying particular attention to the species under examination. Figure 1 highlights the species-specific differences seen in terms of intestinal GLP-1 expression. Figure 2 frames GLP-1 in what is now a recognized gut-brain-islet axis.

After authoring this review, a recently published study restates the importance of intestinally derived GLP-1. Mice genetically deficient for PG only in the distal intestine, show an almost complete loss of plasmatic GLP-1, with an impaired glucose tolerance (Song et al., 2019). This evidence reinstates the known link between intestinal GLP-1 and circulating levels of active GLP-1$(7\text{-}36)_{NH2}$. This evidence needs to be put into the context of novel studies that in addition to the vastly recognized hallmarks of hyperinsulinaemic resistance, and hyperglycaemia, implicate gut-derived Glucagon in addition to raised plasmatic GIP levels which in turn are known to stimulate Glucagon, further compounding on the hyperglycaemic status (Lund, 2017; Lund, Vilsboll, Bagger, Holst, & Knop, 2011). Nonetheless the degree by which

intestinal GLP-1, or other less understood PG derived peptides play a mechanistic role in the human metabolism in health and disease is not yet conclusive and still debatable. Indeed, the translatability of metabolic responses seen in genetically engineered animals as in this study, present important limitations; firstly because of the metabolic adaptations that congenital genetic deficiencies often lead to compensatory expression of other gut hormones and receptors, and secondly because human tissues, such as the pancreatic islets and the gut, are known to bear much higher concentrations of Insulin, GLP-1 and Glucagon (Song et al., 2019) in addition to a different tissue architecture (Brereton, Vergari, Zhang, & Clark, 2015).

The species-specificity of the pathophysiological impact of tissue specific expression of GLP-1 remains open to debate. Nonetheless, restoring or at least improving the physiological release of GLP-1 from the intestine in diseases like type 2 diabetes or obesity, still represents a novel approach holding vast potential. Increasing the physiological release of intestinal GLP-1 harnesses the yet unclear physiology of all GLP-1 metabolites, and other PG derived peptides, possibly minimizing the side-effects seen with available GLP-1 mimetics, leading to a wider therapeutical window.

As summarized in Table 1, the activity of multiple receptors has been proven to translate into the release of GLP-1 and many other hormones co-secreted with it, such as peptide YY (PYY) or Insulin-like peptide-5 (INSL-5). In Figure 3 the recognized topographical localization of the most important receptors is shown to highlight our current understanding of possible therapeutics aimed at increasing GLP-1 expression.

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REVIEW
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# Dissecting the Physiology and Pathophysiology of Glucagon-Like Peptide-1

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An aging world population exposed to a sedentary life style is currently plagued by chronic metabolic diseases, such as type-2 diabetes, that are spreading worldwide at an unprecedented rate. One of the most promising pharmacological approaches for the management of type 2 diabetes takes advantage of the peptide hormone glucagon-like peptide-1 (GLP-1) under the form of protease resistant mimetics, and DPP-IV inhibitors. Despite the improved quality of life, long-term treatments with these new classes of drugs are riddled with serious and life-threatening side-effects, with no overall cure of the disease. New evidence is shedding more light over the complex physiology of GLP-1 in health and metabolic diseases. Herein, we discuss the most recent advancements in the biology of gut receptors known to induce the secretion of GLP-1, to bridge the multiple gaps into our understanding of its physiology and pathology.

**Keywords: glucagon-like peptide-1, metabolic disease, type 2 diabetes, enteroendocrine cell system, GPCR, L-cells, microbiome, α-cells**

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## INTRODUCTION

The gastrointestinal (GI) tract is a complex organ that monitors the body's energetical state and provides it with water and macro and micronutrients extracted from the ingested food. Along its length, the enteroendocrine cells (EECs) constitute a complex endocrine organ that communicates with the central nervous system (CNS) and the enteric nervous system (ENS) to orchestrate the homeostatic balance of the body in response to the GI luminal content.

This enteroendocrine system has traditionally been divided into 12 different cell types, based entirely on their hormonal content and cellular morphology. This endocrine organ is not organized in a glandular structure; on the contrary, it is dispersed heterogeneously, mainly as single cells, along the epithelium of the GI tract, from the stomach to the rectum with a defined cephalocaudal, crypt-to-villus in the small intestine and crypt-to-surface distribution in the colon (1, 2).

Despite representing just 1% of the adult gut epithelium, in the last decade it has become clear that the EECs constitute the largest endocrine organ in mammalia (3). Recent analysis of the expression of specific hormones at the cellular level, demonstrated that the EECs subdivision introduced above is outdated. Each enteroendocrine cell co-secretes multiple hormones with spatio-temporal, crypt-to-villus, and rostro-caudal variability, leading to the formation of overlapped gradients of individual hormones along the GI tract; the concept of well-defined subclasses of cells committed to express a specific subset of hormones independent of their location is currently untenable, thus detailed description of the topographical location of the cells needs to be implemented for future clarity (4).

Collectively, the EECs are responsible for the production of more than 30 different hormones that help to orchestrate the fate of the intermediary metabolism; acting upon different organs such as the pancreatic islets, the hypothalamus or the stomach, for the release of insulin, to regulate food intake or gastric emptying respectively (5–8).

Surprisingly, this heterogeneous and highly plastic population of cells is known to differentiate from a single staminal progenitor that gives also rise to enterocytes, goblet and paneth cells (1, 9).

It has been known for more than a century that the gut is capable to stimulate the endocrine portion of the pancreas and even improve the hyperglycaemic state of diabetic patients (10, 11). In 1932, the Belgian investigator LaBarre referred to these "factors" extracted from the intestinal mucosa as "incrétine," deriving it from: INtestinal seCRETion of insulin (12). In the 60s, different authors demonstrated that oral glucose was capable to induce a 2-fold increase in insulin compared to an in-vein isoglycaemic administration (13).

In the last three decades, the incretin-effect has been attributed primarily to two peptide hormones, the gastric-insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1), excreted primarily by duodenal (K) and ileo-colonic (L) enteroendocrine cells respectively (14). Indeed, type 2 diabetes (T2D) is a metabolic disease reported to involve an impaired intestinal release of GLP-1 and its co-secreted peptides oxyntomodulin and glicentin (15–17), together with an insulinotropic resistance to GIP in the pancreas (18) which lead to a deficient incretin system, purportedly causing the disease (19, 20). Despite being still largely unknown how hyper caloric diets are disrupting the incretin signaling, some authors have shown that even circadian rhythms disruption, and the saturated fat palmitate, are significant stressors capable to hamper GLP-1 secretion (21, 22).

Obesity and Type 2 diabetes are chronic diseases for which the most effective treatment is bariatric surgery. These invasive gut surgical procedures, aimed to reduce absorptive surface area of the proximal GI tract, such as Roux-en-Y gastric by-pass (RYGB) or Sleeve Gastrectomy (SLG), are associated with an improved glycaemic control, weight loss, and often with complete remission from T2DM (23).

Despite this, the complete remittance of a great fraction of RYGB patients represents a fascinating new case-series that points at the importance of the EECs and its modulation of the whole-body metabolism (24). As such, the study of this complex endocrine organ, might help us to create new pharmacological tools to amend the specific molecular axis that drive T2DM and the associated co-morbidities known to affect the cardiovascular (25, 26) and renal system (27, 28).

A panoply of contradictory studies have attempted to establish what is the possible role of GLP-1 or other gut peptides in the rapid, and long-lasting remittance from T2D after bariatric surgery, but no consensus about the identity of the molecular players has yet been reached (29–39).

Since 2005, there are on the market only two classes of drugs that attempt to bolster glucagon-like peptide-1 signaling, GLP-1 receptor agonists and DPP-IV inhibitors, for a supra-physiological GLP-1 activity. Unexpected safety-issues and important side-effects (40) prove that the peripheral hijack of this peptide is not sufficient, and does not replicate the remittance seen in bariatric surgery.

This review summarizes the most recent studies that reframe our understanding of the physiology of GLP-1 in health and disease.

## CHEMOSENSATION IN GLP-1-PRODUCING CELLS

Intestinal proglucagon expressing cells were historically named L-cells more than 4 decades ago because of their large 500 nm secretory granules seen under electron microscopy (41). Today, we know that these are nutrient-responsive enteroendocrine cells that secrete a variety of peptide hormones, primarily derived from the proglucagon gene (*GCG*) (42). Once translated, the 180 amino acid long GCG protein is processed by two proteases, Psck1 and Psck3, to give GLP-1, GLP-2 but also the less studied and understood glicentin and oxyntomodulin (43). Other peptide hormones, such as insulin-like peptide 5 (INSL5) (44, 45), PYY (46), GIP and neurotensin (17, 47) can be co-expressed with the *GCG* products depending on the topographical localization of the cell; surprisingly, it appears that GLP-1 and PYY can be excreted independently possibly due to the existence of compartmentalized secretory vesicles (48).

There appear to be considerable species-specificity in terms of anatomical localization of GLP-1 production as summarized in **Figure 1**. Independently of other hormones, in mice the distal colon and rectum show the higher levels of GLP-1 per gram of tissue. Conversely, in rats the distal ileum and in pigs the caecum are the anatomical regions with the highest amounts of GLP-1 (49). In humans, the density of GLP-1 and PYY positive cells increase steadily along the small intestine, decreasing in the colon, and then raising again reaching a maximum density in the rectum with the highest values of around 150 GLP-1-expressing cells per square millimeter. Curiously in type 2 diabetes, an equally distributed gradient of GCG and PC1/3 mRNA appears upregulated, but with normal GLP-1$^{+}$ cell densities, indicating a possible translational resistance (51).

The L-cells derived cocktail of hormones is believed to play pivotal roles in digestion, for example slowing down the GI motility (PYY) and suppressing the appetite *in vivo* (GLP-1, oxyntomodulin, PYY), apparently in response to direct sensing of the gut luminal content via G-protein coupled receptors or through neuronal circuits (43, 52).

Current *in vitro* technologies are not capable to support for long-term *ex vivo* the growth of isolated GLP-1 producing-cells. The available knowledge about the biology of GLP-1 is primarily drawn upon studies operated with the murine-derived GLUTag or STC-1, and the human-derived NCI-H716 cell lines. It is important to understand that these *in vitro* models express a different hormonal cocktail and respond to different chemical stimuli than intestinal L-cells *in vivo* (53, 54). Primary cultures are another useful short-term system; nonetheless GLP-1-producing cells amount to only 1–2% of the whole cultured mucosal population, with considerable intra and inter-assay variability (53).

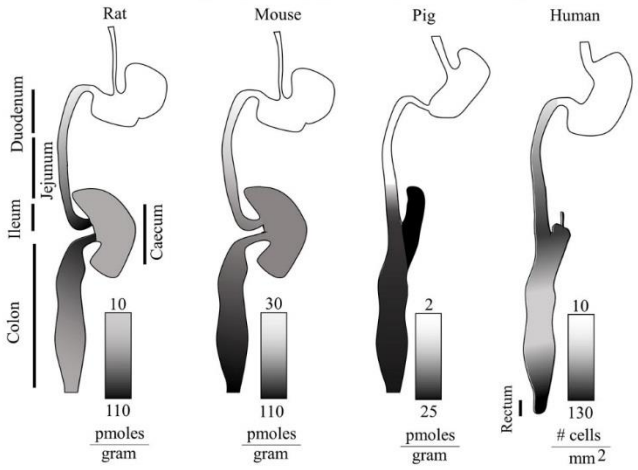


**FIGURE 1 |** Intestinal glucagon-like peptide-1 expression across species. Total GLP-1 expression along the rat, mouse, pig and human intestinal tracts (relative lengths not to scale) is displayed with gradients as individually indicated in figure. The rat GI tract shows the highest levels of GLP-1 in the ileum and proximal colon. On the other hand the murine gut, displays the highest GLP-1 levels in the distal colon. The porcine intestine shows highest levels in the caecum and distal colon, and virtually none in the proximal small intestine. In humans, a steady increasing gradient along the small intestine is followed by a decrease in expression in the colon, and a second steeper gradient culminating in the rectum with the highest GLP-1 expression (49–51).

The more physiologically relevant studies make use of *in vivo* transgenic mice, *ex vivo* perfused intestines or, more recently, crypt organoids derived from human, mouse or porcine guts (55).

*In situ* immunostaining and FACS studies have demonstrated that the hormonal secretome of GLP-1-secreting-cells is anatomically dependent. In the upper gut where these cells are more sparse and rare, GLP-1 is co-expressed with GIP, a K-cell feature, but also with cholecystokinin (CCK) and Neurotensin (NT). Conversely in the colonic mucosa, GLP-1 co-localizes with PYY, CCK and the orexigenic Insulin-Like peptide 5 (INSL5) (4, 43, 45, 53, 56, 57). Interestingly, colonic L-cells possess twice as much total GLP-1 compared to L-cells from the upper GI tract (53). Furthermore, considering the differential response to glucose, it is clear that the physiology of this population of EECs is distinct, and evolved under a different evolutionary pressure dictated by the exposure to a different luminal content (53, 58).

L-cells are known to modulate the release of their hormonal cargo in response to the activation of a plethora of receptors capable to sense fats, carbohydrates, proteins and many other compounds. Enteroendocrine cells, like other endocrine, muscle and neuronal cells, are electrically excitable. Membrane depolarization, triggered by a ligand-bound receptor, results in a spike of intracellular calcium ($Ca^{2+}$) which leads to the fusion of the endocrine granules with the lateral and the broader basal side, resulting in the discharge of a hormonal cargo in the capillaries of the mucosa.

Surprisingly, the EECs in the colon have been demonstrated to physically connect through a basal process named Neuropod, with afferent nerve cells residing in the lamina propria, defining a neuroepithelial circuit that expands the physiology of these cells (59). In fact, the idea of a direct neuronal regulation has been demonstrated decades ago in rats, where a bilateral vagotomy massively downregulates circulating PYY and GLP-1 levels after a glucose load (60). Furthermore, intracerebral acute, but not chronic administration of GLP-1 in mice, improves pancreatic glucose stimulated insulin secretion (61).

## GPCRs AS MOLECULAR TASTANTS

G-protein coupled receptors (GPCRs) are evolutionary ancient proteins spanning seven times across the plasma membrane of virtually any known cell type. In metazoans, these proteins evolved into thousands different molecular transducers capable to translate the presence of extracellular molecules into intracellular cascades of messages amplified by different G-proteins, which in turn enforce a myriad of different cellular processes via secondary messengers (62). The transmembrane domain of these chemosensors being exposed to a tighter evolutionary pressure lead to a relative evolutionary stability of the same 3-dimensional structure. On the contrary, the extracellular facing portion is what primarily defines the identity of a myriad of different receptors, capable to sense a panoply

of molecular entities ranging in size from a single atom to hundreds aminoacids long proteins. The intracellular portion of these nano-sensors, has evolved in humans in a complex hub that triggers multiple molecular cascades that results in short-term and long-term modifications of the target cell and even the whole-body metabolism.

Different receptors, expressed by the same cell type or tissue, can trigger the same molecular cascade. With this notion, the study of these molecular transducers has been approached by some authors in recent years from a top-down point of view, whereby sub-type specific, allosteric positive or negative modulators (PAM, NAMs), as well as direct agonists, are utilized as tools for pathway dissection and analysis (63, 64). In the last decade, technological advancements in techniques such as circular dichroism (65), Cryo-electron microscopy (Cryo-EM) (66) and crystallography (67) have expanded our understanding of the physiology of multiple chemosensors expressed by L-cells, which led to the discovery of new molecular tools with possible future clinical applications in diseases such as type 2 diabetes (64, 68–70).

The expression of different GPCRs to restricted anatomical regions, such as the enteroendocrine cell system, is a finely tuned system that evolved in metazoan. Macronutrients, bile acids (BAs), and microbiota-derived compounds activate many of these GPCRs expressed by GLP-1 expressing cells (71). Nonetheless, not all intestinal stimuli signals through these chemosensors; for example glucose induces the release of GLP-1 from human duodenum and ileum via electrogenic transporters (SGLT1) and voltage-gated Calcium and Sodium channels responsible for the membrane depolarization and hormonal release (53, 72).

The main G protein-coupled receptors which activation appears to cause the release of GLP-1 are: GPRC6A (73), GPR40-41-42-43-93-119-120 (43), GPR142, GHS-R1A (74), Tas1R2-Tas2R3(T1R2-T1R3) (75), GPBAR1 (TGR5), and CasR (6, 76, 77) (**Table 1**). The functional differences seen between Jejunum-Ileal and colonic GLP-1 producing cells, could be explained by a different pool of GPCRs, or possibly by the presence of heteromers displaying a more complex pharmacology than with each individual receptor.

A summary of the recognized main activities of all the major GLP-1-secreting receptors, including the GIPR (93, 94), is shown in **Table 1**.

Many of these chemosensors are also expressed by other enteroendocrine cells, so that the same dietary ligand traveling along the GI tract, leads to the release of multiple hormones.

There are some receptors, such as GPRC6A, with a pleiotropic distribution and still a limited understanding of its physiology. GPRC6A is highly expressed in GLUTag cells, and its activation by L-ornithine has shown to induce GLP-1 secretion (102). Nonetheless, mice deficient for the receptor, show no difference in responsiveness to both L-ornithine and L-arginine (103).

## THE PHYSIOLOGY OF GLP-1

In the last three decades a major tenet seeing GLP1 $(7\text{-}36)_{NH2}$, GLP1 (7-37) and the Gastric Insulinotropic Peptide (GIP) as the major contributors of the physiological incretin effect has reached widespread consensus (104). The remaining Glucose-stimulated insulin secretion (GSIS) appears to be enhanced by nutrients, hormones such as CCK, bile acids and endogenous ethanolamides. Animal models show compensatory mechanisms by which, in absence of a major incretin axis, other minor pathways are promoted in the β-cells to maintain their metabolic activity; namely proteins such as GPR119, or the CCK A receptor itself are upregulated, implying a highly plastic metabolic adaptation (105).

Multiple cell types found in the enteroendocrine cell system, the pancreatic islets or the brain have been shown to express the GCG product, a 180 aminoacids long peptide known as proglucagon (PG) (106, 107), which gets trimmed tissue-dependently into at least 6 different bio-active peptides, namely glicentin, oxyntomodulin, glucagon, miniglucagon, GLP-1 and GLP-2 (108, 109). The post-translational processing of the preproglucagon gene into the individual peptides is controlled by two distinct serine proteases, specifically prohormone convertases named Psck1/3 and Psck2, also known as PC1/3, or just PC1, and PC2 respectively (107, 108, 110). PC1/3 and PC2 are responsible for the metabolism of a plethora of peptide pro-hormones, including insulin and GCG among others (111). In particular PC1/3 expressing cells, such as intestinal L-cells and pancreatic β-cells, produce GLP-1, GLP-2 oxyntomodulin and glicentin (110, 112), while PC2 action on PG results in the production of glucagon and its active metabolite mini-glucagon (113, 114). Differential expression of PC genes regulates the hormonal output, and indeed it has been proven that both are expressed along the intestine, with PC1/3 positive cells found more distally than PC2 expressing cells (51), likely secreting glucagon (115). Indeed, the RYGB surgery removes the biggest pool of PC2/glucagon expressing cells from the exposure to nutrients, possibly contributing to the surgical success.

Active GLP-1(7-37), in human and mice is largely metabolized by the enzyme peptidyl-glycine α-amidating monooxygenase (PAM) into the equally active GLP-$1(7\text{-}36)_{NH2}$ (49, 116). Both these peptide species are trimmed at their N-term, and inactivated by the ubiquitous protease dipeptidyl-peptidase-IV (DPP-IV), found in the intestinal capillaries, vena porta and liver. Indeed, it has been estimated that just 10-15% of the secreted GLP-$1(7\text{-}36)_{NH2}$ reaches the systemic circulation (117), with some authors reporting meager peripheral meal-induced changes in both healthy and diabetic people (118). Furthermore, the DPP-IV product, GLP-$1(9\text{-}36)_{NH2}$, is trimmed into GLP-$1(28\text{-}36)_{NH2}$ and GLP-$1(32\text{-}36)_{NH2}$ by another ubiquitous protease, known as NEP24.11, CD10 or also Neprilysin among other names (119, 120).

Indeed, these once thought inactive metabolites of the recognized GLP-1 receptor agonist GLP-$1(7\text{-}36)_{NH2}$ have recently shown to possess multiple beneficial properties. The 9 aminoacids long GLP-1(28-36) protects β-cells from glucolipotoxicity (121), diet-induced steatosis of the liver (122), improves hepatic glucose tolerance in diabetic mice (122–124). Similarly, the 5 aminoacids long GLP-$1(32\text{-}36)_{NH2}$ improves glucose disposal, increases energy expenditure and protects β-cells in a diabetic environment *in vivo* (125–127). Indeed GLP-1(9-36) pharmacodynamics studies in human might be partially explained by the activity of its metabolites (128).

**TABLE 1** | Demonstrated primary effects of the major GLP-1-stimulating receptors.

| Receptor | Ligand | Effect | Experimental condition | References |
|---|---|---|---|---|
| FFAR1/GPR40 | Palmitate | Insulin ↑, glucagon ↑, somatostatin↑ | *Ex-vivo* human islets | (78) |
| | Free fatty acids | GLP-1 ↑, GIP ↑ | *In-vivo* mouse | (79) |
| | Long chain fatty acids | CCK ↑ | *Ex-vivo* murine duodenal I cells | (80) |
| FFAR2/GPR43 | Inulin | PYY ↑ | *In-vivo* diabetic mouse | (81) |
| | Propionate | PYY ↑, GLP-1 ↑ | *Ex-vivo* murine colonic Primary cultures, & *in-vivo* murine and rat | (82) |
| FFAR3/GPR41 | Propionate | PYY ↑, GLP-1 ↑ | *Ex-vivo* murine colonic primary cultures | (83) |
| FFAR4/GPR120 | α-Linolenic acid | GLP-1 ↑ | *In-vivo* mouse | (84) |
| | Lard oil, corn oil | GIP ↑, CCK ↑ | *In-vivo* mouse | (85, 86) |
| GPR119 | Oleoyl-LPI, OEA | GLP-1 ↑ | *In-vitro* murine GLUTag, *ex-vivo* human colon | (87, 88) |
| | AR231453, AR435707, AR440006, OEA, 2-0G | PYY ↑, GLP-1 ↑, GI motility ↓ | *Ex-vivo* murine gut, *in-vivo* healthy and diabetic mouse, *ex-vivo* human colon | (89, 90) |
| | Hypergl* + AR231453 | Insulin ↑ | *In-vitro* murine MIN6 | (88) |
| | Hypergl. * Compounds A/B$^{\Delta}$ | Insulin ↑ | *Ex-vivo* rat pancreas | (91) |
| | Hypogl.** Compounds A/B$^{\Delta}$ | Glucagon ↑ | *Ex-vivo* rat pancreas | (91) |
| | DS-8500a | Insulin ↑, glucagon ↑, GLP-1 ↑, GIP ↑, PYY ↓ | Type 2 diabetic humans | (92) |
| GIPR | Hypogl.** + GIP | Glucagon ↑ | Type 1 diabetic humans | (93) |
| | Hypergl.* + GIP | Insulin ↑, somatostatin↑ | Healthy humans | (94) |
| | GIP | IL-6 ↑ | *Ex-vivo* human, and murine α-cells | (95) |
| GLP-IR | GLP-1 | Insulin ↑, somatostatin↑, glucagon ↓ | *Ex-vivo* healthy murine pancreas | (96) |
| | GLP-1 | Appetite ↓ | *In-vivo* intracerebral rat | (97) |
| | GLP-1 | GLP-1 ↑ | *In-vitro* murine α-TC 1-6 | (98) |
| | Exendin-4 | Glucagon ↓ | *Ex-vivo* healthy rat pancreas | (99) |
| | Exendin-4 | Glucagon ↑ | *Ex-vivo* diabetic rat pancreas | (99) |
| TGR5 | Hypergl.* + INT-777$^{\dagger}$, or LCA§ | GLP-1 ↑, insulin ↑ | *Ex-vivo* healthy human, and murine diabetic islets | (100) |
| | Taurodeoxycholate | GLP-1 ↑ | *Ex-vivo* murine primary ileal cultures | (101) |

*Analytes are indicated as up (↑) or down (↓) regulated. All in-vivo, or in-human studies, indicate peripheral plasmatic levels. *(Hypergl.) and **(Hypogl.) indicate conditional presence/hyperglycaemia, or absence of glucose/hypoglycaemia. §(LCA) lithocolic acid, †(INT-777) semisynthetic bile acid, (GSIS) Glucose-stimulated insulin secretion. Δ(Compounds A and B) are experimental GPR119 agonists described by Li et al. (91).*

These metabolites have possibly important implications for any future treatment of metabolic pathologies such as type 2 diabetes, where our understanding of the pharmacokinetic and pharmacodynamics in humans is virtually absent (128).

In healthy humans, intact GLP-1(7-36) $_{NH2}$ is mainly released by intestinal EECs after the ingestion of food, especially meals rich in fat and proteins (14, 129). Other stimuli, such as physical activity, are also capable to raise its plasmatic levels for up to 90 min after exercise (130).

This hormone generates both short-term and long-term pleiotropic effects. GLP-1 stimulates the β-cells to produce Insulin, blocks pancreatic α-cells' glucagon release via somatostatin (96), slows down gastric emptying (131), improves peripheral glucose tolerance (132), suppresses appetite in the hypothalamus and amygdala (97), increases β-cell mass, GSIS, and elicits protection from glucolipotoxicity (133) and apoptosis (134). Curiously, it also regulates bone physiology (135), and shows anti-inflammatory properties (136).

On the other hand, the most abundant DPP-IV-processed metabolite GLP-1 (9-36)$_{NH2}$, has also been reported to have biological activities, protecting human aortic endothelial cells and cardiomyocytes *in vivo* in dogs (137) and *ex vivo* in mice (138) and rats (139), even in the absence of a GLP-1 Receptor (139, 140). Some authors postulate the existance of an unknown GLP-1(9-36)$_{NH2}$ receptor (141, 142), because indeed this cleaved peptide is found in peripheral blood at one order of magnitude higher concentrations than "active" GLP-1 (7-36)$_{NH2}$ and shows cardioprotection, antioxidant properties (138) and appears capable to also inhibit hepatic neoglucogenesis (141).

GLP-1 (7-36)$_{NH2}$ itself is known to have general protective and modulating cardiovascular effects (143), as shown by different commercial GLP-1 mimics with proven cardioprotection type 2 diabetes (144).

In healthy fasted individuals, it is recognized that peripheral plasmatic active GLP-1 $(7\text{-}36)_{NH2}$ plasmatic levels hover around 5 pM, but within 5–10 min after an oral glucose load, they start to rise, up to a maximum of less than 10 pM after 40–90 min, and slowly descend back to baseline values in 150 min. On the other hand, the cleaved GLP-1 $(9\text{-}36)_{NH2}$ summed to the GLP-1 $(7\text{-}36)_{NH2}$ to give what is normally referred to as total GLP-1 levels, raise up to more than 40–60 pM (108). In perspective, GIP and Insulin show much broader dynamic ranges, with post meal levels reaching 300 and 400 pM respectively, from their baselines <20 pM within 30 min post glucose ingestion (108, 145). Curiously, some bariatric RYGB patients experience up to a 10-fold increase in post-meal active GLP-1 plasmatic levels (from fasting 5 pM to post-prandial 30–65 pM) (146), and have a 2- to 3-fold higher glucose-stimulated Insulin secretion (147), which in some diabetic patients results in GLP-1-mediated hyperinsulinemic hypoglycaemia that requires GLP-1 antagonism or surgical reversal of the intestinal anatomy (148).

Different authors consider the success of surgical intervention a consequence of a major change in gut hormonal profile, primarily a supra physiological post-prandial GLP-1 secretion (29, 30). This reasoning fits with the observation that type 2 diabetic patients display a shorter post-prandial peak of GLP-1, hence they are deficient for the longer response seen in healthy individuals. Multiple groups describe diabetic patients with lower plasmatic GLP-1 but heightened GIP levels and β-cell resistance to the stimulatory effect of both GLP-1 and GIP (18, 149–153).

Nonetheless, different animal models deficient for GLP-1 signaling, in addition to human studies, prove the dispensability of GLP-1 for surgical success (31–34), questioning the causative nature of GLP-1 for the reported metabolic benefits.

On the other hand, PYY has been proven to be upregulated, and necessary, for RYGB mediated restoration of the diabetic islets, and overall cure of diabetes in rats (35) and humans (154).

Another important source of endogenous GLP-1 is the brain, a tissue where it acts as a neurotransmitter. Indeed central GLP-1 production appears essential, since peripheral GLP-1 is assumed to not be able to cross the blood-brain barrier (BBB). In particular, neurons of the hindbrain found in the nucleus-tractus solitarius (NTS) secrete GLP-1 and activate hypothalamic neurons of the paraventricular nucleus (PVN), resulting in satiety (155, 156). Indeed it is clear that PC1/3 dominant neurons of the NTS express also other the PG peptides oxyntomodulin, glicentin, and GLP-2 together with GLP-1 (157). Although expressed at much lower levels, PC2 activity has also been recognized in these neurons, and traces amounts of glucagon might have important implications.

NTS neurons-derived GLP-1 appears to reach out to multiple locations within the central nervous system (CNS), which have been proven to express the receptor, and be activated after a central administration of GLP-1 receptor agonists. These areas include the NTS itself, the supraoptic nuclei, the arcuate nucleus (ARC) and the area postrema (AP) other than corticotropin-releasing hormone (CRH) PVN neurons (158, 159). Beyond satiety, this signaling appears to be a key factor for neuroprotection (160) insulin sensitivity and glucose metabolism (158).

Curiously, the feeling of satiety, is also achieved by another neurotransmitter, the Cocaine- and amphetamine-regulated transcript (CART) (161). This peptide, acts also as a hormone, and is expressed by both β-cells and intestinal GLP-1 and GIP producing cells causing GLP-1 secretion *in vivo* via a yet unknown GPCR (162).

It is not entirely clear to what extent endogenous GLP-1 activates all the reported GLP-1 receptor expressing neurons and to what extent it depends on the CART peptide especially in type 2 diabetes or obesity. Nonetheless, some commercial mimics of GLP-1, such as Liraglutide, even when administered peripherally, appear to cross the BBB and activate neurons within the ARC resulting in GABA dependent inhibition of neuropeptide Y (NPY) and agouti-related peptide (AgRP) secretion. This signaling has proven to be essential for the Liraglutide mediated weight loss in rats (163). GLP-1R expressing hypothalamic neurons have proven dispensable for the beneficial metabolic activity of both BBB permeable Liraglutide and Exending-4 (164).

Singularly, BBB impermeable mimics of GLP-1 have still shown to activate GLP-1 Receptor expressing neurons (165), but they require a functional gut-brain axis through the vagus nerve (166). In particular, vagal afferent neurons expressing the GLP-1R are necessary for GLP-1 mediated induction of satiety (167) but not glucose lowering effects (168).

The complex inter-organ pharmacokinetic of GLP-1, compounds into a convoluted pharmacodynamics encompassing multiple metabolic systems.

Indeed the GLP-$1(7\text{-}36)_{NH2}$ receptor, a GPCR, is found to be expressed by a wide range of tissues and cells such as: α, β, and δ-cells (169), sinoatrial node myocytes, arterial smooth muscle cells of lungs and kidneys, megakaryocytes, macrophages, monocytes, lymphocytes, gastrointestinal tract mucosa [mainly Brunner's gland in the duodenum, but also in the parietal cells of the stomach, jejunum ileum and the nerve plexus around the small and large intestine (170, 171)], central nervous system [neocortex, cerebellum, thalamus, amygdala, area postrema, hypothalamus, hippocampus, nucleus tractus solitarius (158)], peripheral nervous system (myenteric plexus) and in the skin (14, 172–176).

Counterintuitively, mice completely defective for the GLP-1 receptor were reported to be protected from high-fat diet-induced peripheral Insulin resistance (177) and, consistently with this, central inhibition of GLP-1R signaling with the antagonist exendin 9-39 improves glucose tolerance and glycaemia (178). Conversely, mice defective for both the receptors for glucagon and GLP-1, or GLP-1 and GIP, show a highly plastic entero-pancreatic system that adapts and gives these animals no overt phenotype in terms of glucose homeostasis (105).

Nonetheless, the pharmacological activation of the GLP-1R is clinically beneficial (179), offering an improved glycaemic control with lower cardiovascular morbidity and without the risk of hypoglycaemia associated with some current anti diabetic drugs (173). Furthermore, being an appetite suppressant, GLP-1 signaling also helps to lose body weight, especially if in combination with metformin. Conversely, anti-diabetic drugs such as sulfonylureas, or Insulin, are known to induce not only weight gain (180, 181), but also an increased risk of

hypoglycaemic events (182). Pharmacological activation of the GLP-1 Receptor has also shown to help exogenous insulin in the control of glycaemia in patients with type 1 diabetes, by slowing the gastric emptying and blocking glucagon secretion (183, 184).

Currently, six different peptide GLP1-Receptor agonists are on the market, with more in clinical trials. In particular, two short-acting formulations of Lixisenatide and Exenatide and four long acting preparations of Exenatide, Liraglutide, Dulaglutide and the most recent and successful Semaglutide, were approved in October, 2017 for the North American markets by FDA[1] (25, 185). The first GLP-1 analog to be approved by FDA in 2005 for the management of Type 2 diabetes was the chemically synthesized Exenatide under the name of Byetta (186), a formulation of the DPP-IV resistant peptide discovered in the gila monster *Heloderma suspectum* saliva in 1992 (187). Despite the longer half-life in serum, Byetta needs to be injected twice a day. In the last decade, formulations with extended release entered the market with once-weekly self-administrations pens.

Pleiotropic beneficial effects have been reported for this class of drugs. Beyond the improved glycaemia control, essential for the short term treatment of diabetes (188), different GLP-1RAs are powerful clinical tools for the management of diabetic kidney disease (DKD) (28, 189) non-alcoholic steatohepatitis (NASH) (190), neuroinflammation (191), obesity and cardiovascular disease (192–195).

Although GLP-1RA are improving the lives of patients affected by type 2 diabetes or the metabolic syndrome (196), the physiology of GLP-1 is far from being clear.

More recent data suggest how the unimolecular co-activation of GLP-1 and GIP receptors, has powerful anti-diabetic effects superior to either agonism (197). Furthermore, oxyntomodulin is a natural dual-agonist of GLP-1 and glucagon receptors and displays anti-diabetic properties in humans (198, 199). Upon this finding, a tri-agonist peptide, targeting the receptors of GLP-1, GIP, and glucagon was created (200). The *in vivo* effects of this drug are unparalleled, even superior to what can be achieved with the dual agonists for either combination. The synergistic activation of these three important receptors is capable to revert diet-induced obesity, cognitive impairment and T2D in mice models, warranting future human studies (201, 202).

## EXPANDING THE PHYSIOLOGY OF GLP-1

When examining the physiology of glucagon-like peptide-1, it is important to consider that there is an expanding body of evidence that questions its systemic endocrine physiology (203, 204). Pancreatic α-cells have been demonstrated to express and secrete not only GLP-1 (205, 206), but also PYY (35) GIP (207, 208) mini-glucagon (209) or even Xenin (210) together with glucagon (**Figure 2**). The key protease responsible for the processing of the proglucagon peptide into GLP-1 is Psck1/3, which has shown to be upregulated in α-cells during hyperglycaemic, hyperlipidemic, or inflammatory conditions to promote glucose-induced glucagon suppression, a compensatory response to a metabolic insult as in type 2 diabetes (205). Insulin itself has shown to modulate PC1/3 expression to possibly aid its own metabolic activity (211).

Recently, the whole dogma of the role of intestinal GLP-1, envisioning the traveling from the gut to the liver and ultimately reaching the pancreatic β-cells to bind its GLP-1R has been questioned in transgenic mice (204). Indeed, since both DPP-IV degrades and NEP24.11 degrade GLP-1 within seconds, the possibilities of any intestinal GLP-1 to reach the system circulation and then the islet microcirculation are doubted. Besides, it is important to consider that intestinal GLP-1 has a local concentration in the nM range (10–100 pico moles per gram of tissue, see **Figure 1**), further advocating that the main action of this protein have evolved to be locally restricted.

Animals deficient for the GCG gene in the intestine, still experience a normal incretin effect disrupted with the GLP-1R antagonist Exendin (9-39) (204). This indicates that it is the intra islet, α-cell derived GLP-1 that shows the meal-induced insulinotropic properties. A critic to the use of a murine model deficient for intestinal GCG products, would be that other gut hormones might compensate for the lack of a functional GCG gene in that tissue, hence explaining the normalized incretin effect. Indeed other gut hormones such as GIP must be responsible for the incretin effect to a higher degree than once thought. Nonetheless, it is also clear that intra-islet GLP-1R signaling is essential for GSIS, with more evidence that an intra-islet paracrine GLP-1 signaling is physiologically present (212, 213) and necessary for β-cell health under metabolic (214).

In contrast, mice deficient for GLP-1R only in β-cells have a normal incretin response and oral glucose tolerance, indicating the dispensability of intra-islet signaling of GLP-1 for the incretin effect. Interestingly, these same animals have an improvement of their glucose tolerance in response to oral DPP-IV treatment, but not to subcutaneous GLP-1 mimics, indicating how the former relies completely on localized, non β-cell GLP-1R (215).

There are still multiple gaps into our understanding of how different GLP-1 producing tissues communicate, especially in the brain to islet axis. It is known that acute, but not chronic, central GLP-1 receptor activation directly modulates glucose-induced Insulin secretion implicating a direct brain to islet neuronal communication (61).

On the other hand, chronic GLP-1 activity in α-cells increases its own secretion, feeding an autocrine loop that gets overstimulated with the use of exogenous synthetic GLP-1R agonists [(98); **Figure 2**]. Curiously in diabetic rats, it has recently been shown that this loop might indeed induce the production of more glucagon than in healthy animals (99).

It has been known for more than two decades and has been confirmed more recently, that an infusion of GLP-1(7-36)$_{NH2}$ has insulinotropic and glucagonostatic effects. This is seen when the plasmatic levels are above 50–60 pM, equivalent to more than five times the levels seen post-prandial in healthy individuals challenged with a bolus of glucose, or 10-fold their basal levels (153, 216), adding further doubt to the physiological hormonal dogma of intestinal GLP-1. Considering the mounting

[1]http://press.novonordisk-us.com/2017-12-5-Novo-Nordisk-Receives-FDA-Approval-of-OZEMPIC-R-semaglutide-Injection-For-the-Treatment-of-Adults-with-Type-2-Diabetes

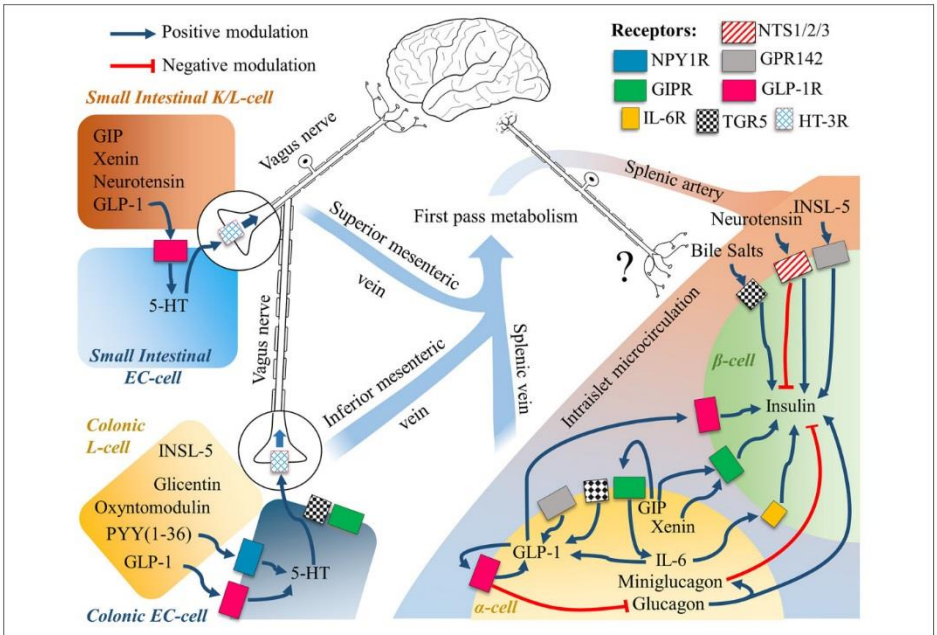


**FIGURE 2 |** The gut-brain-islet axes of GLP-1. The intestinal EECs secretome is subject to first pass metabolism, while intraislet signaling relies on paracrine signaling. Intestinal cells are known to communicate with the Enteric Nervous System, and the Central Nervous System through the Vagus Nerve. Neuronal engagement between the gut lumen and the islets of Langerhans is a possible compounding explanation to the incretin effect, whereby the mechanistic of the single molecular players are still largely unknown. See text for further details.

evidence, it is clear that we need to understand what hormonal and/or neuronal signals are bridging the gut luminal content to the insulin secretion explaining the incretin effect. Given that Intestinal oxyntomodulin, glicentin, glucagon and GLP-1 expression have proven to be dispensable in mice (204); other intestinal hormones such as GIP, PYY, Neurotensin, INSL-5 or the GIP co-secreted Xenin (217) might play an important role (**Figure 2**). Currently, not much is known about the physiology of Neurotensin, INSL-5 and Xenin. The first two have been reported to be co-expressed with GLP-1 in the small and large intestine respectively, with Neurotensin being reported also in pancreatic β-cells (210), while Xenin in a sub population of duodenal GIP positive cells and α-cells. Neurotensin levels are correlated with leptin (218), rise in response to fatty meals, signals through two different G-protein coupled receptors known as NTSR1 and 2, and a third single transmembrane receptor, NTSR3, also known as sortilin (219). All of these receptors are expressed by pancreatic β cells, where their activation appears to mediate insulin release at low glucose levels and blockage at high levels (219, 220), (see right side of **Figure 2**). On the other hand, INSL-5 targets a GPCR known as GPR142, also known as RXFP4, a receptor found to be expressed by the NCI-H716 cell line (54), and both α and β-cells in the pancreas, and its activation directly stimulates the expression of GLP-1 and insulin, representing a possible new pharmacological tool for the treatment of type 2 diabetes (77, 221), and supporting a possible role for INSL-5 in the incretin effect. Xenin is another gut-derived food-induced peptide known to potentiate GIP activity (222, 223). Considering that α and β-cells express GIPR (224) and that the GIP-potentiating activity of Xenin has been reported to be lost in human diabetics (223), it appears to be a critical player in this disease, likely involving the activity of GLP-1.

In addition, both *in vitro* and *in vivo* Interleukin-6 (IL-6) has shown to be a powerful GLP-1 secretagogue, capable to positively modulate both the proglucagon gene, and the expression of PC1/3 in α-cells and intestinal L-cells (225, 226). Indeed, GIP has shown to not only be co-expressed with GLP-1 and glucagon in α-cells (207); it also stimulates in an autocrine/paracrine fashion the expression of IL-6 in the same α-cells, thus indirectly acting as a GLP-1 secretagogue (95).

IL-6 has shown also to induce the secretion of intestinal GLP-1, indirectly via the release of adipocytes derived Leptin (227).

Curiously, it was recently reported that this pro-inflammatory cytokine, IL-6, similarly, but independently from GLP-1, slows gastric emptying (228). Furthermore an inflammatory status, as seen in pathologies such as type 2 diabetes, might compromise the gut mucosal permeability, leading to the exposure of intestinal EECs to luminal LPS, and a TLR4-mediated release of GLP-1 (229). This is consistent with the knowledge that GLP-1, as well as glucagon, has shown to possess powerful anti-inflammatory properties *in vivo*, an area that hold with vast therapeutical potential (136, 230).

Ghrelin is another possible player, since it has been proven to be expressed not only in the gut, but also in a distinct subpopulation of islet cells named ε-cells (231) and, being known to be a stress-induced (232) GLP-1 secretagogue (233, 234), it might play an important role in the intra-islet signaling.

Recently, it has been demonstrated that mice with a deletion of the GLP-1 receptor only in β-cells, are resistant to the beneficial anti-diabetic effect of a vertical sleeve-gastrectomy (36), suggesting how GLP-1 activity in β-cells is key to the bariatric surgery success. It is not known if intra-islet α-cells production of GLP-1 is affected by the surgical procedure or, more importantly, how this axis is impaired in the metabolic syndrome, type 2 diabetes and related pathologies.

It appears that only in RYGB and SG patients intestinal derived GLP-1 has a true endocrine role, while in healthy individuals, localized, paracrine and neuronal signals primarily define the GLP-1 physiology.

It is therefore clear that currently available GLP-1RAs, mimicking on the peripheral action of GLP-1 $(7\text{-}36)_{NH2}$, not only ignore the yet unknown physiology of GLP-1 $(9\text{-}36)_{NH2}$ or its metabolites, but they also fail to address the tissue specific physiology of GLP-1 $(7\text{-}36)_{NH2}$, while pushing to supra-physiological limits the endocrine GLP-1 receptor axis, likely explaining the reported side-effects and only partial success in the treatment of T2D.

In addition, it is important to notice that the ubiquitous DPP-IV protease targets not only GLP-1 but also oxyntomodulin, GIP and PYY among other proteins (235). Specifically, the GLP-1 co-secreted cousin PYY(1-36), agonist of the vasoconstrictive Y(1) receptor, is physiologically trimmed by DPP-IV to give rise to the appetite-suppressant, anti-diabetic and blood-brain barrier permeable PYY(3-36) agonist of Y(2) receptor (220). It is therefore clear that pharmacological DPP-IV blockage disrupts this axis and induces hypertension (236).

Recent studies provide new evidence supporting the paracrine nature of intestinal GLP-1, whereby Serotonin-(5-HT)-secreting enterochromaffin (EC) cells are directly stimulated by locally produced GLP-1, which in turn stimulate afferent Vagal nerves (**Figure 2**) bridging the gut to brain axis. Accumulating evidence suggest that, especially in the colon, EC cells express multiple receptors for the microbiome metabolites, representing a new important link bridging the microbiome to the brain (237, 238).

A better way to amend the pathophysiology of GLP-1 reported in diabetes or other diseases, would be to induce tissue specific *de novo* GLP-1 production, leading to a more physiological and likely safer, short and medium distance signaling. Numerous attempts have been made with multiple GLP-1 secretagogues such as GPR119 agonists (239) but so far no compound has reached the market because of bioavailability issues and systemic off-target toxicity. One possible way to minimize the side-effects of the single drugs is to combine them to achieve synergistic effects, as reported recently with a combination of a DPP-IV inhibition, SSTR5 antagonism and GPR40 and TGR5 agonism, capable to raise circulatory active GLP-$1(7\text{-}36)_{NH2}$ levels to more than 300-400 pM in mice (240).

## SWEETNESS IN THE GUT

Studies *in vitro* and *ex-vivo* with isolated human primary cells suggest that there are two temporally distinct pathways that lead to the glucose-stimulated release of GLP-1, similarly to what happens in β-cells with the 1st or 2nd phase insulin release. A quick mechanism independent of the cell energetical state and a slower one, metabolism dependent, mediate the release of this incretin (53, 72).

The 1st phase in the pathway of glucose signaling, sees the electrogenic sodium-coupled glucose transporters 1 (SGLT1) mediated uptake of two $Na^+$ ions for every internalized glucose molecule (53). This depolarization is propagated through voltage-dependent Calcium and Sodium channels, which currents lead to the discharge of the hormones containing vesicles (72).

The 2nd phase is exemplified by the absorption of simple sugars, such as Glucose or Fructose, via the facilitative transporters GLUT2 and GLUT5 respectively, which leads to an increased internal metabolism mirrored by intracellular ATP levels. This state leads to the blockage of ATP dependent potassium channels and the subsequent membrane depolarization, followed by the secretion of the hormonal cargo.

Mace et al. (241) demonstrated how diazoxide, a $K^+$ATP channel opener, completely abolished the glucose-dependent incretin release while a channel blocker, tolbutamide, exacerbates it in terms of secreted GLP-1, GIP and PYY.

More recent data, question the first mechanism in enteroendocrine cells. Glucose mediated GLP-1 release happens in humans only in the proximal and distal small intestine and independently of ATP mediated potassium channels closure. Furthermore, concentrations of up to 300 mM glucose do not induce GLP-1 secretion from colonic human mucosa because GLP-1 producing L-cells barely express SGLT1 (43, 53, 58, 72).

Consistently, the use of α-methyl-D-glucopyranoside (MDG), an acaloric substrate of SGLT1, within 5 min triggers the release of GLP-1 as glucose does, demonstrating how it is the sodium current that triggers the release of the incretin, and not the metabolic ATP-driven arrest of potassium currents and following calcium spike (58).

The pharmacological blockage of SGLT-1 with phloridzin, in a rat small intestine perfused system, results in just a halved secretion of GIP, GLP-1, or PYY, and the addition of phloretin,

a GLUT2 inhibitor, brings these values down to basal levels. In fact, this double blockage of SGLT1 and GLUT2, completely inhibits the responsiveness to other stimulants as well, such as sucralose, glycylsarcosine, OEA, propionate and taurocholate. The activity of the calcium channel CasR is also essential for the responsiveness to free aminoacids (241).

All these observations are challenged by longer term *in vivo* studies. Blockage of SGLT-1 markedly improves glucose-stimulated GLP-1 release if a 3-h long period is considered.

The rationale given by Oguma et al. (242) is that SGLT-1 is expressed mainly in the small intestine, hence its inactivation results in heightened luminal glucose that travels down to the colon where it someway stimulates GLP-1 release. Given the fact that SGLT-1 is barely detectable in colonic proglucagon positive cells and that potassium channels in this tissue are unresponsive to sulfonylureas, the molecular sensor(s) that causes the release of GLP-1 *in vivo*, remains elusive.

Another enigmatic G protein is α-gustducin, a key element in sweet-taste transduction pathways downstream of the heterodimer formed between the GPCRs Tas1R2 (T1R2) and Tas1R3 (T1R3).

Its expression has been reported in colonic L-cells and appears to be responsible for the glucose-stimulated release of incretins (243, 244). This is confirmed by the impaired glucose-stimulated release of GLP-1 in mice lacking either T1R3 or α-gustducin (244).

Interestingly, this axis is also activated by the disaccharide sucrose and by the non-metabolizable and therefore anergic sucralose (243). Of note also Aspartame, Acesulfame K, Glycyrrhizin and Saccharin bind the sweet receptor heterodimer Tas1R2/3 and they have shown to stimulate GLP-1 secretion in the human duodenal adenocarcinoma-derived HuTu-80 cell line (245, 246). Despite this report, other groups weren't able to replicate these results (53). Indeed, it was shown that proglucagon expressing cells, derived from the colon of Venus mice cultures, were not responding significantly to Sucralose (1 mM) in terms of both released GLP-1 and intracellular Calcium. Conversely, proglucagon negative cells responded to the sweetener. More doubts about the role of Tas1 receptors were raised after the demonstration that oral gavage with sucralose, saccharin, stevia, acesulfame potassium or tryptophan do not cause a gut incretin release in Zucker diabetic fatty rats (247).

## LONG AND MIDDLE CHAIN FATTY ACID RECEPTORS

The study of the receptome of enteroendocrine cells, has provided invaluable pharmacological insight with the discovery of proteins capable to sense multiple compounds once thought to be only nutrients.

A prime example is given by two GPCRs, GPR40 and GPR120, also known as Free Fatty Acid Receptor 1 (FFAR1) and 4 (FFAR4) respectively. These chemosensors are two major molecular players in the detection of dietary, medium (C8-12) and long (C14-22) chain fatty acids (LCFA) (84, 248).

GPR40 is primarily expressed by the pancreatic β-cells, where it plays a pivotal role in FFA-mediated insulin secretion (249) but also in α-cells (78, 250), CCK (80), GIP (251), and GLP-1 (79) producing cells in the gut and in hypothalamic neurons (248, 252, 253). Animals deficient for this receptor are protected from obesity-induced hepatic steatosis, hyperinsulinemia, hypertriglyceridemia and hyperglycaemia. More than a decade ago a study showed that GPR40 mediates the long-term FFA-induced lipotoxicity seen in the diabetic islets (254); nonetheless, these findings are still under debate today. Recent data are still highly polarized, with some authors supporting (255), and others disproving this (256), or even indicating that GPR40 protects β-cells from lipotoxicity (257) rendering difficult to draw any conclusive mechanistic involvement in healthy and diabetic individuals. Nonetheless, the activation of this receptor with FFAs has demonstrated to induce the secretion of incretins (79, 258) glucagon (78, 250) and partially glucose-stimulated insulin (259, 260) reducing food intake, and lowering body weight in animals models (261). Mice without a functional GPR40 display an impaired CCK and GLP-1 secretion after an oil gavage, while surprisingly animals deficient for GPR120 display a normal corn oil-induced GLP-1 secretion (80, 262).

GPR40 is coupled to both Gq and Gs proteins and *in vivo* studies suggest how signaling through both these cascades elicits the most powerful GLP-1 secretion (258). Ligands that bind GPR40 and activate predominantly only the Gq pathway are not good GLP-1 secretagogues. Indeed recently it has been shown that dietary triglycerides appear to induce the secretion of GLP-1 via GPR40 in synergy with the Gs activating GPR119 (263). Nonetheless, chylomicrons have been reported to be powerful GPR40-Gq activators and GLP-1 secretagogues, acting from the basolateral side of the intestinal mucosa (264).

The two synthetic GPR40-specific compounds AM-1638 and AM-5262, have been found to act as double Gq and Gs agonists but also as positive allosteric modulators, capable to enhance the GLP-1-secreting capabilities of Gq-only agonists such as dietary docosahexaenoic (DHA) and α-linolenic acid (ALA), independently of the orthosteric site (265).

GPR120 shows very little sequence similarities to the other free fatty acid receptors but, likewise, is found to be expressed by the enteroendocrine cell system, especially in the colon (see **Figure 3**), but also in the lungs (267), white and brown adipose tissue (274, 275), hypothalamic microglia (253), macrophages and, contrarily to GPR40, not in β-cells but in somatostatin producing δ-cells (276). Both small intestinal GIP and colonic GLP-1 secreting cells express GPR120, and the molecular cascade triggered by this receptor has been shown to mediate dietary incretin release directly or indirectly through CCK (84–86). Interestingly, both of these two receptors are expressed only by a fraction of hormone positive EECs; in particular, it has been reported that only 3% of GLP-1 positive cells express GPR40, and 23% GPR120 (266).

GPR120 displays a ligand preference similar to GPR40; a broad range of long chain fatty acids signal through it, with some ligands eliciting more robust calcium responses than others (84). Multiple dietary compounds have shown to be powerful agonists of GPR120, such as pinolenic acid, a poly-unsaturated fatty acid

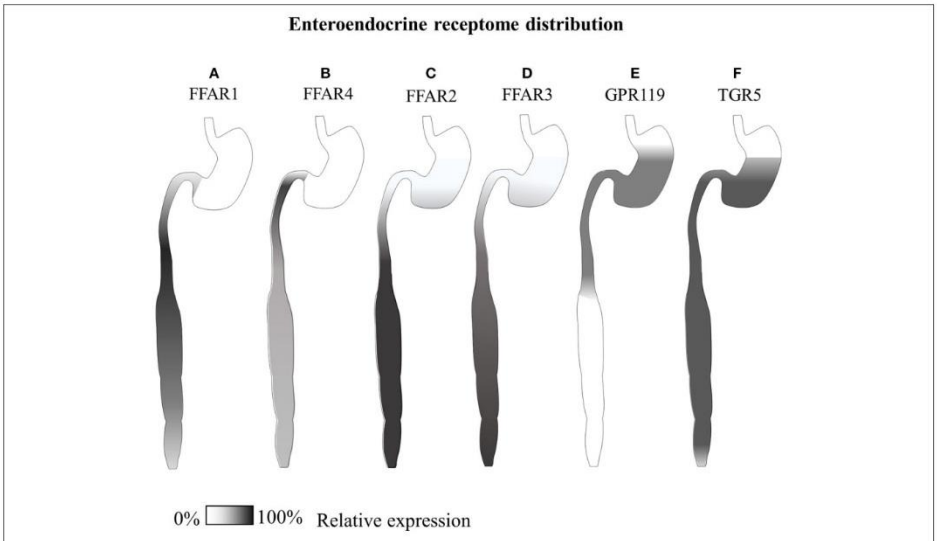


**FIGURE 3 |** Gastrointestinal GLP-1-secreting receptome distribution. Summary of available expression studies of different GLP-1-secreting receptors along the gastrointestinal tract. **(A)** GPR40/FFAR1 has been reported to be expressed in the small intestine in different EECs, with overall higher transcript levels than GPR120, and superior co-localization with GIP in the distal small intestine (79, 80, 266). **(B)** GPR120/FFAR4 has shown co-expression with both proximal small intestinal GIP$^+$ and large intestinal GLP-1$^+$ cells (85, 266–268) **(C)** GPR43/FFAR2 and **(D)** GPR41/FFAR3 are co-expressed by all types of enteroendocrine cells, from the stomach to the rectum, especially in the colon (83, 269, 270). **(E)** Reports of comparative GPR119 transcript are contradictory, while immunohistochemical data indicate co-localization with a minor fraction of CCK and GLP-1 positive cells mainly in the stomach and small intestine (266, 271). **(F)** TGR5, has been reported equally distributed along the whole gastrointestinal tract of dogs (101, 272, 273).

(C18:3 *trans, cis, cis* Δ 5, 9, 12) found in pine nut oil (277), or the yeast derived phytosphingosine (278).

In macrophages and adipose tissue, GPR120 mediates ω-3-mediated anti-inflammatory and insulin sensitizing effects (279, 280). Contrarily to GPR40, the genetic deficiency of GPR120 is more dramatic. Knockout animals show hyperinsulinemia and insulin resistance, hyperglycaemia and osteoarthritis (281), hepatic steatosis and therefore obesity. Furthermore, an absence of GPR120, results in an overactive glucagon signaling, explaining the hyperglycaemia (282). Indeed, in humans, a single aminoacid mutation of the receptor that hampers its signaling is associated with obesity and insulin resistance (283). Expectedly, GPR120 agonism shows powerful anti-diabetic, anorexic, and hepatoprotective properties in multiple animal models (275, 284–287), at least partially mediated by GLP-1 (288).

Considering the overlap of natural ligands of GPR40 and GPR120, it has been difficult to study them individually and understand their individual physiology, while recent data indicate that indeed these two receptors work synergistically, to exert anti-diabetic activity *in vivo* from the gut (289), and the brain (253).

Despite these advancements, in clinics there are currently no available drugs targeting GPR40 and GPR120. TAK-875, the best candidate for GPR40 which showed promising GSIS capabilities up to Clinical Phase III for the treatment of T2D, had to be halted because of hepatotoxicity and alteration of bile salts composition (290).

Despite these setbacks, encouraging animal data warrant future efforts for the development of new drugs capable to activate synergistically both GPR40 and GPR120 and mediate, through GLP-1 and other intestinal, pancreatic and cerebral peptides, better treatments for multifactorial chronic metabolic diseases.

## SHORT CHAIN FATTY ACID RECEPTORS

In 1997, four 7 α-helixes transmembrane receptors, GPR 40, 41, 42, and 43 were mapped on the same locus found on the long arm of chromosome 19 (291). Soon after, different groups identified GPR 43 and 41 as the receptors for free fatty acids, which were then chronologically renamed FFAR2 and FFAR3 respectively (292–294).

Both these receptors are activated by similar types of short chain fatty acids (292), and both these signal through an inhibitory G type protein, but FFAR2 is also capable to signal

through Gq/11 proteins (293) by which it has shown to mediate GLP-1 and PYY secretion *in vitro* and *in vivo* (82, 295).

Along the gastrointestinal tract, both GPR 41 and 43 have been reported to be co-expressed, with FFAR2/GPR43 at higher levels and overall number of cells, especially intraepithelial leukocytes, while FFAR3/GPR42 is found on submucosal neurons [see **Figure 3**, (83, 295–297)]. Indeed FFAR2 holds promise for the management of Inflammatory Bowel Disease (IBD) (298) a possible side-effect of anti-diabetic treatment with DPP-IV inhibitors (299).

Feeding rats with fructo-oligosaccharide as a source of SCFAs has also shown to upregulate FFAR2 (270). Recently, both the receptors have shown to heteromerize *in vitro,* eliciting synergistic signaling and β-arrestin-2 recruitment (300). Furthermore FFAR2 activation *in vivo* with an inulin-enriched diet in mice results in PYY release and proliferation of L cells *in vitro* (81). Nonetheless, there is still some controversy on the *in vivo* involvement of FFAR2 and FFAR3 in GLP-1 modulation (301, 302), with some reports indicating that blockade of GPR43 *in vitro* releases GLP-1 (303) and others indicating different mechanisms of action, with FFAR2 releasing PYY from intestinal L-cells (81), while FFAR3 restricted to submucosal neuronal activity (295) despite its apparent expression by the majority of enteroendocrine cells (83).

In pancreatic β-cells, both GPR43 and GPR41 are expressed, and the latter antagonizes GSIS (304).

Adding complexity to the study of these receptors, there is extensive species-specificity, so that animal findings result in poorly translatable data, requiring the generation of complex human-murine chimera currently under intense study (305, 306).

Nonetheless, considering that the half-maximal effective concentration ($EC_{50}$) for Acetate, Propionate, and Butyrate is around 0.5 millimolar upon both GPR41 and GPR43 (292) and that the SCFA concentration in the human ileum and colon lumen is superior to 100 millimoles per kg (307–309), it is likely that both receptors are constitutively active. Obese patients, have been reported to produce more SCFAs in their intestines (310), but indeed meaningful diet-induced shifts in SCFA production fluxes have proven not sufficient to modulate peripheral levels of GLP-1 and PYY (311).

GPR42 is another G-Protein-Coupled-Receptor that was initially considered to be an inactive pseudogene derived from GPR41. In 2009, 29% of 202 human alleles of GPR42 were shown to have an inactivating single nucleotide polymorphism (SNP) at W174, and 61% with an arginine in like GPR41, resulting in a fully functional receptor, differing from it by only 5 aminoacids (312). A more recent study highlights how GPR42 is not only functional, but displays a pool of haplotypes in a great proportion of humans, with a distinct pharmacology (313).

## GPR119

GPR119, also known among other names as glucose-dependent insulinotropic receptor (GDIR), was independently discovered less than two decades ago by several groups around the world and deorphanized soon after with the discovery of Oleoylethanolamide (OEA) as its first endogenous ligand (314–316).

Recently our group has demonstrated that indeed OEA is just a partial agonist of GPR119, and the biological ligand of this receptor is the lysophospholipid Oleoyl-Lysophosphatidylinositol (Oleoyl-LPI) (87). This bioactive lipid induces a powerful GPR119 mediated-GLP-1 secretion *in vitro* and *ex-vivo* from intestines of wild type, but not GPR119 deficient mice. This peculiarity is not shared by LPI species with different aliphatic chains, which have been described as the ligands of GPR55 (317).

This GPCR is primarily expressed in the pancreas by α-cells, β-cells and γ-cells (271, 318, 319), and is found at lower concentrations along the GI tract, especially in the stomach and duodenum, where counterintuitively only a minor fraction of CCK, and GLP-1 expressing duodenal enteroendocrine cells display GPR119 (266, 271). This receptor is also expressed, and hence can be studied, *in vitro,* by the human enteroendocrine cell model NCI-H716 or by the murine GLUTag cell line (320). Heterologous expression *in vitro* unveiled its constitutive activity capable to raise intracellular cAMP levels through Gαs (321) and lead to the secretion of GLP-1 and PYY (89). Rodents, contrarily to humans, express GPR119 also in some regions of the brain (316). The activation of this receptor is known to mediate glucose-stimulated insulin secretion and a glucose-independent release of incretin hormones by intestinal enteroendocrine cells (88).

Long-chain fatty acids and phospholipids like lysophosphatidylcholine (LPC), other compounds such as retinoic acid (RA) and multiple N-acylethanolamines (NAE) such as N-oleyldopamine (OLDA), palmitoylethanolamide (PEA), or oleylethanolamide (OEA), all act as endogenous ligands of GPR119. OEA is a more potent GPR119 agonist than its glycerol ester 2-Oleoyl Glycerol (2-OG) found in olive oil (322).

Indeed, oleic acid is internalized via CD36 and converted to OEA in the duodeno-jejunal enterocytes, which in turn causes satiety directly via PPAR-α (323) or indirectly through an incretin secretion mediated via GPR119 in the gut (324). Curiously, fat-induced OEA synthesis is a fairly conserved pathway in metazoan, being present in fish and extremely slow-metabolism reptiles such as pythons (325, 326).

Triglycerides, with medium length fatty acids such as 1,3 Dioctanoyl- 2 Oleoyl-glycerol, can also cause the release of GLP-1 in humans. However, this happens via the metabolized 2-OG component, since dietary medium chain fatty acid do not cause any appreciable release of incretins (322).

Counterintuitively, long term olive oil feeding does not improve glucose tolerance or insulin responses in diabetic rats (5). Indeed, more recently it has been reported that a high-fat diet enriched in oleic acid leads to an impaired endogenous OEA and other N-acylethanolamides intestinal production in mice (327), suggesting that a chronically resistance is taking place within the OEA synthesis pathway.

Surprisingly, a daily activation of GPR119 with OEA or other synthetic agonists, increases β-cell responsiveness in islets transplanted into STZ-induced diabetic mice (328).

The importance of GPR119 in the fat-induced incretin secretion is demonstrated by the impaired incretin signaling displayed by transgenic animals deficient for this protein only in PG expressing intestinal cells. Male and female mice, completely loose the GLP-1 response to an oral gavage of olive and corn oil (329).

More recently, it was reported that whole-body GPR119$^{-}$knockout mice are protected from high-fat induced glucose intolerance and insulin insensitivity. Interestingly, the specific ablation of GPR119 only in β-cells does not affect glucose tolerance nor insulin secretion. In fact AR231453, a selective GPR119 agonist, improves glucose tolerance and insulin sensitivity in both WT and Gpr119$^{\beta cell-/-}$, suggesting how insulin release is independent from pancreatic GPR119 but depends on gut incretin release (330).

Curiously, GPR119 activity appears to be directly dependent on the PYY receptor NPY1 (331). This phenomenon is independent of DPP-IV, the GLP-1 receptor, or the PYY related peptide NPY.

Furthermore, GPR40 also shows synergism with GPR119, mediating a more than additive GLP-1 response to triglycerides in the large intestine (263).

Agonism of GPR119 in both healthy or diabetic and obese mice, is known to improve glucose tolerance (90), or even prevent atherosclerosis in mice (332), while at the same time inducing the secretion of glucagon under low glucose levels avoiding hypoglycaemia (91); therefore since 2008, multiple agonists have been synthesized (239, 254, 333), as well as unimolecular dual DPP-4 inhibitors and GPR119 agonists (334). Despite the good results seen in rodents, species-specific pharmacology might be to blame (335).

Up to now all the prospective GPR119 agonists were plagued by low bioavailability, lack of efficacy and more importantly, cardiotoxicity which has stopped all human studies before any large scale Phase III clinical trials (239).

Despite the multiple failures, the compound DS-8500a is showing promising glucose lowering properties in Phase II clinical trials without any apparent toxicological issues in clinical trials (92).

## TGR5

Bile acids (BAs) are cholesterol-derived molecules produced in the liver and temporarily stored in the gallbladder. When food is ingested, BAs are released into duodenum to solubilize dietary lipids under the form of micelles, a necessary step for the maximization of the surface-to-volume ratio of fat droplets, aiding interface-acting lipases.

Indeed the release of lipids from micelles has directly proven to release GLP-1 and GIP via the FFAR1 in the duodenum (264).

This release of bile acids, mainly cholic (CA) and chenodeoxycholic (CDCA) acid derivatives, happens through the relaxation of the smooth muscle sphincter upon CCK signaling (336) or indirectly through a similar VIP action on the sphincter of Oddi (337).

Historically described as mere fat-solubilizing agents, these amphipathic compounds were recently recognized as key signaling molecules capable to modulate the host metabolism directly acting as ligands of intestinal GPCRs (101, 338, 339), or after being metabolized by the colonic microbiota into secondary bile acids, mostly deoxycholic and lithocholic acid(340).

The chemosensor believed to be the main receptor of bile acids is TGR5, also known as GPR131 or GPBAR1 among other names. This receptor has been reported to be expressed by colonic GLP-1-secreting enteroendocrine cells and pancreatic α-and β-cells (100, 101), with some controversy regarding the presence in murine islets (339).

TGR5 activity appears to not have been lost in type 2 diabetic humans whereby the infusion of CCK, or rectal taurocholate, causes GLP-1 and insulin release via the TGR5 axis in colonic L-cells and pancreatic β-cells respectively (341, 342).

This notion is in stark contrast to the well-known anti-diabetic properties of BAs sequestrants, (343) and some, have proven to elicit GLP-1 secretion via TGR5 mediated PC1/3 upregulation (344). A likely explanation is that the BAs bound to a sequestrant into the intestinal lumen can't be absorbed and hence travel more distally in the GI tract where the complexes are still capable to activate the TGR5 expressing colonic L-cells. Furthermore, the lower systemic levels of bile salts prompt the liver to produce more bile, which in turn feeds more TGR5 agonism into the colon (343).

This chemosensor is expressed by the pancreatic α-cells where its signaling activates Gs proteins and induces the secretion of GLP-1 directly through Epac proteins and indirectly via CREB mediated expression of Psck1, while in β-cells mediates insulin release [(100); **Figure 3**].

TGR5 is the target of different BAs, but the most potent endogenous agonist has shown to be lithocholic acid (LCA) and its taurine conjugates with activity at nanomolar concentrations (273, 339). Secondary bile salts, metabolized by the microbiota, exhibit less potency toward this receptor.

Despite this promising anti-diabetic activity of TGR5 mediate by GLP-1 (345), its pharmacological activation in diabetic patients has shown side effects at the level of gallbladder and heart, hampering its clinical use (346).

Another bile salts chemosensor is the nuclear farnesoid X receptor (FXR) (347) which activation, contrarily to TGR5, blocks the release of GLP-1 in the colonic L-cells (348), while in the liver induces glycogenesis helping to improve glucose homeostasis. This counterintuitive pharmacology has been confirmed *in vivo* whereby the administration of the FXR agonist GW4064 by mouth drives hyperglycaemia and obesity (349) while intraperitoneal injection exerts protection from it (350). Consistently, an indirect inhibition of intestinal FXR through microbiota modulation, or genetic deletion of intestinal FXR, corroborate this phenome displaying protection from high-fat diets induced obesity and fatty liver disease (351).

This could explain why bile acid sequestrants support a positive glucometabolic homeostasis. Indeed, the insoluble complexes of bile salts can activate lumen-facing TGR5 receptors, while they cannot cross plasma membranes to activate intracellular GLP-1-suppressant FXR receptors.

FXR is a very important receptor, part of a negative feedback in the liver, whereby the binding of bile salts, especially

chenodeoxycholic acid, represses the *de-novo* synthesis of bile salts (352, 353). Indeed, there are multiple primary or secondary bile acid chemosensors in the liver (348, 354) or scattered along the gastrointestinal tract (355), where they ensure a direct negative feedback aiding detoxification (356) and protecting from hepatotoxicity and carcinogenicity displayed by some secondary bile salt such as lithocholic acid.

Accumulated evidence, indicate how bile acids are important modulators of the whole body metabolism, bridging the microbiome to the brain, likely being key signaling molecules in the pathogenesis of obesity and type 2 diabetes. Indeed remittance from diabetes experienced by RYGB or SG patients, has been attributed to the elevation of circulating bile acids (37, 38, 357), warranting further investigation, especially the development of gut-restricted TGR5 agonists (358).

## TRPV1 AND THE TRP CHANNEL FAMILY

The transient receptor potential vanilloid 1 (TRPV1) is a tetrameric non-specific cationic channel found in most of mammalian sensory neurons (359). Each of its constituting monomers crosses the plasma-membrane six times and both the N and C-term face the cytoplasmic side, where they make up 70% of the receptors' entire volume (360). This chemosensor, together with other 27 non-selective cationic channels, is part of a larger family named transient receptor potential (TRP) channel superfamily and is known to play an important role in the metabolic syndrome (361, 362).

TRPV1 is primarily activated by vanilloids and capsaicinoids including Capsaicin (360), eliciting the sensation of spiciness; multiple stress-related stimuli cause its activation and opening with subsequent membrane depolarization. For example cigarette smoke, excess of protons (pH < 5.9) (363), temperatures above 43° (360), certain animal toxins (364, 365), ATP (366) or even cannabinoids such as Anandamide (367) and cannabidiol (359, 368), are all stimuli known to activate this sensor. Indirect stimulation has also been demonstrated by bradykinin (366), NGF (366), PGE2 (369), PGI2 (369) and agonists of Protease-activated Receptors (PARs) (370).

TRPV1 has been shown to be expressed in the brain, β-cells (371), nociceptor C fibers, dorsal root ganglia, hepatocytes, spermatozoa (372), airway neurons (373), bladder and urothelium (374), blood vessels, and the whole gastrointestinal myenteric plexus (375), especially in colonic and rectal neurons (376). Consistently, TRPV1 is also found to be expressed by the murine enteroendocrine cell line model STC-1 and its agonism induces the release of GLP-1 *in vivo* (377).

This receptor has recently seen an increasing interest since its activation has been found to have pleiotropic beneficial metabolic effects (378).

Indeed, it has been known for more than a decade that capsaicin is capable to elicit a glucose-stimulated insulin release *in vivo* (379). A crossover study operated on 30 human healthy subjects (380), showed a slight increase in plasmatic GLP-1 and a slight decrease in ghrelin levels 30 min after a Capsaicin enriched meal (containing 1,030 mg of 80,000 Scoville heat units red pepper); Peptide YY changes were not statistically significant. Despite these promising results, TRPV1 knockout mice display contrasting phenotypes with the report of opposite phenotypes. One author describes an obese insulin and leptin resistant mouse (381), while another group report animal protected from diet-induced obesity (382).

Considering all the recent findings, drugs targeting TRPV1 would be beneficial for the management of obesity (383) metabolic syndrome (384) and type 2 diabetes (385). Nonetheless, considering the EECs receptome responsible for gut-peptide modulation, TRPV1 has received much less attention, with a yet largely unexplored physiology.

## THE MICROBIOTA

Animals' GI tract is known to host a population of hundreds of different species of bacteria (386), viruses and fungi, estimated to equal in number the cells that constitute the human body (387). These microorganisms thrive in the colon's lumen, where they secrete small molecules ultimately affecting the host immunity (240) and metabolism (388).

The relative abundance of different microbial species is known to depend on the presence of specific nutrients (389); hence, considering that an imbalance in the microbiota correlates with chronic inflammation pathologies of the bowel, or even Type 2 diabetes, it is likely that dietary components indirectly influence the occurrence of these pathologies via the microbiota (390, 391).

The human colonic microflora is known to produce high concentrations of Short-Chain-Fatty acids (SCFAs), among other metabolites, from the anaerobic fermentation of dietary indigestible carbohydrates, or even derivatives of bile salts (389). In fact, the SCFAs Acetate, Propionate and Butyrate are the principal luminal anions in humans and other mammalian's colon (309, 392), with some inter-species variability. Rats show higher levels of fecal Acetate, 75 mM vs. human's 50 mM, Propionate, 27 vs. 11 mM and Butyrate, 16 vs. 5 mM respectively. On the other hand, surprisingly similarly to humans' colonic and fecal values, rumens of herbivores, such as sheep or cows, also contain high levels of acetate, propionate and butyrate, with reported concentrations of 65, 21, and 18 mM, respectively (308). These levels appear to be independent of dietary proteins or fibers; conversely, it is the caloric intake that affects the relative composition and concentrations of SCFAs (308). These metabolites have been found to target specific receptors among the repertoire expressed by the EECs, triggering a hormonal response. It is estimated that in humans almost all fermented SCFA are absorbed by the colonocytes and only 5% are excreted with stool, equivalent to 5–30 millimoles per day. Indeed, it is not practically feasible to measure intraluminal production fluxes of various metabolites *in vivo* in humans; therefore, most studies focus on the easiest but less informative quantification of fecal SCFA content (393).

Despite the most recent studies of transgenic and germ-free animals, it is still largely unknown by what degree hormones such as GLP-1, and all its related peptides, depend on the microflora, especially in pathologies such as type 2 diabetes.

Recent high-throughput pharmacogenomic studies have deepened our understanding of the molecular players in this human-microbiota relationship. Recently it was shown that a new class of N-acyl amides is produced by the microbiota, and target GPCRs expressed by the enteroendocrine cells, modulating GLP-1 expression and overall glucose metabolism. In particular, N-oleoyl serinol (N-OS) is described as a potent GPR119 agonist, acting in the lower micromolar range with twice the efficacy of the endogenous ligand OEA (394).

From the evolutionary perspective, dietary components, together with the microbiota-fermented products, have activated the enteroendocrine system for billions of years, since the dawn of metazoan. Considering the vast and continuous pool of metabolites produced and modulated by the microbiota, the distinction between orthosteric and allosteric ligand becomes blurred; different molecules are likely working in synergy to elicit a specific hormonal response.

Modulation of the microbiome has shown promising results in the treatment of type 2 diabetes. For example, recently it was reported that a rhubarb extract, Rhein, increasing the intestinal population of Bacteroidetes, mediates an increase in ileal GLP-1 producing cells, peripheral GLP-1$(7\text{-}36)_{NH2}$ levels and improved glucose tolerance in diabetic db/db mice (395). Consistently, STZ-treated rats, are protected from oxidative and inflammatory stress when treated with Liraglutide, and *Bacteroides*, as well as *Lactobacilli* strain populations appear to be restored (396).

In the last decade, the scientific community has just started to unveil the molecular pathways produced by this long-lasting symbiosis. It appears that SCFAs not only induce the release of GLP-1, they also represent a mitogenic signal. Rats fed oligofructose, a substrate for the colonic microbiota which leads to higher SCFAs levels, possess an increased number of colonic L-cells (397). This has been confirmed *ex-vivo* in human and mouse small intestinal crypts organoids (398).

Other compounds such as bile salts and xenobiotics (399), are known to be metabolized and excreted by the microbiota, affecting the host physiology. Indeed, the pharmacokinetic and pharmacodynamics of any drug taken by mouth should be appraised considering the role of the microbiota, as the varied efficacy of some chemotherapeutics such as 5-FU has been proven to directly depend on this host-microbiota metabolism (400). Even though the anatomical intestinal rearrangement of RYGB and SG patients is known to affect the microbiota, this doesn't appear to result in a different bile acid metabolism in a rat model (401).

We are at the beginning of a new branch of medical practice, tailored not only to the single person genome, but also to the microbiome.

Future human studies will help us to better understand the big picture of this relationship, to hopefully provide mechanistic knowledge upon which new treatments could be created, such as microbiome-directed gene-therapies for the management of metabolic diseases.

## CONCLUSION AND PERSPECTIVE

GLP-1R-independent signaling of GLP-1, its intra-islet axis, and its once-thought inactive metabolites, all represent new important additions to our understanding of this peptide in health and disease.

Omnivores' gastrointestinal tract has co-evolved in strict relationship with a dynamic microbiota and a complex seasonal and regional diet, resulting into a robust and flexible system tightly interconnected via multiple neuroendocrine axes with different organs.

In nature, dietary fats are scarce energy-dense nutrients primarily found in fish and meat. This evolutionary pressure over millions of years has shaped a system for the attentive sensation, assimilation and storage of precious bioactive molecules in all superior animals.

Sensation happens at multiple levels with a plethora of somewhat redundant intestinal receptors (402), specifically in the enteroendocrine cell system. This redundancy can be seen in transgenic animals, whereby the genetic absence of a single chemosensor doesn't always result in a phenotype, probably due to metabolic compensation from similar and overlapping pathways.

Virtually all macronutrients are absorbed in the small intestine, where maximal activity of the EECs is ensured, while the colonic and rectal GLP-1 secretion is enforced in response to secondary metabolites even hours after the meal ingestion. This pattern is disrupted in bariatric patients undergoing RYGB surgery, where a remodeled GI tract delivers more nutrients to the large intestine, and changes gut-secretome, including its microflora.

Attempts to mimic this altered meal processing, such as proximal blockage of nutrient absorption resulting in increased delivery of nutrients in the distal intestine, have shown some promising results in healthy and diabetic humans (403). Although this is more challenging with fats because dietary lipids require partial digestion by lipases to become efficient secretagogues (404, 405). However, distant delivery of free fatty acids, or even Oleoyl-Glycerol and sodium taurocholate have shown negligible effects on peripheral levels of GLP-1 or PYY, satiety and glucose tolerance (311, 406, 407). Similarly, distal delivery of the best known aminoacidic GLP-1-secretagogue, glutamine, has proven ineffective at ameliorating glucose tolerance in both healthy and diabetic subjects (407–409).

Furthermore, a recent report (410) examined the effect of RYGB on lean pigs, and indicates how it is the post-operative GLP-1 $(9\text{-}36)_{NH2}$ levels that raise, while surprisingly the "active" $(7\text{-}36)_{NH2}$ peripheral levels were reduced.

Indeed, most authors focus only on the peripheral levels of only one of these two peptide species, vastly excluding GLP-$1(28\text{-}36)_{NH2}$ and $(32\text{-}36)_{NH2}$ activity, rendering the overall understanding of each individual GLP-1 species, in both health and disease, difficult to discern.

Technical advances ELISA, capable to specifically dissect these peptide species locally and peripherally, will help us to shed new light into this complex physiology (411).

Conclusively, bearing in mind that insulinotropic or incretinotropic effects are not secondary to any single receptor modulation, whereby pools of different luminal stimuli act synergistically on tens of different chemosensors during their intestinal transit and absorption, while interacting with the microflora metabolism, rendering the restoration of a healthy

physiology in diabetic patients with the pharmacological correction of a single axis, highly improbable.

The final dissection of the molecular axis causative of either metabolic syndrome will need more evidence regarding the localized and inter-neuronal physiology of GLP-1 in physiological and pathological statuses. To ultimately tease apart any possible cause from secondary events, species-specific biology will also need to be carefully dissected and interpreted.

## AUTHOR CONTRIBUTIONS

SP researched and interpreted all the data from available scientific literature on the PUBMED database, organized, wrote and revised the whole manuscript. SP also conceptualized and drew all the figures assembling the final formatted review. MF conceived, organized, wrote and revised the whole manuscript.

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### 1.2 Synopsis of Review 2

Pancreatic Ductal Adenocarcinoma (PDAC) is one of the most lethal forms of cancer, and preventive measures represent the best approach to contain its socio-economic burden. Obesity and diabetes have been proven to share a complex and mutual pathological relationship with PDAC. In recent years, multiple metabolic axes have been implicated in this pathological triad, including the modulation of GLP-1.

A dysfunctional metabolism, as seen in obesity and T2D, represents a key risk factor (Table 1) for the development of PDAC, explaining the increase in incidence for this lethal disease recorded in the last few decades. This surge is predicted to increase in the coming decades given the increasingly obese world population, forewarning a dire healthcare emergency. PDAC is a deadly disease because it metastasizes quickly, before manifesting itself with any symptoms. Preventive measures aimed at diagnosing PDAC in its early, operable stages represents a challenging task that we cannot yet robustly achieve. Multiple blood markers have been described in the past decades as possible proxies of PDAC, nonetheless screening systemically the general population is not a feasible approach due to yet unacceptable specificity and sensitivity. This approach on the other hand, might become acceptable in high-risk populations such as obese diabetics. Understanding the molecular players behind the causative links connecting metabolic pathologies to PDAC, represents the best direction aimed not only to define high-risk populations, but also discover novel diagnostic and therapeutical targets. Fat tissue, once thought to be a homogeneous and passive repository of energy, has been shown in recent years to be a multi-faceted organ capable to not only secrete a panoply of hormones that modulate the whole-body metabolism, but also to support the growth of cancerous tissue. Recent studies have drawn an important distinction between subcutaneous adipose tissue (SCAT) and visceral adipose tissue (VAT), in particular the latter, under the form of pancreatic fat fraction (PFF) appears to be the key pathological driver bridging obesity, and obesity to PDAC. As summarized in figure 1 of the presented Review 2, the pancreatic microenvironment is composed of multiple novel cellular players, including the VAT adipocytes. The microbiota is another equally complex, and yet only partially understood symbiotic organ of our body. Composed of a heterogeneous population of microorganisms, the microbiota dynamically bridges at the molecular level, our diets to the enteroendocrine cells and overall metabolism, and defines our predisposition to

PDAC. Recent evidence indicates that the microbiota is not only physically constrained into the intestinal lumen, it is also found within pancreatic ducts playing a key role in the pathological pancreatic microenvironment as depicted in figure 2.

### 1.2.1 Significance

This manuscript collates and depicts a complex picture, helping to draw a more holistic view on the theragnostic potential of blood-based markers in combination with imaging techniques in high-risk populations. In this updated pathophysiological model, well-known cellular players of the PDAC, such as stellate cells or different types of immune cells, are communicating via soluble factors paracrinally and endocrinally, engaging different types of fat tissue, the microbiota and gut-derived hormones such as GLP-1. In particular, as summarized also in figure 2, pancreatic cancerous cells are directly inhibiting, through exosomes, the secretion of GLP-1, as well as indirectly via increased expression of DPP-4, raising the question whether supplementation with pharmaceutical GLP-1 might ameliorate the diabetic condition and indirectly possess anti-cancerous properties. Indeed, the use of GLP-1 receptor agonists has shown some controversial yet debated pro-tumoral effects. Nonetheless, more recent longitudinal evidence drawn from more than 50,000 patients with T2D, disproves any causative link with pancreatitis or any form of pancreatic cancer (Cao, Yang, & Zhou, 2020). This evidence yet supports the use of drugs acting on the gut as GLP-1 secretagogues. With our current understanding of the PDAC microenvironment, patients experiencing novel-onset diabetes (NOD) can be considered individuals at high-risk. NOD patients might be monitored for other novel, yet correlative seemingly benign signs and symptoms of PDAC, such as body weight loss, or dyslipidaemia which might justify more invasive imaging techniques such as Endoscopic Ultrasound (EUS). Longitudinal human evidence linking mechanistically blood-based biomarkers in the dynamic evolution of obesity, diabetes and PDAC is yet lacking and will be needed to assess their positive predictive values.

The importance of human evidence is underpinned by the species-specific pathophysiology of this disease. Different animal models have been utilized to study the multi-faceted pancreatic microenvironment in obesity or diabetes, but none appears to comprehensively phenocopy the human triad of obesity-T2D-PDAC.

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Review

# The intricate relationship between diabetes, obesity and pancreatic cancer

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ABSTRACT

Pancreatic cancer is one of the leading determinants of global cancer mortality, and its incidence is predicted to increase, to become in 2030 the second most common cause of cancer-related death. Obesity and diabetes are recognized risk factors for the development of pancreatic cancer. In the last few decades an epidemic of diabetes and obesity has been spreading worldwide, forewarning an increase in incidence of pancreatic cancer. This review considers the most recent literature, covering the multiple molecular axis linking these three pathologies, aiming to draw a more comprehensive view of pancreatic cancer for a better theragnostic stratification of the population.

## 1. Introduction

Since its initial recognition in the 20th century, Pancreatic Cancer has always been considered a virtually incurable disease; likewise, the prognosis has not changed much in recent years, compounded by a worldwide increase in incidence [1,2]. Pancreatic Ductal Adenocarcinoma (PDAC) is the most common malignancy of the exocrine pancreas, accounting for > 90% of cases, with a very poor prognosis. In this review we will focus exclusively on PDAC.

Increasing evidence indicate the presence of a pathological link between obesity, diabetes and PDAC. This manuscript reviews the most recent findings, discussing and reframing the pathological picture of high-risk populations for potential PDAC-preventive strategies. As summarized, novel blood-based therapeutical and diagnostic markers, such as exosomes, warrant further clinical and interventional investigation in obese and diabetic patients. These blood-based, and tissue specific biomarkers, are essential tools for a better enrichment and stratification of high-risk populations. In this review, we discuss the promises and the pitfalls of personalized screening of individuals on the path to developing PDAC, with the ultimate aim of reducing the foreseeable burden of these chronic diseases.

## 2. PDAC risk stratification

PDAC risk factors can be divided into inherited and non-inherited. The latter can be further divided in modifiable and non-modifiable risk factors. PDAC is known to be associated with several recognized and modifiable risk factors such as obesity, tobacco smoking, heavy consumption of alcohol and different dietary components [3]. Non-modifiable risk factors include age, diabetes, and chronic pancreatitis. These environmental stressors not only increase the likelihood of developing PDAC, but they also underline the development of other chronic metabolic pathologies, such as type 2 diabetes (T2D) and cardiovascular diseases, offering possible insight into this complex pathological relationship known to be enforced via an altered expression of a vast gamut of genes. Smoking is one of the major risk factors for PDAC and smokers can be more than twice as likely to contract PDAC compared to non-smokers (Table 1); nonetheless, compared to other smoking-related cancers, no carcinogen-related mutational signatures have been identified in PDAC [4]. Around 5–10% of PDAC patients have a familial history of the disease characterized by at least two first degree relatives affected by PDAC [5]. Several known hereditary syndromes and genes are associated with an increased risk of PDAC such as *BRCA1, BRCA2, CDKN2A* and DNA mismatch repair genes *MLH1, MSH2,* and *MSH6* (Table 1).

Epidemiological evidence indicates that, for many patients, pancreatic cancer is secondary to metabolic pressures, caused for example by obesity and T2D; conversely, patients-bearing PDAC are at much higher risk of developing diabetes [6]. This strong correlative evidence has been supported by recent studies, offering new avenues for clinical interventions to curb the incidence of PDAC, as well as new insight into the morbidity spectrum associated with metabolic diseases. It is a well-established fact that obese individuals are at higher risk of developing pancreatic precancerous lesions [7]. In particular, the burden of excessive amounts of adipose tissues, especially in early adulthood, raises

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**Table 1**
**PDAC risk factors.** Multiple congenital, and environmental factors are known to increase the risk of PDAC development. A combination of reported confidence intervals is listed for each recognized factor. Data reformatted from [35,36,104,105].

| Risk factor | Overall confidence interval |
|---|---|
| Genetic | |
| STK11/LKB1 | 44–261 |
| BRCA1 BRCA2 PALB2 | 2–3.5 |
| P16INK4A/CDKN2A | 12–47 |
| CFTR | 3.5–6.6 |
| APC | 4.46–6 |
| MLH1, MSH2, MSH6 | 0–8.6 |
| Pathologies | |
| Pancreatitis | 3.5–13 |
| Obesity | 1.3–1.5 |
| T2D overall | 2.0 |
| NOD (< 1 year) | 3.5–8 |
| T2D (> 2 years) | 1.7–2 |
| Long-lasting T2D (> 5 years) | 1.5 |
| Environmental | |
| Smoking | 1.6–2.2 |
| Alcohol | 1.5 |
| Animal fat-rich diet | 1.5 |

the risk for PDAC and overall mortality [8]. Recent evidence expands this tenet, suggesting that the risk is bequeathed to progenies from either obese parent [9]. Similarly, the chronic glucose intolerance seen in maternal diabetes is associated with increased obesity and internal visceral adipose tissue (VAT) in the offspring, which then display an increased risk for PDAC [10]. Taking into consideration that obesity is a risk factor for pancreatic cancer and given its worldwide pandemic status, it is imperative to find new ways to screen this high-risk population for PDAC.

## 3. Pre-clinical models

The preclinical study of the complex relationship between obesity, diabetes and PDAC, is complicated by the lack of animal models translating the human pathology. Animals models of obesity, or diabetes, do not normally develop PDAC. Currently, the most common in vivo system used for the study of PDAC is the transgenic KPC mouse, bearing oncogenic K-RAS G12D and p53 R172H in the pancreas. These animals, despite being successfully used for the study of novel therapeutics in PDAC, have been recently shown to be devoid of any sign of para-neoplastic diabetes [11]. KPC mice fed either standard chow or western high-fat high-sugar diets, did not show any worsening of their glucose tolerance; on the opposite, animals with advanced stage PDAC displayed an improved glucose tolerance. Surprisingly, although the obesogenic diet increased the morbidity of PDAC, the animals with more abdominal fat showed the longest overall survival, even on a standard chow diet. This increase in survival was not maintained when the animals were stratified by whole body weight, in disagreement with human data. As the KPC mouse represents a very aggressive and advanced model of PDAC it is likely that environmental stressors might have a limited impact on the disease progression. In this respect, the KC mouse, a transgenic model that only expresses the oncogenic K-RAS G12D allele in the acinar cells of the pancreas, might represent a more useful animal model. In the KC mouse, an obesogenic high-fat diet significantly increases the incidence of neoplastic growths of the pancreas [12], confirming the link between obesity and its potential contribution to PDAC development and progression while, interestingly, the protection from the same obesogenic diet with metformin has antitumoral properties [13]. In the last decade, several studies have demonstrated that obesity potentiates pancreatic cancer growth and dissemination in animal models [14–17]. Incio et al. in particular proved how dietary-mediated obesity aggravated PDAC aggressivity, via PlGF and VEGFR1 activity and modulating the tumoral immune microenvironment [17]. Moreover, in vitro data indicate that soluble factors produced by adipocytes promote epithelial-to-mesenchymal transition (EMT) and aggressiveness in pancreatic cancer cell lines expressing constitutively activated KRAS [18].

An animal model accurately and comprehensively replicating the human pathophysiology of obesity, diabetes and PDAC in one microenvironment is not yet available; this is likely explained by the species-specific biology, and congenital nature of these animals. A recent report tries to overcome these problems with the use of Tamoxifen inducible Cre recombinase; Talbert and colleagues describe the KPP mouse, expressing the same KRAS-G12D dominant protein, while lacking a functional *Pten* gene in pancreatic acinar cells. The KPP mouse represents a useful pre-clinical model for the study of cachexia [72], a condition of muscle wastage and extreme anorexia seen in most PDAC patients. Nevertheless, the glycaemic status of this model is not reported, making the KC mouse model more indicated for studies exploring the diabetes-PDAC interaction. New studies are necessaries for the assessment of the interaction between modifiable risk factors, such as diet-induced obesity, or hyperglycaemia and genetic predisposition.

## 4. Obesity and PDAC

The link between obesity and PDAC provides a way forward to better define and stratify high-risk populations, in particular through the characterization of anatomically different fat deposits, tissues known to be distinct metabolic organs. A first functional distinction that can be drawn is between the intraperitoneal inter, and intra-organ fat, often loosely referred to as VAT, and the sub-cutaneous adipose tissue (SCAT), with the former being associated with worse prognosis in multiple carcinomas including the pancreas [19].

These different physiologies are also explained by their anatomical location, with the VAT being in close proximity with virtually all internal organs. Indeed, in pathologies such as obesity and T2D the inter-organ VAT infiltrates different tissues including the pancreas and the liver, negatively impacting their physiologies.

Accordingly, the pancreatic fat fraction (PFF) has shown to be the common denominator between obesity, T2D, chronic pancreatitis and PDAC [20–22]; moreover, in obese patients, the PFF correlates with PanIN formation and PDAC development, while being inversely correlated with patients survival [23,24].

The ability to distinguish VAT from SCAT, both at the clinical and molecular level, is therefore essential to depict a more holistic view of PDAC, aiding in a better stratification of patients for more tailored interventional strategies. It is now clear that different adipose tissues communicate locally, and distally through the bloodstream, using a complex array of cytokines named adipokines, that can target different tumour microenvironments. Obesity is indeed a known pro-inflammatory insult, with proven capacity to stimulate Tumor Associated Neutrophils (TANs), that activate Pancreatic Stellate Cells (PSCs), which are in turn responsible for the typical desmoplastic fibrotic reaction seen in PDAC, resulting in a poorly perfused and difficult to treat, tumour microenvironment [25]. An additional way of communication from the adipocytes has been recently demonstrated, occurring via an upregulated and distinct type of lipid-filled extracellular vescicles (EVs) that modulate adipose tissue macrophages, or distant organs, establishing a pro-tumoural environment [26] (Fig. 1).

Few molecular pathways defining the effect of VAT on the tumour microenvironment have been described in different types of cancer, with inflammation being a recognized pathological driver of PDAC [27]. For instance, it has been reported that adipose stromal cells, the progenitors of differentiated adipocytes residing in the VAT surrounding a prostatic tumour, directly committ cancer cells to an EMT transition, possibly via CXCL12, increasing chemoresistance and overall morbidity [28]. A similar interplay is possibly underpinning the

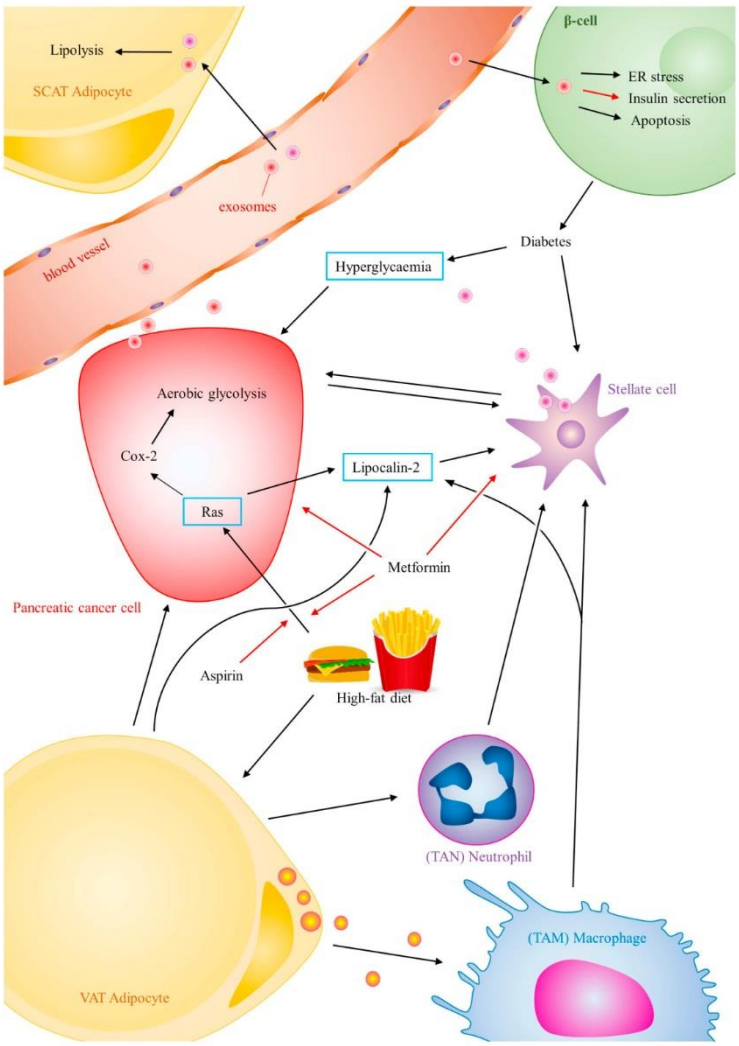


**Fig. 1. The pancreatic cancer microenvironment in obese and diabetic individuals.** Different environmental stressors support the growth of a dysplastic pancreatic cancer cell (shown at the centre-left in red), and its epithelial-to-mesenchymal transition (EMT). Obesogenic diets, rich in fat and ω-6 lipids, have recently been shown to directly sustain a Ras-mediated aerobic glycolysis supporting cellular proliferation reliant on glycolysis [32]. A western-type, high fat diet is a pro-inflammatory and pro-oncogenic stimulus [16,29,30], that can at least partially be blocked with metformin [13]; a drug that has also shown to inhibit the PSCs-mediated oncogenic desmoplastic reaction [101], and directly reduce tumoral metabolic plasticity [102]. Animal data also indicates that aspirin protects from HFD-induced PDAC [95]. Visceral fat directly supports PDAC development with soluble factors such as Lipocalin-2, and indirectly involving TANs, TAMs and PSCs-mediated inflammation [17,23,25,27,32]. The cancerous pancreatic cells, as well as PSCs, have proven to secrete exosomes containing adrenomedullin capable to induce peripheral lipolysis [79–81]. Moreover, the diabetic state, through hyperglycaemia or hyperinsulinaemia, supports the metastatic dissemination and growth of the primary tumour, especially through pancreatic stellate cells (PSCs) [42,43,45,50,63]. Adipocytes have also been proven to bey key players, secreting paracrine Wnt ligands with direct pro-oncogenic properties [18], in addition to pro-inflammatory capacities mediated by recruited TANs, and TAMs in special nanovescicles [25,26]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

development of PDAC, making this axis a valuable target for future therapies aimed at its inhibition.

Several studies have also investigated the effect of different diets, or selective dietary components on cancer development and progression. For instance, it has been reported that processed meat, or a hyper caloric diet rich in fats, especially omega-6 lipids, directly correlates with more pancreatic intraepithelial lesions (PanINs), resulting in increased invasiveness supported by an inflamed microenvironment [29,30].

Recently, Wang et al. [31] demonstrated that a high-fat diet (HFD), but not a high-carbohydrate diet, upregulates KRAS-G12D and drives a shift toward an aerobic glycolysis in the KC mouse model, resulting in more invasive PanINs and ultimately PDAC. Importantly, the observation that such overactivity of KRAS-G12D depends on the key inflammatory mediator cyclooxygenase-2, further provides a promising novel therapeutical target for the indirect inhibition of KRAS activity.

Lipocalin-2, a protein known to be involved in the development of multiple malignancies, is also a key player in the microenvironment underlying PDAC development. Lipocalin-2 was recently shown to be an important master regulator of the VAT hypertrophy detected in HFD fed animals; its genetic deletion curbs PDAC development in K-RasG12D expressing transgenic mice [32]. Although more studies are required to better understand the clinical importance of these pathways, the available data indicates that the pharmacological modulation of specific types of VAT represents a novel promising approach holding powerful theragnostic potential.

## 5. Diabetes-derived PDAC

The hyperglycaemic status observed in T2D patients is the consequence of excessive hepatic gluconeogenesis, hampered incretin activity and peripheral uptake of glucose, all compounded by an impaired insulin signalling. The pathophysiology of diabetes has many pro-tumoral features, and represents a recognized risk factor for the development of PDAC. Indeed, patients are more likely to develop PDAC within few years from their diagnosis of diabetes, rather than later [6]. To understand the rationale behind this differential risk, and better stratify high-risk populations, it is important to frame it in the context of the metabolic dynamics of type 2 diabetes. Before being diagnosed, patients are often asymptomatic for up to a decade, with a silent glucose intolerance controlled by a growing hyperinsulinemia. Only after a few decades of overt, uncontrolled hyperglycaemia, diabetics become hypoinsulinemic, with ever-present increased levels of glucose in their tissues. Nonetheless, in this timeframe often encompassing at least two decades, the first years appear to be critical, making novel onset diabetes (NOD) patients > 50% more likely to develop PDAC than long-term diabetics [33–37].

Indeed, extensive epidemiological data link hyperglycaemia to PDAC risk [39,40], and recent studies have started to unveil a novel axis that could offer a broader picture of this relationship, offering possible therapeutical targets.

For instance, a study led by Rahn et al. [41] sheds some light on the strong pro-tumoral effects of hyperglycaemia; the authors showed how high blood sugar in mice supports the activation of the transforming growth factor β-1 pathway, causing reduced E-cadherin expression in the ductal epithelial cells, resulting in a more mesenchymal and pro-metastatic morphology.

Mechanistic evidence between high glucose levels and genomic instability has been recently found in pancreatic cancer. High glucose levels increase post-translational O-GlcNAcylation leading to nucleotides imbalance and genomic instability ultimately supporting KRAS mutations [42].

This is corroborated by evidence in patients with resected PDAC, whereby glycated haemoglobin (HbA1c) levels higher than 9.0% are worse prognostic markers than the diabetic status [43].

Beyond hypoglycaemia, recent evidence [44] indicates that lipotoxicity, the accumulation of lipid derivatives in non-adipose tissues, as seen in T2D, activates pancreatic stellate cells (PSCs) residing in the islets, which then impair the viability of pancreatic β-cells, leading to insulin deficiency, while stimulating PDAC cells proliferation and invasion, both in vitro and in vivo in a xenograft mouse model [45,46]. Importantly, such pro-diabetic action could be prevented by the over-expression of sterol regulatory element-binding protein -c1 ex vivo in rat islets [44], possibly suggesting a strategy to counteract diabetes onset in this context. This study however contrasts with older evidence in diet-driven diabetic rats, where the use of the anti-fibrotic agent pirfenidone was shown to impede the migration and activity of PSCs but did not improve the diabetic state [47]. Activated PSCs are also well-known cellular players responsible for the highly desmoplastic microenvironment characterizing PDAC. Although more evidence is needed to pinpoint their pro-fibrotic nature specifically in T2D, studies in orthotopic mouse models of PDAC implicate the HGF/cMET pathway as a viable target in multidrug therapy [48]. PSC are indeed key proxies acting at the interface of multiple cell types, including the immune system, mechanistically linking T2D to pancreatic cancer via their pro-inflammatory nature [34].

Beyond the pathology of T2D itself, PDAC is an iatrogenic condition resulting from diabetes pharmacologic management. Strong correlative evidence indicates that independently from the obesity status, hyperinsulinemia is an unfavourable predictive marker in any type of cancer mortality [49,73]. In diabetics, insulin is either overproduced endogenously to compensate initial peripheral resistance, or is introduced exogenously as a treatment in later stages of the disease. Recent studies have proven that this hormone supports a proliferative niche via the activation of PSCs [50], or directly, in synergism with Insulin-like growth factor 1 (IGF-1), via the activation of the ERK1/2 pathway [51,52], a critical axis in PDAC development as recently demonstrated in human primary, and metastatic tumour tissues [53]. Indeed IGF-1 has proven to be a valid blood marker capable to discriminate diabetic patients bearing PDAC from pancreatitis [54]. The hyperactivity of ERK1/2 is in turn associated with the pathological development of insulin resistance [55], bridging again the pathologies of PDAC and T2D.

Amongst the novel anti-diabetic drugs, glucagon-like peptide-1 (GLP-1) receptor agonists have also similarly been associated with increased risk of PDAC, although the causality is yet unproven [56].

Overall, these studies indicate a complex, inter-cellular network between diabetes and PDAC, unveiling contrasting possible effects of different anti-diabetic drugs on cancer development and progression.

## 6. Diabetes as a clinical manifestation of PDAC

The strong correlation between diabetes and PDAC is indeed a two-way self-reinforcing relationship. As recently reviewed by Singhi et al. [38], patients diagnosed with PDAC are much more likely to have different degrees of glucose intolerance, whereby only less than a tenth displays a normal fasting glycaemia at diagnosis. This strong correlation between diabetes and pancreatic cancer is indeed a two-way self-reinforcing relationship. Most PDAC-bearing patients are chronically hyperglycaemic, and tend to lose both body weight and their pancreatic β-cell activity with the progression of the disease, resulting in overt late-stage diabetes over time.

Indeed, fasting blood glucose is now recognized as an easy-to-measure blood-marker for a better stratification of populations at high-risk for PDAC. Impaired glycaemia has recently been shown to be the clinical manifestation of less differentiated tumours of 1 to 2 $cm^3$ in volume, detectable up to 30 months before PDAC diagnosis [57]. New studies have defined these periods more precisely, showing how patients bearing PDAC experience a loss of body weight averaging 4 kg in the 18 to 6 months prior to diagnosis, and 8 kg in the 6 months preceding diagnosis. Moreover, the same individuals experience reduced levels of their serum lipids, including low-density lipoprotein, high-density lipoprotein, triglycerides and cholesterol [58,59].

Reportedly healthy patients, experiencing unexplained weight loss

and hyperglycaemia, could be further stratified with the use of other blood markers, that albeit yet correlative in nature, could improve the stratification strategy of patients.

## 7. Metabolic abnormalities in PDAC

As described in the previous chapter, patterns of metabolic changes are normally occurring in the prediagnostic phase of PDAC, offering a diagnostic window for the early detection of an ideally localized resectable and curable disease. In a recent comprehensive study, the temporal profile of metabolic parameters has been annotated in a cohort of PDAC patients compared to controls [58]. The authors identified three distinct metabolic phases, starting around 3 years before diagnosis with new onset hyperglycaemia (Phase I, hyperglycaemia). Subsequently, a decrease in circulating lipids coupled with weight loss, occurs 1.5 years before classic diagnosis (Phase II, pre-cachexia). In the last phase (Phase III, cachexia), occurring around 6 months before diagnosis, a further general decrease in lipids, SCAT, VAT and muscle is seen, whereas fasting glucose continues to increase. SCAT loss is accompanied by an increase in body temperature suggesting that this is a consequence of the browning of white SCAT, a known mechanism of SCAT reduction in cancer [66]. Indeed, the vast majority of PDAC patients reports weight loss that meets the criteria of cachexia at diagnosis [67]. Cancer-associated cachexia is hard to treat [68], being a major cause of mortality responsible for at least 20% of cancer deaths has an impact not only on survival but also on quality of life [69]. Cachexia is a recurrent problem in cancer patients, and it is most commonly seen in PDAC patients; characterized by a reduced calorie intake due to loss of appetite, and coupled with increased energy expenditure [70], it results in pronounced muscle wasting and general inflammation induced by a combination of tumour-derived factors and host cytokines secreted by different tissues and cells of the tumour microenvironment [68].

Non-canonical IκB kinases (IKKs) TBK1 and IKKε are another molecular bridge linking this meta-inflammation to cancer, obesity, and diabetes. [74]. Recent studies have shown in blood samples of PDAC patients, reduced expression of pancreatic polypeptide (PP) and glucose-dependent insulinotropic peptide (GIP) in response to a meal [60,61], or increased activity of the GLP-1 degrading serine exopeptidase dipeptidyl-peptidase-IV (DPP-IV) in NOD patients [63]. Further stratification could also be implemented considering increased plasmatic levels of the antigen CA19-9. However, the sensitivity and specificity of this blood marker is still controversial, marred by unacceptably high levels of false negatives, due to 5–10% of patients not producing it for genetic reasons, or false positives indicating benign conditions [65]. Nonetheless, some authors have recently described its use in diabetic patients, defining a cut-off value of 75 U/ml offering a yet unprecedented sensitivity of 69.5%, and specificity of 98.2% [64].

Despite these encouraging results, as discussed recently by Singhi et al. [38], there is currently no recognized marker that can diagnose a silent PDAC as all available studies use cohorts of patients either carrying PDAC or not.

Diagnosing a clinically silent PDAC requires the validation of a panel of markers in prospective longitudinal studies, whereby different high-risk populations are followed for multiple years. Nevertheless, current evidence already warrants the clinicians' attention in NOD patients reporting weight loss [57,58]. It is in this growing high-risk population that all the potential PDAC specific biomarkers must be investigated to improve the likelihood of early detection while maximizing both sensitivity and specificity. Given this complex picture, it is important to further segment cohorts based on the underlying pathology, distinguishing obesity from T2D or NOD, and considering the overall internal anatomy of PFF, VAT, and SCAT.

## 8. Extracellular vesicles as proxies of the tumour microenvironment

EVs are emerging as key players in multiple neoplastic microenvironments [75]. EVs are divided functionally and morphologically into microvesicles, apoptotic bodies, both derived from the plasma membrane, and exosomes, formed from late stage endosomes with a unique cell-specific cargo.

Recently, PDAC cells have been shown to secrete exosomes capable to suppress the gut-derived insulin-secretagogues GIP and GLP-1 via micro RNAs (miRNAs) in vitro, which compounded by the increase in DPP-IV activity mentioned in the previous section, results in lower levels of GIP reported in PDAC patients.

These miRNA-containing exosomes (*exo*-miRNA), are delivered to the gut via pancreatic juices, and exacerbate the diabetic state often seen after PDAC diagnosis [76]. Some of the glucose lowering properties of metformin can also ascribed to its GLP-1 secreting capabilities [77]. Nonetheless this loss of GLP-1 activity seen in PDAC appears benign given the retrospective evidence also mentioned in section 5; iatrogenic GLP-1 receptor over-activation is associated with PDAC development, especially in the short term [57], although the timing also needs to be considered to discern either causality.

Overall exosomes are valuable blood-markers and, as recently reported, they are capable to distinguish patients with non-malignant pancreatitis from PDAC. Nakamura et al. isolated exosomes from pancreatic juices of patients with either pancreatitis or PDAC, and showed elevated levels of the *exo*-onco-miR-21 and exo-miR-155 while, interestingly, their levels in the whole pancreatic juice were non-discriminatory [78]. Overall, cytology of the pancreatic juice, combined with exo-miR-21 and exo-miR-155, are reported to offer an accuracy of 91% for the diagnosis of PDAC.

Furthermore, both PDAC cells and PSCs are known to secrete exosomes containing adrenomedullin and CA19-9, with the ability to induce peripheral lipolysis [79,80] (Fig. 1), as well as endoplasmic reticulum-stress, to β-cells, resulting in impaired insulin secretion and ultimately leading to cellular death and overt diabetes [81]. This molecular axis explains the weight loss that precedes the diagnosis of NOD and underpins the diagnostic potential of CA19-9 in the plasma as described in the previous section 7.

## 9. Microbiota, immune system and pancreatic cancer

Recent evidence indicates that the human intestinal microbiota can migrate into the pancreatic ducts, establishing an intra-pancreatic microbiome that has been reported to support the development of PDAC and other malignancies in multiple mouse models [82]. The majority of PDAC patients possess an intra-pancreatic microbiota [83], primarily Gram negatives of the phyla *Proteobacteria*, with a marked decrease in their gut levels compared to healthy controls [84].

The PDAC-associated pancreatic and intestinal microbiomes were proven to be pro-tumoral in animal models, while their ablation by oral antibiotics was sufficient to slow down PDAC progression in KC mice [85].

The pro-oncogenic microenvironment supported by this microbiome is shown to involve immune tolerance through the hyperactivation of multiple pattern recognition receptors in monocytic cells [85].

Surprisingly, in addition to a dysbiotic intestinal and pancreatic microbiota, a recent study indicates that the oral microbiota of PDAC patients is also disease-specific, holding a promising non-invasive theragnostic potential [85].

Data also indicate that PDAC treatments can affect the gut microbiome and affect the progression of the disease. For instance, Panebianco et al. [86] have recently shown that treatment with Gemcitabine increases the presence of *Akkermansia muciniphila*, *Escherichia coli*, and *Aeromonas hydrophila* in a mouse xenograft model, while

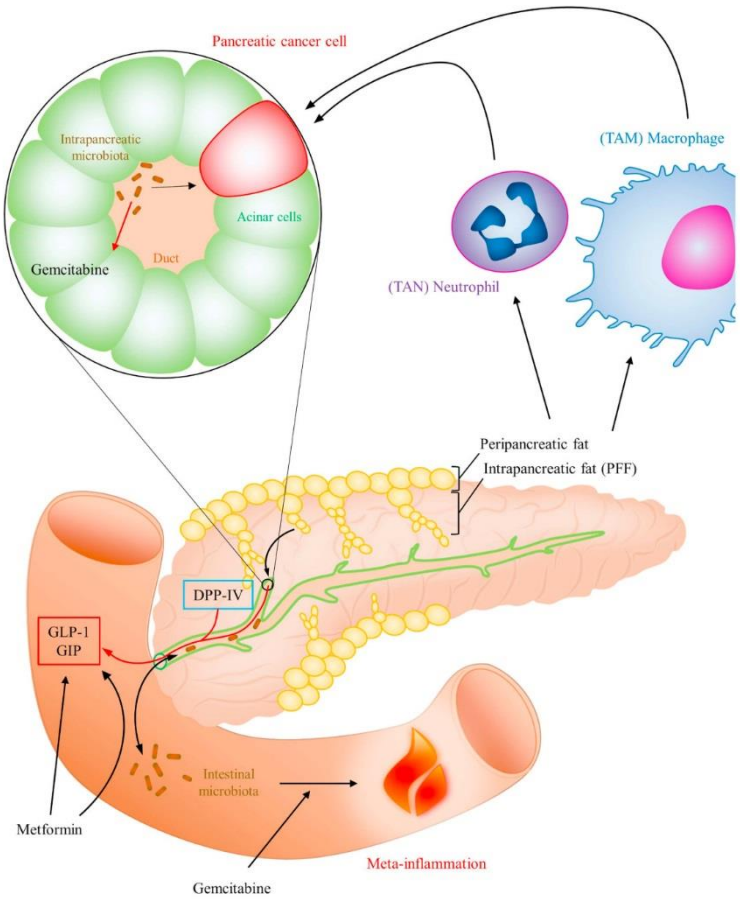


**Fig. 2. The role of microbiota in PDAC associated with diabetes and obesity.** A key molecular player bridging the environment, with obesity, diabetes and the PDAC is the microbiota. A section of the duodenum and the pancreas is shown, highlighting the main pathological axis seen in obese, diabetic patients with PDAC. Tumour bearing patients display high levels of *Proteobacteria* in their pancreatic ducts, capable to exert both pro-tumoral, and chemoresistant properties to PDAC cells [83,90]. Gemcitabine has also shown to support a pro-inflammatory intestinal microbiota [87]. An obesogenic environment supports the growth of visceral, inter-organ and intra-organ fat, which elicits pro-inflammatory and pro-tumoral effects mediated by TAMs, and TANs [14,15,17,23–26]. PDAC derived exosomes containing different miRNAs, have shown to lower the levels of the incretins GLP-1 and GIP [76], a finding compounded by the elevated DPP-IV activity seen both in plasma and in PDAC tissues [63]. Conversely, the anti-diabetic drug Metformin has shown GLP-1 secreting capabilities directly from gut L-cells [77], or indirectly through the microbiota [87]. The clinical monitoring of this pathological microenvironment holds strong potential for a better screening of patients bearing a silent pancreatic cancer. Black and red arrows indicate proven positive and negative modulations respectively. Molecular players found to be upregulated, or down-regulated are squared in blue or red respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

decreasing the two phyla of *Firmicutes* and *Bacteroidetes*, supporting a pro-inflammatory microbiome. Reduced levels of Akkermansia muciniphila have also been described in T2D independently from PDAC status [87]. Furthermore, some Gamma *Proteobacteria*, detected in the majority of pancreata of PDAC patients, are able to metabolize Gemcitabine, therefore mediating chemoresistance [84]. These studies not only underline the importance of the microbiota during PDAC development and progression, but they also suggest its central role during drug resistance development [88]. The role of the immune system, specifically the neoantigen number and quality of CD8 positive T-cell infiltrates, represents a critical parameter that long-term PDAC survivors display. [89]. Indeed, a new study provides experimental evidence that the PDAC associated microbiome is not only protumoral [90], but its composition holds prognostic potential, in long-term

survivors was shown to be more diverse, and less immune-tolerant in mouse models.

Another recent study reports that the faecal microbiota of KPC mouse models in the early stages of the disease is enriched in *Proteobacteria* and *Firmicutes*, representing another easy to access tumour marker [91]. As the gut microbiota represents a dynamic ecosystem that shifts under metabolic insults as seen in obesity [92], both the intestinal and intrapancreatic microbiota are key proxies bridging environmental insults, such as diet, to obesity, diabetes and PDAC [93], playing a significant role in their reciprocal triple-causality (Fig. 2). The benefits of metformin can also be ascribed to its interaction with the gut microbiota, increase in Short chain fatty acids (SCFAs) secreting bacteria, conjugated bile acids, and levels of *Akkermansia muciniphila*, which have shown to play in concert resulting in improved incretin secretion, blood lipidomic, gut barrier integrity and consequent reduction of inflammation [88]. This interplay offers an important therapeutical opportunity adding a new layer of complexity to segment high-risk population screenings, while also providing the grounds for its therapeutical manipulation in future pre-clinical and clinical studies of diabetic individuals at high risk of PDAC. The understanding of the interactions of the immune system with different microbial populations throughout the tumoral evolution is of paramount importance for the development of improved future immunotherapies.

## 10. Pancreatic cancer chemoprevention

Chemopreventive strategies could be useful in individuals that are at high risk of developing pancreatic cancer, or presents premalignant lesions [3]. Different natural and synthetic compounds have been suggested as pancreatic cancer chemopreventive agents both in cellular in vitro and in vivo animal models.

As discussed in section 4, high-fat diet is pro-tumoral in mouse models, with proven capacity to generate metabolic shift via hyper activation of mutagenic KRAS [31]. Another study indeed builds upon this concept, demonstrating that simple anti-inflammatory treatment with aspirin is sufficient to protect from this risk in HFD mice [94].

This study implies that other environmental factors, such as smoking, infection and aging, could influence the phenomenon of cell competition which is physiologically present, and sees normal epithelial cells extruding, and eliminating transformed ones. Nonetheless, results on the utility of aspirin to prevent PDAC risk in humans are debated. Indeed, cholesterol lowering statins, but not aspirin, was found by Archibugi et al. to be associated with a reduced PDAC risk [95].

In particular, the use of statins in combination with aspirin did not reduce further the risk compared with statin alone; moreover, the protective effect of statins was dose-dependent and more evident in smokers, elderly and obese patients. More recently, a more comprehensive meta-analysis study compounds on this evidence indicating that statins use, especially with atorvastatin, is associated with a 30% reduction of PDAC risk [96]. However, the use of statins as chemopreventive agents should take into account the confirmed adverse events caused by their use including the effect on incidence of NOD [106].

Another drug actively investigated in cancer clinical trials, in combination with other treatments and for chemoprevention, is the biguanide drug metformin, commonly used for the treatment of T2D patients. Recent meta-analysis studies, as well experimental evidence in animal models, have indicated that metformin, protects from and improves the prognosis of different types of cancer including PDAC [13,71,97], supporting this first line of treatment for any newly diagnosed T2D. Metformin in particular, appears to exert its tumour suppressing properties by phosphorylating 5′ AMP-activated protein kinase (AMPK), and inhibiting the desmoplastic pro-tumoral reaction of activated PSCs [102]. Further compelling evidence supporting metformin comes from another recent study indicating that it is possible to impair the tumour plasticity by using metformin coupled with intermitted-fasting induced hypoglycaemia. This time-dependent treatments simultaneously blocks oxidative phosphorylation and glycolysis, impairing tumour growth in vitro, and in vivo in mice xenografted with patients-derived melanoma cells [103]. However, other studies dispel the benefits of metformin on PDAC survival [98], with others reporting some benefits specifically in post-pancreatitis diabetes mellitus (PPDM) patients with PDAC [99], a particular subtype of diabetes, different from T2D, often referred to in literature as Type-3c diabetes [100].

## 11. Conclusions

Considering the current worldwide epidemic of obesity and T2D, an increase in the incidence of pancreatic cancer can be foreseen. In this manuscript, we have highlighted the most recent studies that link these three pathologies, defining a complex inter-organ microenvironment summarized in Figs. 1 and 2.

With our current understanding of PDAC, we can better stratify patients with NOD, mainly considering a reported body weight loss and multiple blood-based biomarkers that need to be further evaluated to justify the invasiveness of imaging techniques such as endoscopic ultrasounds (EUS). The latter, is indeed the only available tool for the detection of a localized, early-stage malignant neoplasm with volumes inferior to 2–3 mm, invisible to any other high-content technique such as CT or MRI [38].

Major clinical evidence is necessary to evaluate the druggability of this complex inter-organ pathological interplay, in order to formulate the next generation of therapies for improved prevention, detection and cure of PDAC.

To achieve this goal, longer multi-centred prospective studies are required for a more detailed monitoring of obese and diabetic patients, aiming to better stratify their cohorts, including the important variable of dietary intake. As recently reported, fasting-mimicking diets (FMD) have a profound impact on the body [103], offering an important tool worth exploring as part of preventive, and possibly curative, regimens to disrupt the pathological array of pathways described in this manuscript.

An extended blood chemistry analysis should consider circulating adipokines, which are essential to dissect the role of visceral mesenteric fat, peri-pancreatic fat, and even liver steatosis, given their recognized and distinct role in the pathophysiology of T2D and PDAC. The secretome of the microbiota and other tumoral players such as PSCs are also offering valuable opportunities.

This comprehensive approach could result in more personalized and targeted diagnostic and therapeutic strategies, ultimately providing much needed improvement to the current abysmal survival rates for PDAC patients.

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## Author contributions

S.P. collected analysed and interpreted data from available PUBMED literature, conceptualized and wrote the manuscript. M.F. conceived, revised, supervised and wrote the study; both authors approved the final version of the article.

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### 1.3 Synopsis of Editorial 1

The enteroendocrine cell system is a much more complex endocrine organ than once assumed. More than 30 different peptides have been proven to be released into the circulatory system upon different stimuli, coordinating the whole-body metabolism. Beyond the most studied and understood GLP-1, in recent years the biology of multiple gut-derived hormones has been studied. Gastric Inhibitory Peptide (GIP) and PYY, together with GLP-1 have been shown to not only possess anorexigenic, satiety-inducing properties, but to also affect bone metabolism. Serotonin, Ghrelin and INSL-5, as well as cholecystokinin (CCK) have all been shown to mediate gut mechano-sensation, and responsiveness to bitter molecules. Non-caloric sweeteners, once thought to be passively transiting through the intestine, have shown in recent years to not only activate sweet taste receptors and induce the release of GLP-1, but also to impinge on glucose tolerance directly acting on L-cells, and by modulating the microbiota. The physiology of GLP-1 has indeed evolved in strict relationship with many other intestinal and pancreatic hormones. The intracellular synergism of many signalling pathways elicited by many of these hormones has proven in recent years to offer important clinical benefits. For example, co-agonism of both GLP-1R and Glucagon receptors has potent hepatoprotective properties, with encouraging animal data supporting its use in non-alcoholic fatty liver disease (NAFLD).

#### 1.3.1 Significance

This editorial has been authored to collate and discuss nine recently published manuscripts, including Review 1 introduced in chapter §1.1, as part of the research topic of gastrointestinal hormones hosted by the publishing Journal Frontiers in Endocrinology. The studies analysed in Editorial 1, highlight the complexity surrounding the biology and of gastrointestinal hormones, helping us to shape a broader picture of this endocrine organ in metabolic diseases.

frontiers in Endocrinology

EDITORIAL
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# Editorial: Gastrointestinal Hormones

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**Keywords: glucagon-like peptides, gastrointestinal hormone, G protein coupled receptor (GPCR), NAFLD, enteroendocrine cell**

## Editorial on the Research Topic

## Gastrointestinal Hormones

The gastrointestinal tract is a vast organ hosting a broad gamut of hormone secreting cells. After more than a century since the first description in 1906 of metabolically active gut extracts by Moore (1), we are still unveiling novel functions in health and disease for this complex endocrine organ.

In the current Research Topic, we picture our current understanding of the enteroendocrine cell system (EECs), with new layers of complexity, covering not only the physiology and pathophysiology of the most well-characterized gut peptides Glucagon-like peptide-1 (GLP-1) and the Gastric insulinotropic peptide (GIP), but also many other mediators of central metabolism.

In this issues Sun et al. discuss the most recently recognized physiological impact of different gut hormones including GIP, GLP-1, Oxyntomodulin, peptide YY (PYY), serotonin, ghrelin, and the less studied insulin-like peptide 5 (INSL-5). The first four, known primarily for their anorexigenic, satiety-inducing action, while the last two for their orexigenic, appetite-inducing activity, orchestrate our metabolic response to food, maintaining our glucose, and energy homeostasis. The role of serotonin (5-HT) is also highlighted by Beyder, as a key mediator of gut-mechanosensation.

Recent studies have expanded our understanding of the physiology of different gut hormones, namely GLP-1, GLP-2, GIP, and PYY in bone metabolism. Schiellerup et al. discusses the most recent evidence updating us on the potential development of drugs based on these peptides for the management of diseases affecting bone metabolism, such as osteoporosis.

In two other reviews, an updated picture of the gut chemo-sensation of sweet and bitter tastants is dissected. Kreuch et al. delves deeper into the most recent findings surrounding the ability to detect dietary sugars, considering the recent rise in ingested non-caloric sweeteners and their impact on enteroendocrine cells and the microbiome, ultimately affecting satiety and glycaemia. Influence of this complex gut-brain axis holds a yet untapped potential for the management of chronic metabolic diseases such as obesity and diabetes. Similarly, understanding of the bitter sensation in the gut, highlighted by Xie et al., has important clinical implications. The chemo-sensation of bitter molecules beyond the tongue is transduced by different G-protein coupled receptors, namely different species of type 2 receptors (T2Rs) expressed by the enteroendocrine cells. This powerful gut axis is another tool with therapeutical implications for the modulation of different gut hormones, including the anorexigenic GLP-1, PYY, cholecystokinin (CCK), and the orexigenic Ghrelin.

Nonetheless, among all the gut-derived hormones, GLP-1 is the only one, which biology is currently exploited for the treatment of metabolic pathologies such as type 2 diabetes and obesity. Rowlands et al. expand upon the implications of GLP-1 mimicking drugs, consider the molecular targets in both acute and chronic settings, and dissect the tissue-specific downstream signaling pathways behind the recognized cardiometabolic benefits of these therapeutics.

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Drugs activating the GLP-1 receptor are also beneficial in the management of another current epidemic, namely non-alcoholic fatty liver disease (NAFLD). Seghieri et al. delves deeper into this topic by reviewing the clinical benefits of molecules designed to activate both GLP-1 and Glucagon receptors, with superior anti-NAFLD properties. Nevertheless, the physiology and pathophysiology of GLP-1 is indeed much more complex than initially thought, with GLP-1 now thought to not only be acting systemically as a hormone through the bloodstream. Our understanding of gut-derived, and pancreas derived GLP-1, are both analyzed and repurposed by Paternoster and Falasca drawing more attention on the tissue-specific action of different GLP-1 species, including the once thought inactive, and much more abundant, metabolites.

Rehfeld encourages us to broaden the old concept of gut hormones inducing the release of pancreatic hormones, or incretins, currently epitomized mainly by GLP-1 and GIP. The currently recognized physiological axis linking the gut to the pancreas for whole-body metabolic control sees hundreds of different peptides, often with overlapping functions and possibly synergistic actions of yet unknown biology and metabolic impact.

A better understanding of the physiology of EECs and their involvement in a variety of physiological pathways and pathophysiological phenomenon will likely provide a strong platform for future therapeutics designed to address the morbidity of chronic metabolic diseases such as obesity and type 2 diabetes.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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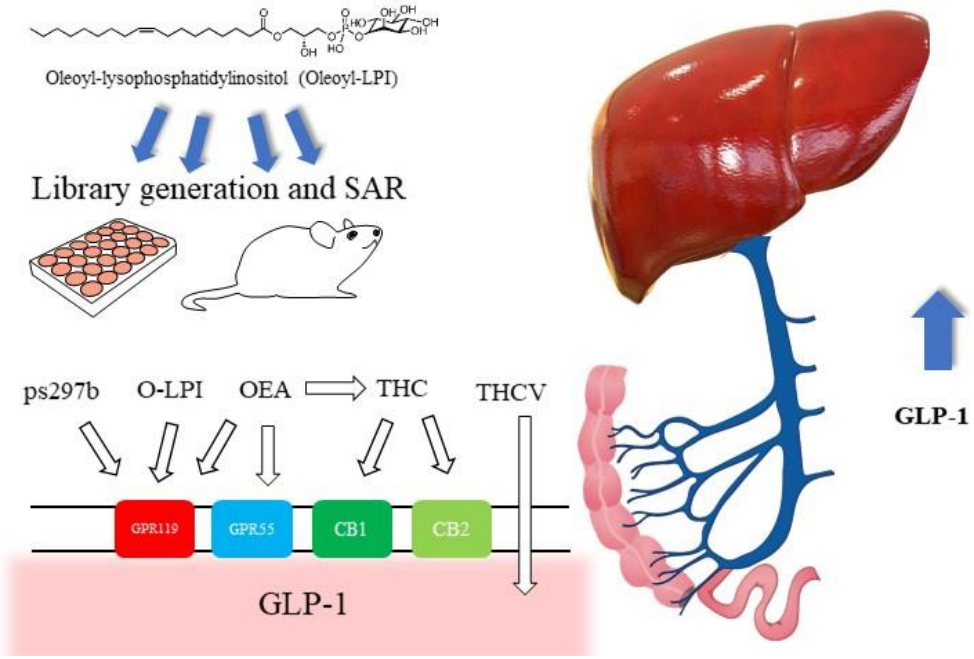
**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## 1.4 Graphical Abstract




The overall aim of this PhD thesis is to dissect and explore the pharmacological properties of multiple natural and synthetic drugs that display GLP-1 secreting properties, further advancing our understanding of this novel therapeutical approach in metabolic diseases.

# Chapter 2

## Chapter 2: Oleoyl-lysophosphatidylinositol as a novel GLP-1 secreting agent

**The content of this chapter presents the following peer-reviewed manuscript for which I share the first name authorship**

Arifin SA*, **Paternoster S***, Carlessi R, et al. Oleoyl-lysophosphatidylinositol enhances glucagon-like peptide-1 secretion from enteroendocrine L-cells through GPR119. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2018;1863(9):1132–1141. doi:10.1016/j.bbalip.2018.06.007

* These authors contributed equally to this work

### 2.1 Synopsis of Manuscript 1

The digestion of food is a complex physiological process orchestrated by multiple organs and tissues that have evolved in concert to maximize the absorption of macromolecules, essential microelements and water.

This multi-stage process has been shaped by our diets throughout millions of years of food scarcity, whereby energy-dense fats of either animal or plant origin represented a superior source of sustainment. This evolutionary pressure, selected for a fine-tuned ability to not only metabolize, but also communicate to the rest of the body the ingestion of a lipid rich meal.

Only a few decades ago, with the development of molecular tools for the detection of specific proteins in our blood, we started to appreciate the complexity and heterogeneity of lipids. It is now recognized that different cell types in different tissues actively use a growing number of lipid-like molecule to communicate via paracrine and endocrine messages, both in health and disease.

Multiple food-derived lipidic molecules, as well as many produced endogenously from different cells across the human body, have been shown in the last two decades to stimulate the release of GLP-1 from the EECs by activating one or more transmembrane receptors**.** Oleoyl-ethanolamide (OEA) and Lyso-phosphatidylcholine (LPC), are two bio-active lipids, of primarily endogenous and dietary origin respectively, that agonise and stimulate the same G-protein coupled-receptor (GPCR) GPR119 (H. S. Hansen, Rosenkilde, Holst, & Schwartz, 2012; Lauffer, Iakoubov, &

Brubaker, 2009; Overton et al., 2006), triggering an intracellular molecular cascade, that ultimately leads to the release of GLP-1.

LPC is just one lipid of a broader family of lyso-phospholipids (LPLs), under which multiple other molecules, such as Sphingosine-1-phosphate (S1P) or lyso-phosphatidyc acid (LPA) have been shown to activate multiple transmembrane receptors, regulating pleiotropic cellular functions such as survival, and angiogenesis (Falasca & Ferro, 2016; Uhlenbrock, Gassenhuber, & Kostenis, 2002). Another less studied and understood subclass of LPLs is represented by lyso-phoshatidylinositols (LPI). Glycerol-based phospholipids, embedded in cellular membranes, can be processed by two different types of Phospholipases A (PLA1 and PLA2), to generate mono acylated phosphatidylinositols with increased solubility in aqueous medium (Arifin & Falasca, 2016). PLA1 enzymes, trim the esther bond in position 1, while PLA2 hydrolyses the chain in position 2; the resulting lysophospholipids will be left with the original aliphatic chain present in the PI. Membranes of animal cells often bear in position sn-1 a saturated chain of stearic acid, and in the central position sn-2 an arachidonic acid.

This important distinction signifies that the products of PLA1 will be primarily 2-arachidonoyl-LPI. However, the fatty acid in this central position is unstable, and quickly isomerizes into 1-arachidonoyl-LPI (A-LPI) (Rytczak, Drzazga, Gendaszewska-Darmach, & Okruszek, 2013). On the other hand activity of PLA2 leads to the formation of Stearoyl-LPI (S-LPI), the most abundant LPI species found in the rat brain at concentration approaching 20 nanomoles per gram of tissue (Oka et al., 2009). Another more recent study further dissects the abundance of LPI species in different mouse tissues and corroborates previous reports showing that S-LPI is the most abundant species found at concentrations ranging from 10 to 50 nanomoles per gram of tissue. Only in the brain stem A-LPI is reported as the main LPI at the relatively lower concentration of 5.5 nanomoles per gram of tissue. On the other hand, a third species of LPI, bearing an Oleoyl chain, ranges from 2 to 5 nanomoles per gram of tissue (Masquelier & Muccioli, 2016). A recent study has also shown that in mice, the levels of both A-LPI and O-LPI decrease with age (Eum et al., 2020). This evidence clearly indicates that multiple LPIs are present in a pharmacologically relevant micromolar range and their concentration can be modulated. PLA1 enzymes are indeed a family of lipid-processing proteins that display different degrees of specificity for

Phospholipids beyond PIs, leading to the formation of different lyso-lipids. Phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholines (PC), and PI can all be metabolized by different PLA1s to 2-arachidonoyl-LPI, but also LPA, LPS, LPC and Lyso-phosphatidylethanolamine (LPE) (Aoki, Inoue, Makide, Saiki, & Arai, 2007; Aoki, Nagai, Hosono, Inoue, & Arai, 2002). In brain tissue the activity of PLA1, also known as DDHD1, appears to be regulated by another class of Phospholipases type D (PLD) (Yamashita et al., 2010) and appears important in multiple neurodegenerative diseases (Inloes, Jing, & Cravatt, 2018). Mammals express also a heterogeneous family of enzymes displaying PLA2 activity, with different localization, modulation and implication in pathologies like cancers (Mariggiò et al., 2006). Amongst all the lyso-phospholipids, LPIs are the less studied and understood. These bio-active molecules are known to modulate important physiological and pathological functions such as tumour promotion (Falasca & Ferro, 2016) in a Ras dependent manner (Falasca & Corda, 1994) or even atherosclerosis in a mouse model (Yan et al., 2019). The specific activity of LPIs was indeed recognized in terms of calcium-mediated insulin secretion more than three decades ago (Metz, 1986, 1988).

Only after almost 3 decades, GPR55 was shown to be the LPI receptor (Oka, Nakajima, Yamashita, Kishimoto, & Sugiura, 2007; Oka et al., 2009), corroborating an intracellular Gαq-Rho-ROCK mediated Calcium signalling pathway. Indeed, agonism of GPR55 appears critical in the progression of pancreatic cancer (PC), whereby cancerous cells have proven to display increased activity of cytoplasmic PLA2 that increases the levels of 1-acyl-LPI, which is then extruded from the cell with the transporter ABCC1, to access and agonise GPR55 ultimately supporting cellular proliferation (Piñeiro, Maffucci, & Falasca, 2011).

Indeed inhibition of GPR55 with the natural antagonist Cannabidiol, has proven to improve the survival of mouse models of pancreatic cancer (Ferro et al., 2018).

Blood levels of LPI have also been correlated with metabolic diseases, such as obesity and diabetes. Elevated levels of LPI, as well as GPR55 expression in visceral fat, have been shown in obese individuals, representing a valuable blood-based biomarkers of early stages of Type 2 diabetes (Moreno-Navarrete et al., 2012; Mousa et al., 2019) albeit with some contrasting reports indicating stable, non-diet inducible LPI levels in obesity (Del Bas et al., 2016). Nonetheless LPIs mediate some of their cellular

function, including Insulin secretion, independently from GPR55 (Liu et al., 2016; Piñeiro & Falasca, 2012), implicating the activity of other receptors.

A possible receptor candidate is indeed represented by GPR119, known to be expressed by pancreatic β-cells, as well as GLP-1 secreting enteroendocrine cells. As mentioned earlier, this receptor is already known to show some promiscuity for its ligands, being activated by OEA and Oleoyl-LPC among other lipids.

Having considered the available evidence, in the study presented in this Manuscript 1, we set out to dissect the pharmacology of LPIs in enteroendocrine cells, and if this would indeed result in possible GLP-1 secreting properties.

LPIs can be differentiated by the nature of their aliphatic chain, which could vary in length and unsaturation degree. The preparations of commercial LPIs used in many of these studies, are sold as heterogeneous extracts isolated from food sources like soybeans, raising important questions regarding the pharmacology of specifically acylated LPI species. Given the LPI preference of GPR55, we used highly purified LPI preparations to discern a similar species-specific pharmacology against GPR119.

Firstly, we screened the GLP-1 secreting properties of Oleoyl-LPI, Arachidonoyl-LPI, Heptadecenoyl-LPI and Stearoyl-LPI, and surprisingly identified Oleoyl-LPI as an efficacious and potent GLP-1 secretagogue in both murine GLUTag and human NCI-H716 cells; this activity was far superior to the previously recognized endogenous GPR119 ligand OEA (Manuscript 1, Figure 1).

OEA is indeed another bio-active compound naturally synthesized within the mucosa of the gastrointestinal tract, and distributed systemically with diurnal fluctuation, to primarily regulate satiety via activation of PPARα (Lo Verme et al., 2005)

Pharmacological inhibition of GPR55 (Manuscript 1, Supplementary Figure 4), in addition to genetic knockdown with siRNAs *in vitro*, as well as *ex-vivo* secretion studies from tissues genetically deficient for either GPR55 or GPR119 (Manuscript 1, Figure 2 and 3), pinpointed the latter as the only receptor capable to relay Oleoyl-LPI-mediated GLP-1 and insulin release.

Having defined the main endogenous ligand of GPR119, we set out to dissect the intracellular cascade underpinning GLP-1 secretion, to better understand its pharmacology.

Within 10 minutes, Oleoyl-LPI activates in a GPR119-dependent manner, both the MAPKKK cascade of MEK1/2-ERK1/2 (Manuscript 1, Figure 4), as well as the cAMP-PKA-CREB pathway (Manuscript 1, Figure 5). The former appears essential for an efficient GLP-1 secretion, while the latter at least partially dispensable in the tested cell lines. Combined pharmacological inhibition of both pathways completely abolishes GLP-1 secretion, suggesting a mechanistic interaction between the two axes. The only partial mechanistic implications of CREB activity, are also corroborated by the similar degree of cAMP that both Oleoyl-LPI and OEA elicit (Manuscript 1, Supplementary Figure 3), while resulting in wildly dissimilar efficacies in terms of secreted GLP-1.

## 2.2 Brief materials and methods

### 2.2.1 Cell culture and hormone secretion studies

Two different cell lines were utilized to model intestinal GLP-1 secreting cells. GLUTag and NCI-H716 cells are widely accepted immortalized cell lines, useful for their easy maintenance in culture, amenable to transient genetic engineering, making possible the study of prospective GLP-1 secreting agents such as LPI species.

For this study, cells were adhered to 24-well plates, and treated for 2 hours with different drugs dissolved in serum-free media. Cleared supernatant were collected and total GLP-1 levels were quantified with a commercial ELISA kit.

The COS-7 cell line transiently transfected with GPR119 was used as an established model to quantify and compare the accumulation of intracellular cAMP.

### 2.2.2 Genetic knockdown with siRNA

In order to validate the tropism of Oleoyl-LPI, the expression of either GPR55, or GPR119 was knocked down with a pool of 4 different oligonucleotides targeting different regions of the same transcript (Supplementary Table 1A). Effective reduction in expression was validated both via Real-Time PCR and Western Blotting.

### 2.2.3 Animal work

To further corroborate the in vitro findings, primary cultures from the colons of mice genetically deficient for either GPR55, or GPR119 were established as previously described in literature (Reimann et al., 2008). The colonic preparations were exposed to Oleoyl-LPI for 2 hours, and secreted GLP-1 was then quantified from clarified supernatants. Similarly, pancreatic islets from GPR119 deficient animals, were exposed to Oleoyl-LPI, and secreted Insulin was quantified with a commercial ELISA kit.

### 2.2.4 Immunoblotting

To validate the efficient genetic knockdown of receptors at the protein level, as well as to assess the activity of specific intracellular cascades, whole cell lysates were screened via immunoblotting. Blotted cellular extracts were probed with commercially available antibodies. Of note, GPR119 membranes were incubated overnight at 4°C with the Santa Cruz goat-derived polyclonal antibody sc-48195 diluted 1:500 in Milk-PBS-T.

## 2.3 Conclusions and significance

This manuscript sheds new light on the molecular pharmacology of GPR119. In particular, we show that the release of both GLP-1 and Insulin via GPR119, is dictated by the nature of the fatty acid chain of the ligand, defining a novel and relevant bio-active lipid with important therapeutical implications. GPR119 agonists are indeed known secretagogues of both GLP-1 and Insulin release (Arifin et al., 2018; Moran, Abdel-Wahab, Flatt, & McKillop, 2014; Oshima et al., 2013), therefore the focus of this study was to qualitatively discern the pharmacological activity of Oleoyl-LPI.

The pharmacological evidence we provide in this manuscript, should be further corroborated in future studies via genetic rescue of GPR119 in different cell lines, as well as pharmaceutical antagonism of the same receptor.

Furthermore, the dose-dependent GPR119-mediated cAMP cascade triggered by O-LPI that we report in COS-7 cells, should be further validated in more physiologically relevant L-cell line models to exclude pharmacologies unique to GLP-1 secreting cells.

Patients affected by diabetes are already using synthetic GLP-1, as well as DPP-4 inhibitors to activate at supraphysiological, and systemic levels the GLP-1 axis. But this comes at the price of important side effects. In this study we show that Oleoyl-LPI, among the LPI species that we tested, represents the prototypical food derived bio-active lipid, that could potentially be taken orally to activate intestinal GPR119 and induce the release of GLP-1, without the need for systemic absorption and distribution.

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# Oleoyl-lysophosphatidylinositol enhances glucagon-like peptide-1 secretion from enteroendocrine L-cells through GPR119

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ABSTRACT

The gastrointestinal tract is increasingly viewed as critical in controlling glucose metabolism, because of its role in secreting multiple glucoregulatory hormones, such as glucagon like peptide-1 (GLP-1). Here we investigate the molecular pathways behind the GLP-1- and insulin-secreting capabilities of a novel GPR119 agonist, Oleoyl-lysophosphatidylinositol (Oleoyl-LPI). Oleoyl-LPI is the only LPI species able to potently stimulate the release of GLP-1 in vitro, from murine and human L-cells, and ex-vivo from murine colonic primary cell preparations. Here we show that Oleoyl-LPI mediates GLP-1 secretion through GPR119 as this activity is ablated in cells lacking GPR119 and in colonic primary cell preparation from GPR119$^{-/-}$ mice. Similarly, Oleoyl-LPI-mediated insulin secretion is impaired in islets isolated from GPR119$^{-/-}$ mice. On the other hand, GLP-1 secretion is not impaired in cells lacking GPR55 in vitro or in colonic primary cell preparation from GPR55$^{-/-}$ mice. We therefore conclude that GPR119 is the Oleoyl-LPI receptor, upstream of ERK1/2 and cAMP/PKA/CREB pathways, where primarily ERK1/2 is required for GLP-1 secretion, while CREB activation appears dispensable.

## 1. Introduction

The hormone glucagon-like peptide-1 (GLP-1) is released from the intestine under postprandial conditions and it stimulates insulin secretion from the pancreatic β-cell in a glucose-dependent manner. Although GLP-1 mimics are already being used in the clinic to treat type 2 diabetic (T2D) patients, these have raised several safety issues such as risk of developing pancreatitis and medullary carcinoma of the thyroid [1]. Therefore, an alternative strategy, based on the possibility to increase endogenous GLP-1 secretion rather than administering exogenous GLP-1, is an attractive therapeutic option. It has been reported that glucose-stimulated insulin secretion (GSIS) of GLP-1 can be further modulated by amino acids, fatty acids and lipids [2–4]. Interestingly, most of the endogenous GLP-1 secretion is suggested to be mediated by G protein-coupled receptors (GPCRs) such as GPR119 [5, 6], GPR40 [7] and GPR120 [8]. Of these three, GPR119 seems to be the most important inducer of gastric inhibitory polypeptide or glucose-dependent insulinotropic peptide (GIP) and GLP-1 secretion after fat ingestion [9]. GPR119 is a member of class A GPCRs, which can bind to long-chain fatty acids including oleoylethanolamide (OEA), 2-oleoylglycerol (2-OG) and lysophosphatidylcholine (LPC) [6]. The expression of GPR119 mRNA has been reported in a number of tissues including brain, heart, spleen and stomach [10, 11]. The highest level of GPR119 expression has been reported in islets of Langerhans, pancreatic β-cell lines, and

*Abbreviations:* GLP-1, glucagon-like peptide-1; GPCR, G protein-coupled receptor; Oleoyl-LPI, Oleoyl-lysophosphatidylinositol; T2D, type 2 diabetes

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intestinal L-cells [12, 13]. Activation of GPR119 has been shown to positively modulate incretins and insulin secretion in humans [4, 14]. In rodents, this effect results in reduction of food intake and body weight gain [15, 16]. GPR119 expression in pancreatic β-cells led to the hypothesis that this receptor could play a role in modulating insulin secretion. Therefore, GPR119 agonists, which have been shown to raise intracellular cyclic AMP (cAMP) levels in vitro in different pancreatic β-cell systems expressing endogenous GPR119, would be expected to potentiate GSIS in a similar manner as GLP-1 and GIP, hormones which also act via GPCRs in β-cells. The insulinotropic actions of GPR119 agonists have been demonstrated in different models of pancreatic β-cells [17]. Although OEA has been identified as the main ligand for GPR119, other phospholipids such as lysophospholipids are potential ligands for this receptor [11]. The lysophospholipid lysophosphatidylinositol (LPI), initially discovered as ligand for GPR55 [18], has multiple GPR55-independent physiological and pathological roles [19].

In this study, we describe the role of a specific species of LPI in the secretion of GLP-1 from enteroendocrine L-cells and primary cell preparations. We further demonstrate the specific role of GPR119 in LPI-dependent GLP-1 secretion. To achieve this, we downregulated GPR119 and GPR55 protein expression in vitro through specific siRNAs and we used colonic primary preparations from $GPR55^{-/-}$ and $GPR119^{-/-}$ transgenic mouse models in ex vivo experiments. Investigation of the Oleoyl-LPI-induced GLP-1 secretion mechanism revealed that GPR119 activation regulates GLP-1 secretion through a pathway dependent on the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and stimulation of a cAMP/protein kinase A (PKA)-dependent pathway.

## 2. Materials and methods

### 2.1. Reagents

Different LPI species were purchased from Avanti® Polar Lipids, USA. Stocks were dissolved in methanol:chloroform (M:C) and stored at −20 °C in nitrogen atmosphere. Oleoylethanolamide (OEA), DPP-IV inhibitor (KR-62436), CID16020046 and PD98059 from Sigma-Aldrich, UK, were dissolved in DMSO. The OEA used for the cAMP experiment, from Cayman Chemical, USA, and H-89 (from Santa Cruz Biotechnology, USA) were dissolved in DMSO.

GeneJET RNA Purification Kit, Maxima Reverse Transcription Kit and Maxima SYBR Green/ROX qPCR Master Mix Kit were purchased from Thermo Fisher Scientific, USA. Primary antibodies used for immunoblotting were: anti-GPR119 from Santa Cruz Biotechnology, USA, anti-GPR55 from Cayman Chemical, USA, anti-α-Tubulin from Sigma-Aldrich, UK, anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) from Cell Signaling Technology, USA, and anti-phospho-CREB (Ser133) from R&D Systems, USA.

Animal use for this study was approved by the Animal Ethics Committee of Curtin University, and all procedures were carried out in accordance to NIH guidelines, No. 8023, revised 1978.

### 2.2. Cell culturing

NCI-H716 cells were maintained in 75 $cm^2$ tissue culture flasks (Corning® Costar®) in RPMI 1640 media containing 2.0 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, and 4.5 g/L glucose and supplemented with 10% FBS. When plating for an experiment, cell adhesion and endocrine differentiation were initiated by growing cells in dishes coated with growth factor reduced Matrigel™ diluted at the ratio of 1:2 (Becton Dickinson, USA) in high-glucose Dulbecco's modified Eagle medium (DMEM), 10% FBS, 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. GLUTag cells were maintained in low glucose DMEM (Sigma-Aldrich, UK) (1 g/L) containing 2 mM L-glutamine, 1 mM sodium pyruvate supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. COS-7 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, USA).

### 2.3. siRNA transfection

GLUTag and NCI-H716 cells transfection was performed according to the "Lipofectamine® 2000 transfection protocol" (Thermo Fisher Scientific, USA) and optimised for a 6-well plate format. Briefly, cells were plated in complete growth medium at a density of $3.0 \times 10^5$/well in a 6-well plate and incubated at 37 °C/5% $CO_2$. Twenty-four hours later, 300 nM of total siRNA pool and 4.0 µl of Lipofectamine transfection reagent were diluted in separate tubes in Opti-MEM™ (Thermo Fisher Scientific, USA) and incubated for 5 min at room temperature (RT). The two solutions were then mixed and incubated for further 20 min at RT to form transfection complexes. Cells were washed twice with PBS, and then incubated for 4 h with the siRNA/Lipofectamine complexes before the addition of Opti-MEM™ containing 30% FBS without antibiotic. Cells were further incubated at 37 °C/5% $CO_2$ for 48 h before the secretion experiment. Cells were harvested at the appropriate time point for protein expression analysis (between 2 and 4 days post transfection). Transfection efficiency was quantified by real-time RT-PCR. Sequences of siRNAs and primers are listed in Supplementary Tables 1A and 1B.

### 2.4. Protein analysis by Western blot

Whole-cell protein preparations were obtained from cells grown in 6-well plates at ~80–90% confluency. After two washes in cold PBS, cells were lysed, on ice, in 150 µl of Triton X-100 lysis buffer, containing phosphatase and protease inhibitors (Sigma Aldrich, UK). Lysates were collected by scraping, and cleared from cellular debris by centrifugation at 20,000*g*, 4 °C for 5 min. The supernatants were then collected and total protein content quantified before storage at −20 °C. Lysates were then analysed by SDS/PAGE.

### 2.5. Immunoblotting and visualisation

Non-specific antibody binding was prevented by incubating the nitrocellulose membranes in PBS-0.05%Tween-20 (v/v, PBS-T) supplemented with 5% milk for 30 min on a rotating plate. The membranes were then washed with PBS-T followed by overnight incubation with the primary antibody diluted in PBS-T on a rotating plate at 4 °C. After 24 h, membranes were washed 5 times for 5 min with PBS-T, and then incubated on a rotating plate with the appropriate secondary antibody for 1 h at RT. The membranes were then washed with PBS-T, and incubated with ECL Prime solution (Amersham Bioscience, UK) or with Immobillon Western Chemiluminescent HRP substrate (Merck Millipore, UK) for 5 min, placed in a dark cassette and exposed to chemiluminescence film, or directly imaged with a ChemiDoc XRS system.

### 2.6. Real time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed according to manufacturer's instructions (Thermo Fisher Scientific, USA). Briefly, 100 ng of cDNA were mixed with 7.5 µl of 2X Maxima SYBR green/Fluorescein qPCR mix, 1 µl of forward and reverse primers (10 µM stock) to a final volume of 15 µl. GADPH cDNAs were also amplified as an internal control. All experiments were performed in triplicate. Data were collected at the extension step. The relative changes in gene expression quantification were calculated using the relative ddCT analysis mode of the ABI 7500 Real-Time PCR system software.

The primer pairs used for the amplification of GPR119 and GPR55 were based on published sequences [11, 20] and [21] respectively (Supplementary Table 1B).

### 2.7. Primary cell studies

Mice were euthanized and 5 cm of colon were used for isolation of epithelial cells following a previously described protocol [10]. Briefly, colon segments were dissected from 10-week-old C57BL/6 male mice. Segments were cut open longitudinally and washed quickly in DMEM (serum-free) to remove luminal content. Colon was chopped into 1–2 mm pieces and digested with 0.5 mg/ml collagenase-P for 15 min. Resulting cell suspensions were filtered through 70 μm nylon cell strainers (BD Falcon, UK), centrifuged at 300g for 5 min, and pellets were re-suspended in DMEM supplemented with 10% FBS. The purified cells were collected and cultured on 24-well Matrigel-coated plates at 37 °C in DMEM (25 mM glucose), 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. On the day of the GLP-1 secretion assay, cells were washed twice with PBS, and then treated with LPI or vehicle M:C for 2 h at 37 °C. Supernatants were collected for GLP-1 quantification using the active GLP-1 ELISA kit from Millipore.

After euthanasia, pancreatic islets were isolated as described in detail elsewhere [22]. Following purification, islets were rested overnight at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$ in RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin.

### 2.8. Secretion studies

Approximately 80,000 GLUTag cells were plated on poly-L-lysine–coated 24-well culture plates and grown to 80% confluence. NCI-H716 cells were seeded on growth factor reduced Matrigel™ (Becton Dickinson, USA) in DMEM medium, supplemented with 10% FBS the day before the experiment. For NCI-H716 cells, ~25,000 cells were needed for the secretion experiment in order to detect a clear stimulation over low basal GLP-1 secretion. In brief, GLUTag and NCI-H716 cells were washed twice and then incubated for 2 h with serum free DMEM containing 1% vehicle (M:C) alone (negative control), 10 μM forskolin as positive control (Sigma-Aldrich, UK), or with different concentrations of Oleoyl-LPI.

For secretion experiments, media and cells were collected separately. In separate studies, GLUTag or NCI-H716 cells were pre-treated for 1 h with vehicle (DMSO) alone (negative control), PD98059 (50 μM; Sigma-Aldrich, UK.), H89 (10 μM; Santa Cruz Biotechnology, USA) followed by treatment with Oleoyl-LPI (20 μM), OEA (10 μM), PMA (10 μM) or forskolin (10 μM) in the presence of DPP-IV inhibitors, for 2 h.

Values of GLP-1 detected in the supernatants were normalized to the number of cells/well. Data were then expressed as percentage of basal GLP-1 secretion (GLP-1 detected in supernatants from cells treated with vehicle alone).

Glucose-stimulated insulin secretion (GSIS) was performed on hand-picked islets placed in groups of five into individual micro centrifuge tubes. Islets were washed twice, then incubated with Krebs buffer (KRB, 115 mM NaCl; 4.7 mM KCl; 1.28 mM $CaCl_2{\cdot}2H_2O$; 1.2 mM $KH_2PO_4$; 1.2 mM $MgSO_4{\cdot}7H_2O$; 10 mM $NaHCO_3$; 10 mM HEPES; 0.5% BSA; pH 7.4) supplemented with 1.1 mM glucose for 40 min at 37 °C. Supernatants were discarded and islets were incubated with KRB supplemented with 16.7 mM glucose in the presence or absence of Oleoyl-LPI for 20 min. Supernatants were collected for insulin determination using Mouse Ultrasensitive Insulin ELISA (Mercodia, Sweden) according to manufacturer instructions and data was reported as picograms of insulin per islet in 20 min (pg/islet/20 min).

### 2.9. cAMP assay

COS-7 cells were transfected with 40 μg GPR119 DNA or pcDNA3 per 175 $m^2$ flask using the calcium-phosphate precipitation method [23]. One day after transfection, 35,000 cells/well were seeded in a 96 well white plate (Perkin Elmer, USA). The cells were washed twice the following day and incubated 30 min at 37 °C with HBSS plus 1 mM IBMX (Sigma-Aldrich, USA). Agonists were added for 30 min in the assay medium. The Hithunter® cAMP XS+ (DiscoveRx, USA) was carried out following the manufactured description [24].

### 2.10. Ex-vivo studies

The GPR55$^{-/-}$ mice were kindly provided by Prof. David Baker (Queen Mary University of London) and GPR119$^{-/-}$ were obtained from Taconic. Littermates were used as wild-type controls.

## 3. Results

### 3.1. Oleoyl-LPI enhances secretion of GLP-1 from enteroendocrine L-cells

In order to determine which LPI species is able to modulate GLP-1 secretion from enteroendocrine L-cells, murine L-cells GLUTag were stimulated with 20 μM of different species of LPI (Arachidonyl-LPI, Stearoyl-LPI, Heptadecenoyl-LPI and Oleoyl-LPI). OEA was used as a control based on previous studies reporting its role in the secretion of GLP-1 [11, 20]. Forskolin and phorbol 12-myristate 13-acetate (PMA) have been reported to potently enhance GLP-1 release in GLUTag [25] and in human L-cells NCI-H716 [26], hence they were also chosen as positive controls in these experiments. Data indicated that Oleoyl-LPI significantly stimulated GLP-1 secretion in GLUTag (Fig. 1A). Importantly, no increase of GLP-1 secretion was detected upon stimulation of GLUTag cells with any of the other LPI species used at the same concentration (20 μM). Dose response experiments further confirmed increase of GLP-1 secretion upon stimulation with Oleoyl-LPI from both GLUTag (Fig. 1B) and NCI-H716 (Fig. 1C) cells.

Interestingly, we observed that Oleoyl-LPI was able to stimulate GLP-1 release much more than OEA in these cells. Dose-response analysis of GLP-1 secretion induced by Oleoyl-LPI and OEA showed similar EC50 values but different maximal efficacies in both GLUTag and NCI-H716 cells (Fig. 1D and E), suggesting that OEA is only a partial agonist of GPR119 compared to Oleoyl-LPI.

Taken together, these data indicate that Oleoyl-LPI is the specific species of LPI which is responsible for the increased GLP-1 secretion from these L-cells.

### 3.2. Oleoyl-LPI-induced secretion of GLP-1 is not regulated by GPR55

LPI has been identified as an endogenous ligand for GPR55 [18] with a role in gut motility and satiety [27]. Although GPR55 is abundantly expressed along the gastrointestinal tract, its potential involvement in GLP-1 regulation has never been investigated. Our observation that Oleoyl-LPI potently induced secretion of GLP-1 raised the question of whether GPR55 was involved in this process. To investigate this, GLUTag and NCI-H716 cells were transfected with a specific siRNA targeting GPR55 or a non-targeting siRNA (siControl). Downregulation of GPR55 was assessed at the protein level by Western blot analysis in both GLUTag and NCI-H716 cells (Fig. 2A and B), as confirmed by densitometry analysis (Fig. 2C and D) and further validated at mRNA levels in GLUTag cells by RT-qPCR (Fig. 2E). Transfected GLUTag (Fig. 2F) and NCI-H716 (Fig. 2G) cells were then treated with Oleoyl-LPI to assess GLP-1 secretion. There were no statistically significant differences in Oleoyl-LPI-induced GLP-1 secretion between cells transfected with siControl or siGPR55, indicating that GPR55 was not involved in mediating GLP-1 secretion upon Oleoyl-LPI stimulation. Similarly, treatment of cells with the GPR55 antagonist CID16020046 did not affect the Oleoyl-induced GLP-1 secretion (Fig. S1).

To further validate these results, we investigated the effects of Oleoyl-LPI on a mixed colonic primary culture from wild type and GPR55$^{-/-}$ mice, isolated as described previously [28–30]. Forskolin was used as positive control in these experiments. Firstly, we observed that two hour treatment with Oleoyl-LPI stimulated GLP-1 secretion in

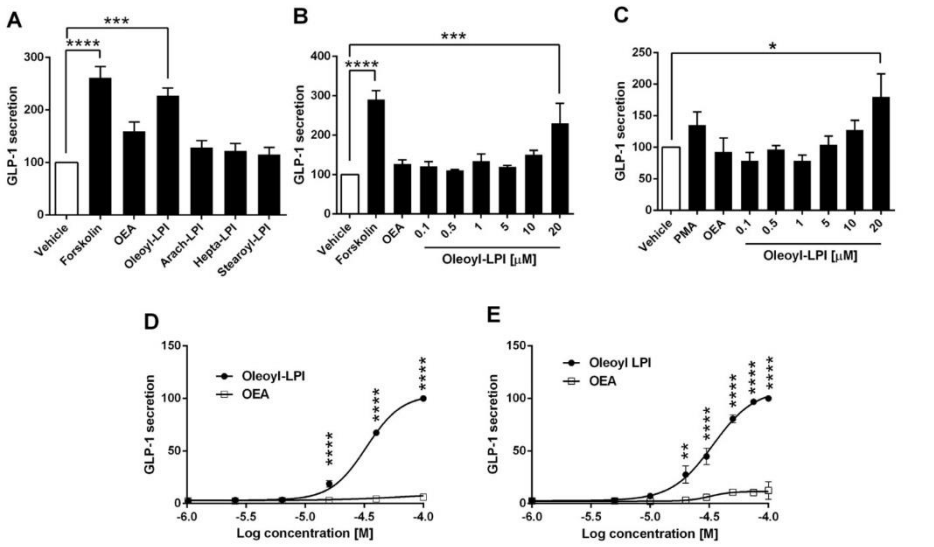


**Fig. 1.** Oleoyl-LPI enhances GLP-1 secretion in vitro.
(A) GLUTag cells were incubated with the indicated species of LPI (20 μM) or vehicle alone for 2 h. Alternatively, GLUTag (B) and NCI-H716 (C) cells were incubated with increasing concentrations of Oleoyl-LPI. In these experiments, OEA and forskolin were used as positive controls. Secretion of GLP-1 was assessed by ELISA as described in the Materials and methods section. Data are expressed as percentage of values from cells treated with vehicle alone.
(D, E) Dose-response curves of Oleoyl-LPI (closed circles) and OEA (open squares) in GLUTag (D) and NCI-H716 cells (E) normalized to Oleoyl-LPI 100 μM. The two compounds display different maximal efficacies but similar potencies, with $EC_{50}$s around 30 μM in both GLUTag and NCI-H716. In all panels data are means ± SEM of at least 3 independent experiments. Statistical significance was assessed via One-way ANOVA followed by a post hoc Dunnett analysis for graphs A, B and C, and Two-way ANOVA with post hoc Sidak test for dose-responses in D and E. Statistical significance is indicated as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

this ex vivo system, confirming our in vitro results.

Importantly, no difference in the Oleoyl-LPI-stimulated GLP-1 secretion was detected between primary cells isolated from wild type or GPR55$^{-/-}$ mice (Fig. 2H). Taken together, these data indicate that the GLP-1 secretion induced by Oleoyl-LPI is not regulated by GPR55.

### 3.3. GPR119 mediates Oleoyl-LPI-induced GLP-1 secretion from L-cells and insulin secretion from pancreatic β-cells

We next determined whether GPR119 was required for the Oleoyl-LPI-stimulated GLP-1 release. To this end, GLUTag were transiently transfected with a specific siRNA targeting GPR119 before stimulation with Oleoyl-LPI. Downregulation of GPR119 completely inhibited the Oleoyl-LPI-induced GLP-1 secretion (Fig. 3A). Western blot, and the correspondent densitometric analysis, indicated efficient downregulation of GPR119 at protein level in GLUTag cells, which was further confirmed by transcript quantification (Fig. 3B). Similarly, our data indicate that Oleoyl-LPI-mediated GLP-1 secretion was dependent on GPR119 in NCI-H716 cells (Fig. S2A). Efficient downregulation of GPR119 in these cells was confirmed by Western blot (Fig. S2B) and RT-qPCR (Fig. S2B) analysis. We next investigated the effects of Oleoyl-LPI on primary cultures of mixed colonic preparation from wild type and GPR119$^{-/-}$ mice. Oleoyl-LPI was unable to induce GLP-1 release in cultures derived from GPR119$^{-/-}$ mice whereas it potently stimulated GLP-1 secretion in wild type mice (Fig. 3C). These data demonstrate that downregulation of GPR119 in GLUTag and NCI-H716 cells, and genetic ablation of GPR119 in mice, impair the Oleoyl-LPI-induced GLP-1 secretion.

LPI has been shown previously to stimulate insulin secretion from pancreatic β-cells [31] directly through a GPR55-independent mechanism [32]. We therefore evaluated the ability of pancreatic islets isolated from wild type and GPR119$^{-/-}$ mice to secrete insulin in response to Oleoyl-LPI in the presence of glucose. We observed that Oleoyl-LPI acutely enhanced insulin secretion by approximately 50% in the presence of 16.7 mM of glucose. Importantly, no secretion of insulin was detected in GPR119$^{-/-}$ islets upon stimulation with Oleoyl-LPI (Fig. 3D), indicating that expression of GPR119 in pancreatic islets is necessary for Oleoyl-LPI stimulation of insulin secretion.

### 3.4. Intracellular ERK1/2 activation is involved in Oleoyl-LPI-induced GLP-1 secretion

It has been reported that phosphorylation of ERK1/2 is involved in GLP-1 secretion mediated by several ligands [8, 33, 34]. In order to determine whether Oleoyl-LPI activated ERK1/2 in the enteroendocrine L-cells we assessed its phosphorylation state over a time course experiment. Oleoyl-LPI stimulation increased phosphorylation of ERK1/2 at residues Thr202 and Tyr204 within 10 min (Fig. 4A). To determine whether GPR119 was involved in this Oleoyl-LPI-mediated ERK1/2 activation, GLUTag cells transfected with siGPR119 or siControl were stimulated for up to 10 min with Oleoyl-LPI, and phosphorylation of ERK1/2 was assessed by Western blot. Data indicated a clear inhibition of ERK1/2 activation in cells transfected with siGPR119, as confirmed by densitometric analysis (Fig. 4B). Similarly, downregulation of GPR119 inhibited ERK1/2 phosphorylation upon stimulation with Oleoyl-LPI in NCI-H716 cells (Fig. S2C). These data indicate that

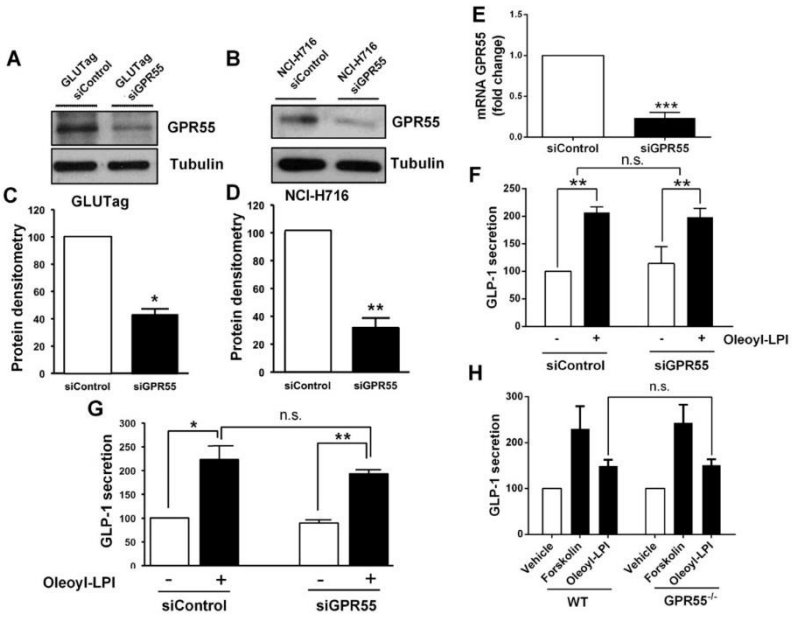


**Fig. 2.** Oleoyl-LPI-induced secretion of GLP-1 is not regulated by GPR55.
Representative Western blot showing downregulation of GPR55 in GLUTag (A) and NCI-H716 (B) at 48 h post transfection with a specific siRNA targeting GPR55 or a non-targeting siRNA (siControl). Tubulin was used as loading control. Corresponding densitometry analysis are shown in (C) and (D) respectively. (E) mRNA levels of GPR55 in GLUTag cells transfected with the indicated siRNAs (48 h post transfection). (F, G) GLP-1 secretion from GLUTag (F) or NCI-H716 (G) cells transfected with the indicated siRNAs and stimulated with 20 μM Oleoyl-LPI (+) or vehicle alone (−) 48h post-transfection. Data are expressed as percentage of values from cells transfected with siControl and treated with vehicle. (H) Secretion of GLP-1 by mixed primary colonic culture from wild type (C57BL/6) and C57BL/6 GPR55$^{-/-}$ mice upon stimulation with Oleoyl-LPI or vehicle alone. Forskolin was used as positive control. Results are normalized to the respective vehicle (control). Data are means ± SEM of at least n = 3 independent experiments. Statistical significance for panels C, D, E was assessed with one-sided Student *t*-tests, and for F, G and H with Two-way ANOVAs followed by Sidak test: significance was assumed for *p < 0.05, **p < 0.01, ***p < 0.001.

Oleoyl-LPI stimulates ERK1/2 activation in L-cells through GPR119. To determine whether the detected activation of the mitogen activated protein kinase (MAPK) signalling pathway was involved in regulation of GLP-1 secretion, GLUTag (Fig. 4C) and NCI-H716 (Fig. 4D) cells were pre-treated with the mitogen-activated protein kinase 1 and 2 (MEK1/2) inhibitor PD98059 for 1 h prior to Oleoyl-LPI stimulation. The inhibitor was kept in the medium throughout the secretion experiment.

Treatment of GLUTag and NCI-H716 cells with PD98059 partially but significantly reduced Oleoyl-LPI-induced ERK1/2 phosphorylation and GLP-1 secretion (Fig. 4C and D). These data indicate that the Oleoyl-LPI-dependent, GPR119-mediated secretion of GLP-1 is partially regulated through activation of ERK1/2.

### *3.5. Oleoyl-LPI stimulates cAMP production in COS-7 cells and activates CREB in enteroendocrine L-cells*

Previous studies have demonstrated that activation of PKA is able to stimulate proglucagon gene transcription and GLP-1 synthesis in GLUTag cells and primary foetal rat intestinal cultures [35, 36]. Furthermore, ligand binding to GPR119 activates stimulatory G proteins that signal via adenylate cyclase to increase cAMP levels and activate PKA [15]. To determine whether Oleoyl-LPI was able to increase intracellular cAMP levels through GPR119, we used COS-7 cells over-expressing GPR119. Treatment with Oleoyl-LPI increased the production of cAMP similarly to OEA with comparable $EC_{50}$s (Oleoyl-LPI 236 nM and OEA 217 nM, Fig. S3). To determine whether the Oleoyl-LPI-mediated secretion of GLP-1 was dependent on increased cAMP levels, GLUTag and NCI-H716 cells were pre-treated with the PKA inhibitor, H-89 (10 μM) before stimulation. PKA inhibition suggested a hampered Oleoyl-LPI-induced GLP-1 secretion, although data were not statistically significant (Fig. 5A and B). On the other hand, simultaneous inhibition of MEK1/2 (using PD98059) and PKA (using H-89) completely abolished the Oleoyl-LPI-induced GLP-1 release in GLUTag cells (Fig. S4). Interestingly, we observed that inhibition of PKA reduced partially the Oleoyl-LPI-dependent ERK1/2 phosphorylation (Fig. S5), suggesting a contribution of the cAMP/PKA pathway on ERK1/2 activation. Taken together these data indicate that total inhibition of Oleoyl-LPI mediated ERK1/2 signalling (as achieved by treatment with PD98059) only partially inhibits GLP-1 secretion whereas partial inhibition of ERK1/2 (as achieved by treatment with H-89) is not sufficient to appreciably affect it. On the other hand, combination of full ERK1/2 inhibition (by PD98059) and PKA inhibition (by H-89) completely blocks the Oleoyl-LPI-dependent GLP-1 secretion.

It is known that the increase of proglucagon gene transcription in L-cells is regulated by a cAMP-response element (CRE) in the 5′-flanking sequence of the gene [37]. CREB is a cellular transcription factor that binds to CRE to regulate the transcription of proglucagon.

Interestingly, we noticed that phosphorylation of CREB at its residue

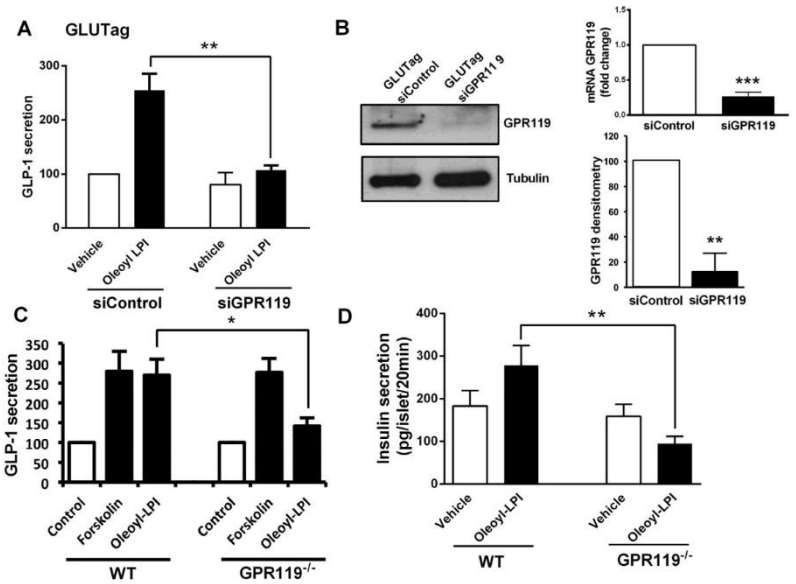


**Fig. 3.** GPR119 mediates Oleoyl-LPI-induced GLP-1 and insulin secretion in vitro and ex-vivo.
(A) Secretion of GLP-1 from GLUTag stimulated with 20 μM Oleoyl-LPI at 48 h post transfection with the indicated siRNAs. Data are expressed as percentage of values from cells transfected with siControl and treated with vehicle and are means ± SEM of at least n = 3 independent experiments performed in duplicates. Two-way ANOVA with Sidak post hoc test $^{**}p < 0.01$. (B) Results from RT-qPCR (top right panel), representative Western blot and densitometry (bottom right panel) confirming downregulation of GPR119 mRNA and protein levels in GLUTag cells at 48 h post transfection. In the blot tubulin was used as loading control. One-tail Student *t*-test: $^{**}p < 0.01$, $^{***}p < 0.001$. (C) 10 μM Oleoyl-LPI-induced secretion of GLP-1 in mixed primary colonic culture derived from wild type and GPR119$^{-/-}$ mice. Results are normalized to data obtained in cultures incubated with vehicle (control). Averages of 3 independent experiments are shown ± SEM.
(D) Following isolation from wild type, or GPR119$^{-/-}$ mice, pancreatic islets were rested overnight. Glucose-stimulated insulin secretion analysis was performed by incubating islets with 16.7 mM of glucose in the presence or absence of Oleoyl-LPI (20 μM) for 20 min. Tissues from 4 different animals were used in independent experiments.
Two-way ANOVA with Sidak post hoc test was used to assess statistical significance in C and D: $^{*}p < 0.05$, $^{**}p < 0.01$.

Ser133 was stimulated by treatment with Oleoyl-LPI in both GLUTag (Fig. 5C) and NCI-H716 (Fig. 5D) cells.

Taken together these data suggest that Oleoyl-LPI stimulates cAMP production that subsequently triggers PKA activation and phosphorylation of CREB through GPR119. Considering that the single blockade of PKA does not effectively modulate exocytosis, but on top of a MEK1/2 inhibition completely abolishes any Forskolin or Oleoyl-LPI mediated GLP-1 secretion, a complex form of synergism between the pathways can be postulated.

## 4. Discussion

It has been reported that both pancreas and gastrointestinal tract express high levels of GPR55, and GPR119. Recently, the lysophospholipid LPI was identified as a ligand for GPR55 and we hypothesized that GPR119 could be another potential LPI receptor. For instance, it has been reported that LPI can activate GPR119 in RH7777 rat hepatoma cells stably expressing human GPR119 [11]. Activation of GPR119 in the pancreas is correlated with enhanced glucose stimulated insulin secretion and activation of this receptor in the gut results in increased secretion of incretin hormones GLP-1 and GIP [4, 38]. These observations suggest that GPR119 activation may reduce blood glucose levels by acting on L-cells to stimulate GLP-1 release that in turn stimulate β-cells to promote glucose stimulated insulin secretion. Therefore, activation of this receptor by Oleoyl-LPI could be an interesting therapeutic target for treatment of T2D. The overall aim of this study was to explore the potential role of LPI in the secretion of GLP-1 by enteroendocrine L-cells through the activation of GPR119, and the mechanism underlying this process.

Our study demonstrated that Oleoyl-LPI increases GLP-1 secretion in-vitro and in ex-vivo preparations of enteroendocrine L-cells. Previous reports have demonstrated that fatty acids containing an oleoyl chain such as Oleoylethanolamide (OEA) and 2-Oleoyl-glycerol (2-OG) can enhance secretion of GLP-1 by enteroendocrine cells [4, 20]. Oleoyl-LPI is an endogenous phospholipid with an inositol head and an acylated Oleoyl chain (18:1). A heterogeneous mixture of different species of LPI, with distinct acyl chains, primarily saturated, was initially described as the endogenous ligand for GPR55 [18]. This LPI-GPR55 binding, similarly to multiple cannabinoids, is reported to activate a downstream Gα13/RhoA/ROCK-dependent calcium signalling [39]. More recently, the presence of the cation channel TRPV2 was found to be necessary for the activation of this pathway [40]. Nonetheless, the stimulation of GPR55 with the synthetic agonist O-1605, fails to mimic the reported LPI activity, indicating the involvement of other molecular players.

Indeed, the data reported by the authors, clearly indicate only a minor involvement of GPR55 in the LPI-mediated secretion of GLP-1, and fail to address the composition of the LPI mixture [40].

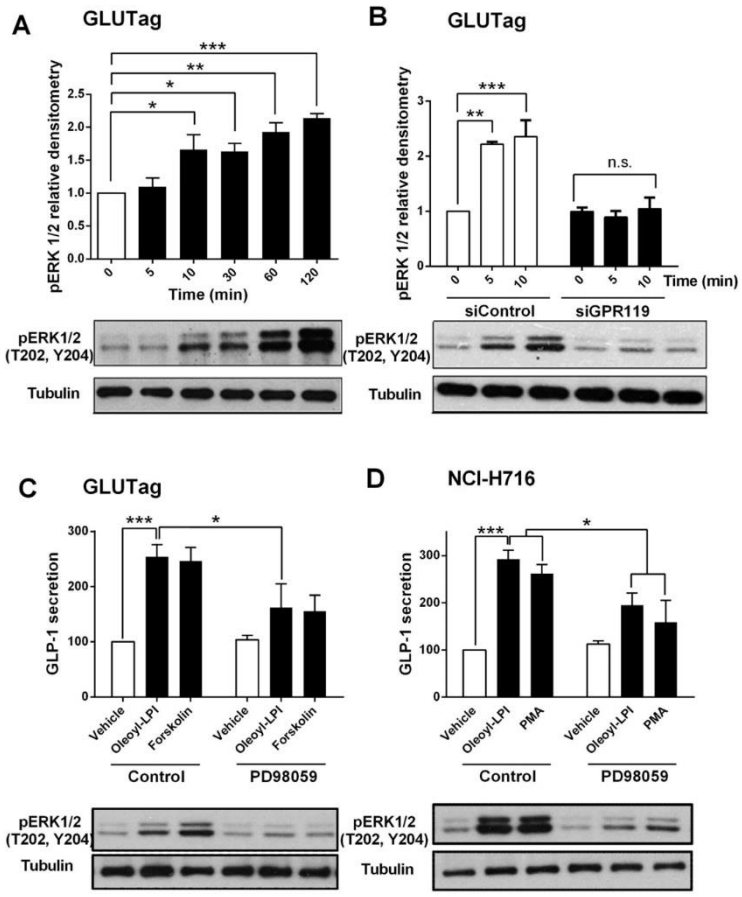


**Fig. 4.** Activation of ERK1/2 is required for the Oleoyl-LPI-induced GLP-1 secretion.
(A) Quantitative analysis of ERK1/2 phosphorylation upon treatment of GLUTag cells with Oleoyl-LPI for the indicated times. A representative blot is also shown (B). GLUTag were transfected with the indicated siRNAs before stimulation with Oleoyl-LPI for the indicated times. Results from densitometric analysis of ERK1/2 phosphorylation and a representative Western blot are shown. (C, D) GLUTag (C) and NCI-H716 (D) were pre-treated with the MEK 1/2 inhibitor PD98059 (50 μM) for 1 h prior to the stimulation with Oleoyl-LPI, Forskolin, PMA or vehicle alone always in presence of the inhibitor. "Control" cells were pre-treated with DMSO. GLP-1 secretion was assessed as above. Data are expressed as percentage of values from control cells treated with vehicle and are expressed as means ± SEM of n = 3 independent experiments. One-way ANOVA post hoc Dunnett test for data in A, and Two-way ANOVA with post hoc Sidak test for Panels B, C and D: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. At the end of the secretion experiments, cells were lysed to confirm efficacy of MEK1/2 inhibitor, assessing ERK1/2 phosphorylation by Western blot analysis. Representative blots are shown. Tubulin was used as loading control.

There is currently no available information regarding the activity of specific LPI species. Here we show that GPR55, contrarily to GPR119, is dispensable for the Oleoyl-LPI-mediated secretion of GLP-1.

Another interesting finding that we report is that Oleoyl-LPI displays a much higher GLP-1 secreting activity than OEA (Fig. 1D and E), despite similar cAMP raising capabilities (Fig. S3). These data appeared to be in contrast with those of Lauffer et al. [20] which concluded that OEA induced GLP-1 secretion when tested at the same concentration (10 μM). Noticeably, these authors maintained the GLUTag cells culture in a higher glucose concentration of 25 mM and not at a level (5.5 mM) normally used for this cell line [25, 35, 37, 41, 42] therefore the possible interference of high glucose cannot be ruled out. A recent study [43] revealed that GLUTag cells cultured in high glucose concentration had increased total production and release of active GLP-1, reactive oxygen species and upregulated proglucagon mRNA expression. Our data further demonstrated that GPR119 is required for the Oleoyl-LPI-mediated GLP-1 secretion (Fig. 3).

Consistent with previous reports of an association between

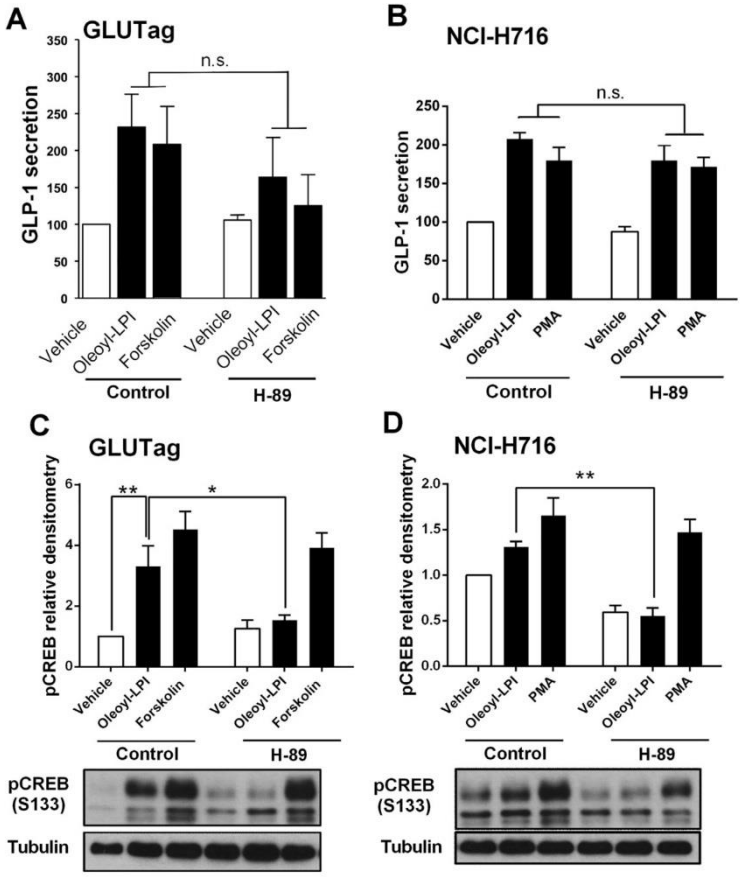


**Fig. 5.** Oleoyl-LPI activates cAMP response-element binding (CREB) in enteroendocrine L-cells.
(A,B) Oleoyl-LPI-, forskolin- and PMA-induced GLP-1 secretion in GLUTag (A) and NCI-H716 cells (B) treated with the synthetic PKA inhibitor H-89 (10 μM). "Control cells" were treated with DMSO. Data are expressed as percentage of "control cells" treated with vehicle and are means ± SEM of n = 3 independent experiments performed in duplicates. Student *t*-test: n.s. $p > 0.05$. (C,D) Representative Western blots and corresponding densitometric analysis of CREB phosphorylation at its residue Serine 133 in GLUTag (C) and NCI-H716 (D) cells. Cells were pre-treated with 10 μM H-89 before stimulation with 20 μM Oleoyl-LPI, 10 μM forskolin or 10 μM PMA in the presence or absence of H-89.
Data are from n = 3 independent experiments. Two-way ANOVA with Sidak post hoc test *$p < 0.05$, **$p < 0.01$.

increased accumulation of cAMP and GLP-1 secretion [25, 44, 45], we hypothesized that Oleoyl-LPI may regulate GPR119 activation and secretion of GLP-1 secretion through the cAMP/PKA/CREB pathway. Pharmacological inhibition of PKA by H-89 was able to reduce Oleoyl-LPI-induced GLP-1 secretion only slightly. Because the reduction of GLP-1 secretion is not statistically significant (Fig. 5), this indicates that Oleoyl-LPI-mediated activation of CREB is partially dispensable for the secretion of GLP-1 in this system.

In addition, our data show that Oleoyl-LPI-induced GLP-1 secretion primarily depends on ERK 1/2 activation. Pharmacological blockade of MEK 1/2 with PD98059 significantly inhibited Oleoyl-LPI mediated GLP-1 secretion (Fig. 4C and D), but synergism with PKA appears essential for maximal GPR119 mediated GLP-1 secretion (Supplementary Fig. 4).

## 5. Conclusion

This study demonstrated that Oleoyl-LPI is the specific species of LPI that is involved in the regulation of GLP-1 secretion from enteroendocrine L-cells. We have identified a signalling pathway involving GPR119 activation, that signals downstream through both the ERK1/2 and the cAMP/PKA/CREB pathway (Fig. 5).

The pharmacology of Oleoyl-LPI could be explored, not only as a specific GPR119 agonist, but also for further development of

metabolically stable mimics for the enhancement of endogenous GLP-1 secretion, and subsequent improvement of glucose homeostasis in pathologies such as T2D.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2018.06.007.

## Conflict of interest

The authors declare no conflict of interests.

## Transparency document

The Transparency document associated with this article can be found, in the online version.

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S.A.A. performed in vitro and ex vivo experiments, S.P. performed experiments, reviewed/edited the manuscript, organized the literature and figures and performed statistical analyses. TM reviewed/edited the manuscript and provided intellectual contribution to the study. J.H.E., P.N. and M.M.R. reviewed/edited the manuscript. R.C. contributed to discussion and reviewed/edited manuscript. I.C. performed experiments and contributed to discussion. M.F. conceived the project, led and supervised the study, performed experiments and wrote the manuscript.

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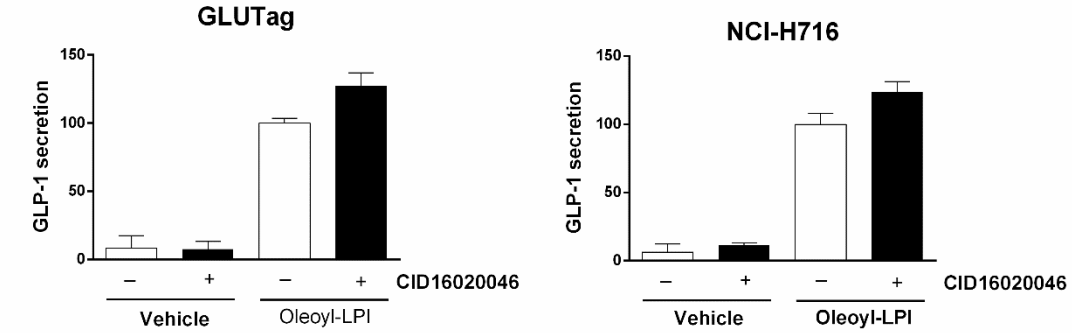
| Target gene | siRNA sequences |
|---|---|
| Human GPR119 | UCAAAGCUCUCCGUACUGU,ACAGGUACCUUGCCAUCAA, CCACUCAUCUAUGCCUAUU,ACCUAGUGCUGGAACGGUA |
| Murine GPR119 | CCUAUCACAUCGUCACUAU,UAACUAGCAUUGUGCAGGU UAUCUUACCUUAUCGGCUU,GGCCGUGGCUGAUACCUUG |
| Human GPR55 | GAAUUCCGCAUGAACAUCA |
| Murine GPR55 | ACAGGGAAGUGGAGAGAUA |

**Supplementary table 1 A**: Sequences of siRNAs used in Knockdown experiments

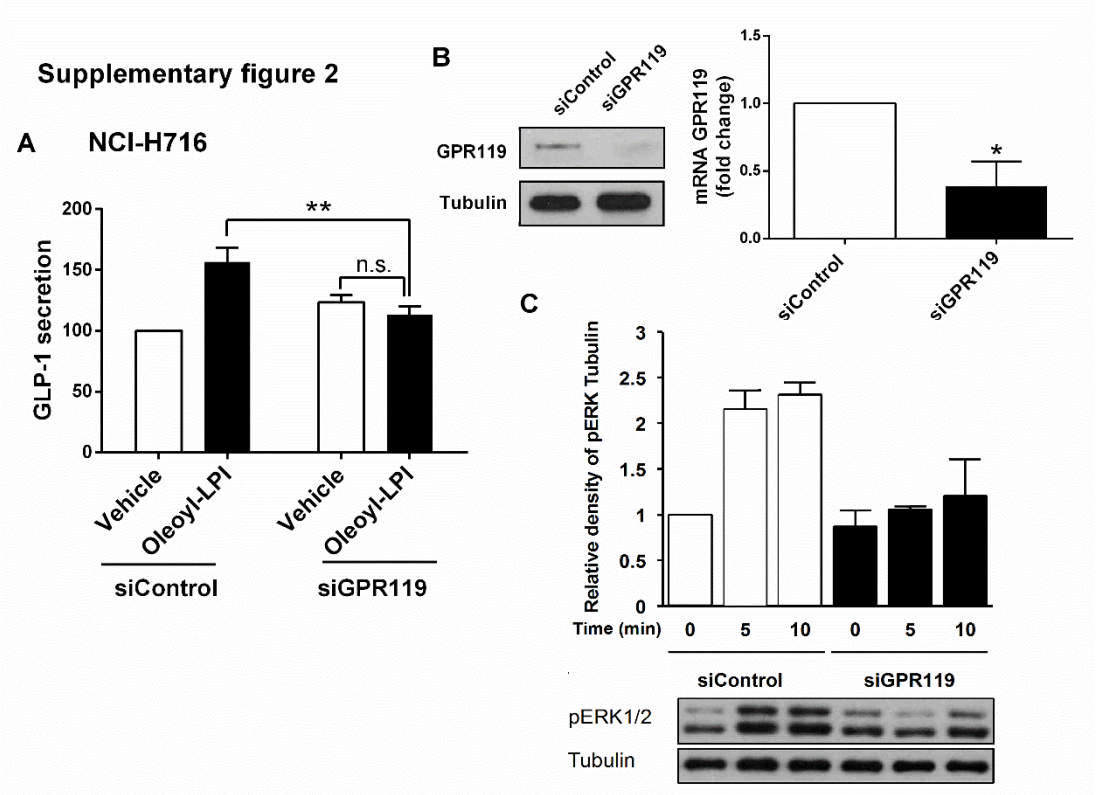
| Target gene | Forward | Reverse |
|---|---|---|
| Human GPR119 | TCTCGGCCCACACAGAAGA | GCTGCGGAGGAAGTGACAAA |
| Murine GPR119 | TGATGGTGTTGGCCTTTGCTTCAC | TGGTAAAGGCAGCATTTGTGGCAG |
| Human GPR55 | GTTTCCATGGGAAAGTGGAA | GGAAGGAGACCACGAAGACA |
| Murine GPR55 | CTATCTACATGATCAACTTGGCTGTTT | TGTGGCAGGACCATCTTGAA |
| Human GAPDH | AGGGCTGCTTTTAACTCTGGT | CCCCACTTGATTTTGGAGGGA |
| Murine GAPDH | GCACAGTCAAGGCCGAGAAT | GCCTTCTCCATGGTGGTGAA |

**Supplementary table 1 B**: Sequences of Primers used in RT-PCR

**Supplementary figure 1**

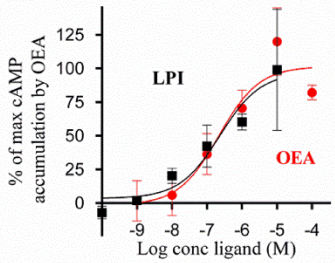



**Supplementary Fig. 1.** Pharmacological inhibition of GPR55 does not inhibit the Oleoyl-LPI-mediated GLP-1 secretion. GLUTag and NCI-H716 cells were pre-treated for 30 min with 5 μM CID16020046, followed by 2 h stimulation with 10 μM Oleoyl-LPI in the presence or absence of CID16020046 (5 μM). Secreted GLP-1 levels were quantified via ELISA. Data are from n = 2 independent experiments.




**Supplementary Fig. 2.** Oleoyl-LPI-induced GLP-1 secretion requires GPR119 in human NCI-H716. (A) Oleoyl-LPI treatment (20 μM) and GLP-1 secretion quantification in NCI-H716 cells transfected with a specific siRNA targeting GPR119. (B) Representative Western Blot and transcript quantification analysis in transfected NCI-H716 cells. (C) siRNA-mediated downregulation of GPR119 inhibits Oleoyl-LPI mediated phosphorylation of ERK1/2. Densitometric analysis of n = 3 independent experiments are shown. Statistical significance was assessed via Two-way ANOVA with post hoc Sidak test in S2A.

**Supplementary figure 3**




| | OEA |
|---|---|
| Sigmoidal dose-response (variable slope) | |
| Best-fit values | |
| Bottom | -1.715 |
| Top | 101.4 |
| LogEC50 | -6.663 |
| HillSlope | = 0.7500 |
| EC50 | 2.173e-007 |
| Std. Error | |
| Bottom | 13.64 |
| Top | 12.33 |
| LogEC50 | 0.4415 |
| 95% Confidence Intervals | |

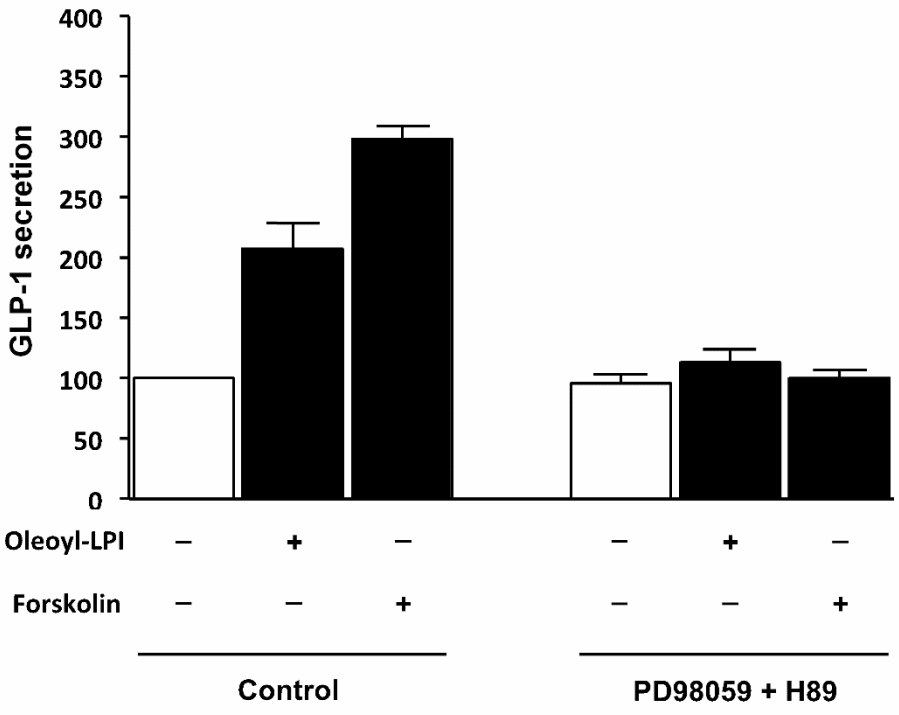
OEA: EC50=-6,66+/-0,44

| | LPI |
|---|---|
| Sigmoidal dose-response (variable slope) | |
| Best-fit values | |
| Bottom | 3.347 |
| Top | 96.83 |
| LogEC50 | -6.632 |
| HillSlope | = 0.7500 |
| EC50 | 2.335e-007 |
| Std. Error | |
| Bottom | 14.22 |
| Top | 22.92 |
| LogEC50 | 0.6631 |

LPI: EC50=-6,63+/-0,66

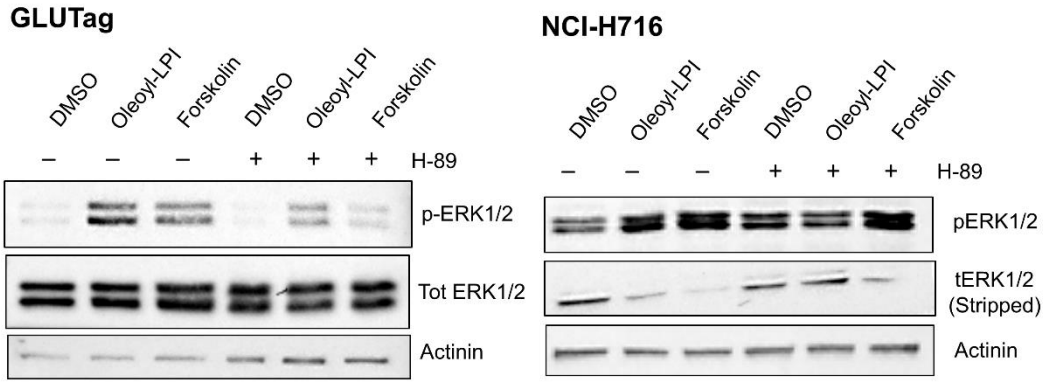
**Supplementary Fig. 3**. Oleoyl-LPI induces cAMP accumulation in GPR119 expressing COS-7 cells. cAMP dose-response curve in GPR119 expressing COS-7 cells treated with increasing concentrations of Oleoyl-ethanolamide (OEA) red circles, or Oleoyl-LPI, black squares. The two drugs display similar Log EC50s.

**Supplementary figure 4**




**Supplementary Fig. 4.** Effect of MEK and PKA inhibition in GLUTag cells on GLP-1 secretion. GLUTag were pre-treated for 1 h with PD98059 (50 μM) alone (A), or in combination with H-89 (10 μM) (B) before treatment with Oleoyl-LPI (10 μM) or vehicle for 2 h. Results of active GLP-1 released are expressed as percentage increase compared to the vehicle (DMSO + M:C). Data are means ± SEM of n = 3 independent experiments performed in duplicate.

**Supplementary figure 5**




**Supplementary Fig. 5**. Immunoblot analysis of PKA-mediated ERK1/2 modulation. A representative blot is shown for GLUTag and NCI-H716 cells pre-treated with 10 μM H-89 for 1 h, and then treated with forskolin 10 μM, or PMA 10 μM, and Oleoyl-LPI 10 μM for 10 min in presence of H-89 10 μM. PKA blockage inhibits Oleoyl-LPI, or forskolin mediated, but not PMA induced, PKC mediated, ERK1/2 activation.

# Chapter 3

# Chapter 3: Structural Activity Relationship study of Oleoyl-LPI based mimetics

## 3.1 Introduction

As discussed in Chapter §1, the ability to improve, or restore the secretion of GLP-1 directly from the intestinal lumen, represents a promising strategy for the management of metabolic diseases. Commercially available GLP-1 mimetics and DPP-4 inhibitors are associated with multiple side-effects (Krittanawong et al., 2018; Lamos, Hedrington, & Davis, 2019), likely due to their non-specific, and supra-physiological systemic activation of the GLP-1 axis. The self-administration of oral drugs makes adherence to prescribed treatment regimens much easier, an important concept that needs to be accounted for, especially in chronic diseases such as diabetes, where patients are known to be more reluctant at self-injecting protein-based drugs such as insulin and GLP-1R agonists (GLP-1RAs) (García-Pérez, Alvarez, Dilla, Gil-Guillén, & Orozco-Beltrán, 2013). New orally available molecules targeting the GLP-1 axis are currently under investigation to simplify the adherence to the treatment regimen. The recently FDA-approved GLP-1 mimetic Semaglutide, has indeed shown encouraging superior glucose and weight lowering properties in late stage clinical trials when compared to other subcutaneously administered GLP-1 mimetics. Nonetheless, up to 80% of the patients experience important gastrointestinal side effects, leaving an important clinical need (R. Pratley et al., 2019; R. E. Pratley et al., 2018).

Another approach, aimed at closing this gap, are non-peptidic small-molecules displaying dual-agonism for both GLP-1R and GIP receptor (GIPR), which have already shown even superior anti diabetic properties in humans (Frias et al., 2018), albeit still being plagued with dose-dependent gastro-intestinal side effects.

Management of the current epidemic of type 2 diabetes, often coupled with obesity and referred to as an epidemic of diabesity, demands novel treatment approaches, to not only elicit a more physiological metabolic homeostasis, but also maximize adherence to treatment.

All drugs currently on the market for the management of diabesity have been developed to act with a systemic pharmacokinetic and pharmacodynamic (PK/PD). Bio-active lipids, of both endogenous, and exogenous origin such as Oleoyl-LPI, offer

a valuable new approach at targeting the easy-to-access biggest endocrine organ in our body, the enteroendocrine cell system. As proven in the study presented in chapter §2, Oleoyl-LPI is an efficient GLP-1 secreting agent with proven activity *in vitro* and *ex vivo*. Nonetheless, its therapeutic use is halted by two main problems, firstly its concentration in food such as Olive oil, is at best in the order of nanomoles per litre (Hatzakis, Koidis, Boskou, & Dais, 2008), making its isolation non practical; secondly when ingested, harsh stomach acids, and pancreatic lipases are physical barriers preventing it to quantitatively reach more distal GLP-1 secreting cells. To overcome these problems, in collaboration with A/Prof. Max Massi, and Dr. Peter Simpson, we have generated a panel of compounds structurally related to Oleoyl-LPI, with a an easy to synthesize chemistry, and a more stable amide-group connecting the aliphatic chain to the hydrophilic head. A library of 18 compounds was screened *in vitro* in 3 different L-cell line models. 3 lead analogues, characterized by an Oleoyl-based chain with an iteration of the same heterocyclic head pharmacophore, were tested *in vivo*, with compound ps297b eliciting at 20 mg/kg a significant increase in the plasmatic levels of glucose-stimulated GLP-1 secretion in diabetic db/db mice. Furthermore, we show that the Oleoyl-LPI analogues ps297b, and ps318a, similarly to their parent molecule, both activate the MAPK-ERK1/2 pathway, with ps318a phenocopying more closely Oleoyl-LPI by also stimulating cAMP/CREB.

We have also examined the direct pharmacological application of Oleoyl-LPI *in vivo*, providing preliminary proof of concept evidence that peripheral levels of total GLP-1 can be elevated even with the low dose of 10 mg/kg. This data supports future studies aimed at improving its therapeutical potential by optimizing its protection from physical barriers with a more targeted distal delivery. This proof of concept evidence provides the groundwork to validate whether a daily small peripheral increase of GLP-1 can ameliorate the pathology of a chronic mouse model of diabesity. Having demonstrated the tropism of Oleoyl-LPI for GPR119 in the study presented in chapter §2, we sought to also validate this receptor tropism for ps297b and s318a. We have generated an in-silico 3D-model of GPR119 interacting with either Oleoyl-LPI or ps297b, highlighting how the Oleoyl-chain is likely interacting with a deep hydrophobic orthosteric site, while the hear pharmacophore engages more freely with superficial residues. Indeed, the length of the aliphatic chain appears critical in this

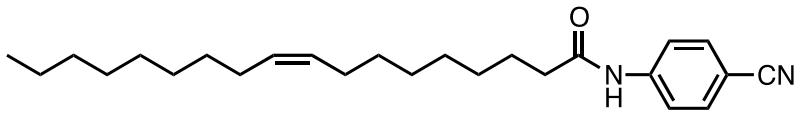
interaction, in line with the known Oleoyl-based structure of all endogenous GPR119 agonist molecules described to date.

Having provided the groundwork for the pharmacology of our efficient GLP-1 secreting compounds and their possible tropism for GPR119, we also sought to validate it experimentally. Nonetheless, downregulation of GPR119 expression *in vitro* with commercial, or in-house RNA interference, proved to be technically challenging, and difficult to implement in the testing of multiple drugs. Therefore, we have also sought to establish a more robust cell line knockout model via CRISPR-Cas9 technique, however this system is yet under current optimization and we are therefore in the process of re-establishing a GPR119-deficient mouse colony. Other receptors such as GPR55 and TRPV2 should also be studied for their possible sensitivity to ps297b and ps318a given their known preference for other LPI species (Harada et al., 2017). Future studies aimed at improving the therapeutical application of the GLP-1-secreting compound ps297b, need to consider an optimization of the delivery of the compound to the distant intestine in chronic mouse models with daily administration, to assess its impact on diet-induced obesity and diabetes, as well as to exclude on-target or off-target toxicity.

## 3.2 Materials and methods

### 3.2.1 Library synthesis and $^{1}$H-NMR characterization

The design of the structures of Oleoyl-LPI mimetic drugs has been a collaborative effort aimed at avoiding the complex synthesis of Myo-inositol found in the hydrophilic head of the natural Oleoyl-LPI. The synthesis and physical characterization by 1H-NMR detailed below, has been undertaken by our collaborators from Curtin University A/ Prof. Max Massi, and Dr. Peter Simpson.

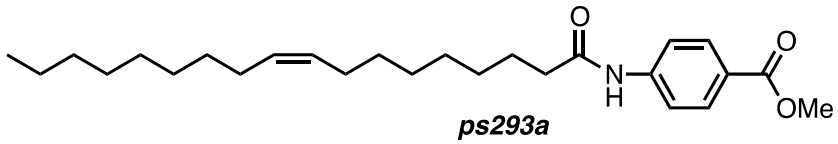



***ps292a***

**Synthesis of ps292a.** Oxalyl chloride (0.71 mL, 8.24 mmol) was added over 10 min to a solution of oleic acid (2 mL, 6.34 mmol) and DMF (3 drops) in DCM (10 mL) at 0 °C, and the solution stirred at room temperature overnight. The solvent and excess oxalyl chloride was removed *in vacuo* and the residue redissolved in DCM (10 mL).

This solution was added dropwise to a solution of 4-aminobenzonitrile (0.61 g, 5.14 mmol) in DCM (10 mL) and $Et_3N$ (1 mL) that had been cooled in an ice bath. Upon complete addition, the solution was stirred at room temperature overnight. The solution was washed with saturated $Na_2CO_3$ solution (3 × 20 mL), $H_2O$ (20 mL), and brine (20 mL), and dried over $MgSO_4$. The resulting residue was subjected to column chromatography, using a gradient elution of EtOAc/petroleum spirits to afford the product as a pale yellow oil that solidified upon cooling. Yield: 1.50 g (76%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3258 br w, 2920 m, 2851 m, 2220 m, 1673 s, 1595 s, 1535 s. $^1$H-NMR δ/ppm ($CDCl_3$): 7.66 (2H, d, $^3J_{H,H}$ = 8.6 Hz, Ar CH), 7.60 (2H, d, $^3J_{H,H}$ = 8.6 Hz, Ar CH), 7.39 (1H, s, NH), 5.29 – 5.40 (2 × 1H, 2 × m, 2 × olefinic CH), 2.38 (2H, m, NHCOC$H_2$), 1.95 – 2.05 (2 × 2H, 2 × m, 2 × $CH_2$), 1.73 (2H, m, $CH_2$), 1.24 – 1.42 (10 × 2H, 10 × m, 10 × $CH_2$), 0.87 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($CDCl_3$): 172.0 (CONH), 142.3 (Ar C), 130.2 (olefinic CH), 129.8 (olefinic CH), 133.4 (Ar CH), 119.5 (Ar CH), 119.0 (Ar C), 106.9 (CN), 37.9 (NHCO*C*$H_2$), 32.0 ($CH_2$), 29.9 ($CH_2$), 29.8 ($CH_2$), 29.6 ($CH_2$), 29.45 ($CH_2$), 29.44 ($CH_2$), 29.38 ($CH_2$), 29.3 ($CH_2$), 29.2 ($CH_2$), 27.4 ($CH_2$), 27.3 ($CH_2$), 25.5 ($CH_2$), 22.8 ($CH_2$), 14.3 ($CH_3$).

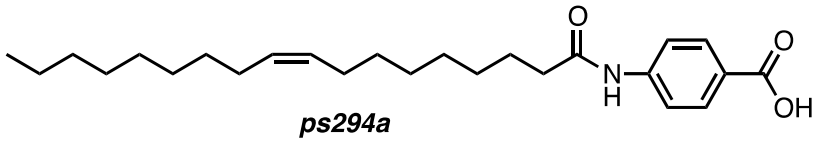
The activity of compound ps292 was not studied in this thesis, nonetheless, being utilized as a substrate for the synthesis of compound ps297b, it is reported for reference.




***ps293a***

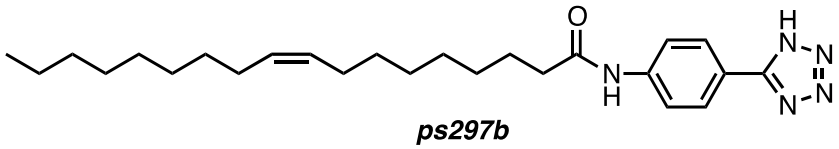
**Synthesis of ps293a.** Oxalyl chloride (0.53 mL, 6.18 mmol) was added over 10 min to a solution of oleic acid (1.5 mL, 4.75 mmol) and DMF (3 drops) in DCM (10 mL) at 0 °C, and the solution stirred at room temperature overnight. The solvent and excess oxalyl chloride was removed *in vacuo* and the residue redissolved in DCM (10 mL). This solution was added dropwise to a solution of methyl 4-aminobenzoate (0.58 g, 3.85 mmol) in DCM (10 mL) and $Et_3N$ (1 mL) that had been cooled in an ice bath. Upon complete addition, the solution was stirred at room temperature overnight. The solution was washed with saturated $Na_2CO_3$ solution (3 × 20 mL), $H_2O$ (20 mL), and brine (20 mL), and dried over $MgSO_4$. The resulting residue was subjected to column chromatography, using a gradient elution of EtOAc/petroleum spirits to afford a pale yellow solid. The solid was triturated with cold petroleum spirits to afford the product

as a white solid. Yield: 1.12 g (70%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3279 br w, 2917 m, 2849 m, 1716 s, 1655 s, 1607 m, 1540 m, 1465 w. $^{1}$H-NMR δ/ppm ($d_6$-DMSO): 10.22 (1H, s, NH), 7.93 (2H, d, $^{3}J_{H,H}$ = 8.8 Hz, Ar CH), 7.76 (2H, d, $^{3}J_{H,H}$ = 8.8 Hz, Ar CH), 5.31 – 5.40 (2 × 1H, 2 × m, 2 × olefinic CH), 3.85 (3H, s, $CO_2CH_3$), 2.37 (2H, m, NHCOC*H*$_2$), 1.96 – 2.06 (2 × 2H, 2 × m, 2 × $CH_2$), 1.62 (2H, m, $CH_2$), 1.21 – 1.39 (10 × 2H, 10 × m, 10 × $CH_2$), 0.88 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 171.8 (CONH), 165.8 ($CO_2$Me), 143.7 (Ar C), 130.2 (Ar CH), 129.62 (olefinic CH), 129.57 (olefinic CH), 123.6 (Ar C), 118.3 (Ar CH), 51.8 ($CO_2$*C*H$_3$), 36.5 (NHCO*C*H$_2$), 31.2 ($CH_2$), 29.1 ($CH_2$), 29.0 ($CH_2$), 28.8 ($CH_2$), 28.7 ($CH_2$), 28.62 ($CH_2$), 28.60 ($CH_2$), 28.56 ($CH_2$), 28.5 ($CH_2$), 26.5 ($CH_2$), 24.9 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).



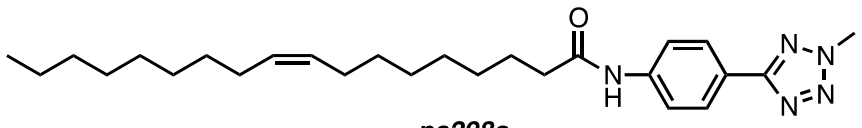

***ps294a***

**Synthesis of ps294a.** A 1M NaOH (0.55 mL) solution was added to a solution of **ps293a** (0.19 g, 0.45 mmol) in MeOH/THF (1:1, 2 mL) and heated at 60 °C overnight. The solution was acidified with 1M HCl, causing a white solid to precipitate. The mixture was stirred for 5 min and the solid was collected, washed with $H_2O$ (4 × 5 mL), MeOH/THF (1:1, 2 × 5 mL), and petroleum spirits (2 × 5 mL), and dried to afford the product as a white solid. Yield: 0.15 g (85%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3317 br w, 2921 m, 2852 m, 1655 s, 1608 w, 1510 m, 1467 w. $^{1}$H-NMR δ/ppm ($d_6$-DMSO): 10.18 (1H, s, NH), 7.90 (2H, d, $^{3}J_{H,H}$ = 8.6 Hz, Ar CH), 7.73 (2H, d, $^{3}J_{H,H}$ = 8.6 Hz, Ar CH), 5.30 – 5.40 (2 × 1H, 2 × m, 2 × olefinic CH), 2.36 (2H, m, NHCOC*H*$_2$), 1.95 – 2.08 (2 × 2H, 2 × m, 2 × $CH_2$), 1.62 (2H, m, $CH_2$), 1.21 – 1.38 (10 × 2H, 10 × m, 10 × $CH_2$), 0.88 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 171.8 (CONH), 167.0 ($CO_2$H), 143.3 (Ar C), 130.3 (Ar CH), 129.67 (olefinic CH), 129.60 (olefinic CH), 125.1 (Ar C), 118.2 (Ar CH), 36.5 (NHCO*C*H$_2$), 31.3 ($CH_2$), 29.1 ($CH_2$), 29.08 ($CH_2$), 28.83 ($CH_2$), 28.68 ($CH_2$), 28.64 ($CH_2$), 28.63 ($CH_2$), 28.60 ($CH_2$), 28.5 ($CH_2$), 26.6 ($CH_2$), 25.0 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).

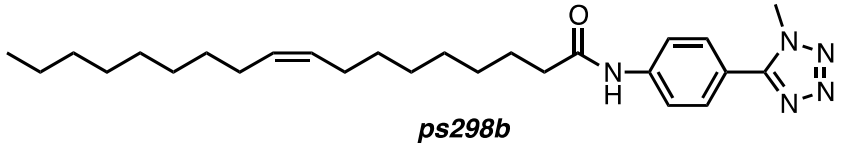



***ps297b***

**Synthesis of ps297b.** Concentrated HCl (0.81 mL, 8.29 mmol, 32%) was added to a solution of $Et_3N$ (1.16 mL, 8.29 mmol) in toluene (20 mL) that had been cooled in an ice bath, and the mixture stirred until no more fumes were observed. $NaN_3$ (0.56 g, 8.29 mmol) and **ps292a** (0.63 g, 1.66 mmol) were added and the mixture heated at reflux for 2 d. The toluene was removed, and the resulting residue suspended in $H_2O$ and acidified with concentrated HCl (~1 mL). The resulting solid was stirred for 5 min and then collected, washed with $H_2O$ (5 × 10 mL), dried, then washed with DCM (3 × 10 mL) and dried, affording the product as a white solid. Yield: 219 mg (36%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3308 br w, 2919 m, 2850 m, 1669 s, 1600 s, 1539 s. $^1$H-NMR δ/ppm ($d_6$-DMSO): 10.20 (1H, s, NH), 8.00 (2H, m, Ar CH), 7.84 (2H, m, Ar CH), 5.30 – 5.42 (2 × 1H, 2 × m, 2 × olefinic CH), 2.38 (2H, m, NHCOC$H_2$), 1.96 – 2.08 (2 × 2H, 2 × m, 2 × $CH_2$), 1.64 (2H, m, $CH_2$), 1.20 – 1.39 (10 × 2H, 10 × m, 10 × $CH_2$), 0.87 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 171.7 (CONH), 154.9 (tetrazolyl C), 141.8 (Ar C), 129.63 (olefinic CH), 129.59 (olefinic CH), 127.6 (Ar CH), 119.2 (Ar CH), 118.4 (Ar C), 36.4 (NHCO*C*$H_2$), 31.2 ($CH_2$), 29.1 ($CH_2$), 28.8 ($CH_2$), 28.65 ($CH_2$), 28.63 ($CH_2$), 28.56 ($CH_2$), 28.5 ($CH_2$), 26.5 ($CH_2$), 25.0 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).



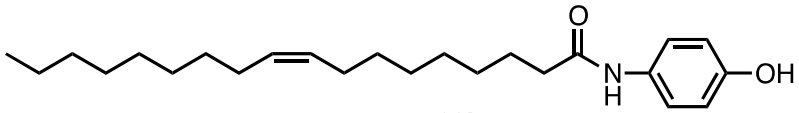
***ps298a***



***ps298b***

**Synthesis of ps298a and ps298b.** Methyl iodide (16 μL, 0.26 mmol) was added to a suspension of **ps297b** (100 mg, 0.23 mmol) and $K_2CO_3$ (65 mg, 0.47 mmol) in acetone (5 mL) and heated at reflux overnight. The mixture was concentrated *in vacuo* then redissolved in EtOAc (10 mL) and washed with $H_2O$ (3 × 10 mL), brine (1 × 10 mL) and dried over $MgSO_4$. The resulting residue was subjected to column chromatography, using a gradient elution of EtOAc/petroleum spirits to afford both N2- and N1-methylated compounds as white solids. Yield: N2 isomer, 54.9 mg (54%); N1 isomer, 18.3 mg (18%). N2 isomer (**ps298a**): FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3282 br w,

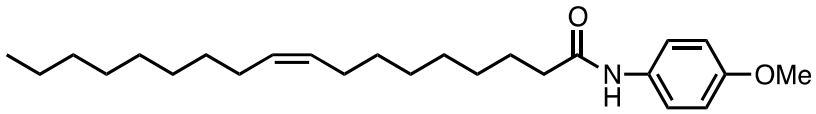
2918 m, 2850 m, 1655 s, 1598 w, 1548 m. $^{1}$H-NMR δ/ppm (d$_6$-DMSO): 10.13 (1H, s, NH), 8.01 (2H, d, $^{3}J_{H,H}$ = 8.7 Hz, Ar CH), 7.81 (2H, d, $^{3}J_{H,H}$ = 8.7 Hz, Ar CH), 5.32 – 5.41 (2 × 1H, 2 × m, 2 × olefinic CH), 4.44 (3H, s, NCH$_3$), 2.37 (2H, m, NHCOC*H*$_2$), 1.97 – 2.06 (2 × 2H, 2 × m, 2 × CH$_2$), 1.63 (2H, m, CH$_2$), 1.22 – 1.40 (10 × 2H, 10 × m, 10 × CH$_2$), 0.88 (3H, m, CH$_3$). $^{13}$C-NMR δ/ppm (d$_6$-DMSO): 171.6 (CONH), 164.0 (tetrazolyl C), 141.2 (Ar C), 129.65 (olefinic CH), 129.60 (olefinic CH), 126.9 (Ar CH), 121.3 (Ar C), 119.2 (Ar CH), 39.5 (NCH$_3$), 36.4 (NHCO*C*H$_2$), 31.2 (CH$_2$), 29.1 (CH$_2$), 28.8 (CH$_2$), 28.7 (CH$_2$), 28.65 (CH$_2$), 28.6 (CH$_2$), 28.5 (CH$_2$), 26.5 (CH$_2$), 25.0 (CH$_2$), 22.1 (CH$_2$), 13.9 (CH$_3$). N1 isomer (**ps298b**): FT-IR (ATR) $\nu_{max}$/cm$^{-1}$: 3300 br w, 2920 s, 2851 m, 1659 s, 1595 w, 1524 m. $^{1}$H-NMR δ/ppm (d$_6$-DMSO): 10.20 (1H, s, NH), 7.87 (2H, m, Ar CH), 7.84 (2H, m, Ar CH), 5.32 – 5.41 (2 × 1H, 2 × m, 2 × olefinic CH), 4.20 (3H, s, NCH$_3$), 2.39 (2H, m, NHCOC*H*$_2$), 1.98 – 2.07 (2 × 2H, 2 × m, 2 × CH$_2$), 1.64 (2H, m, CH$_2$), 1.22 – 1.40 (10 × 2H, 10 × m, 10 × CH$_2$), 0.88 (3H, m, CH$_3$). $^{13}$C-NMR δ/ppm (d$_6$-DMSO): 171.8 (CONH), 153.7 (tetrazolyl C), 141.8 (Ar C), 129.65 (olefinic CH), 129.60 (olefinic CH), 126.3 (Ar CH), 119.0 (Ar CH), 117.7 (Ar C), 36.5 (NHCO*C*H$_2$), 35.1 (NCH$_3$), 31.2 (CH$_2$), 29.1 (CH$_2$), 28.8 (CH$_2$), 28.65 (CH$_2$), 28.63 (CH$_2$), 28.61 (CH$_2$), 28.6 (CH$_2$), 28.5 (CH$_2$), 26.5 (CH$_2$), 25.0 (CH$_2$), 22.1 (CH$_2$), 13.9 (CH$_3$).




***ps300b***

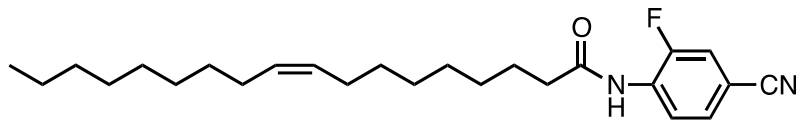
**Synthesis of ps300b.** $^{i}$Pr$_2$EtN (0.49 mL, 2.84 mmol) was added to a solution of oleic acid (0.50 mL, 1.42 mmol) and HBTU (0.59 g, 1.56 mmol) in DMF (4 mL) and stirred at room temperature for 45 min. 4-Aminophenol (0.16 g, 1.42 mmol) was added and the solution was stirred at room temperature overnight. EtOAc (50 mL) was added and the solution was washed with H$_2$O (3 × 20 mL), saturated Na$_2$CO$_3$ (2 × 20 mL), and brine (1 × 20 mL), and dried over MgSO$_4$. The resulting residue was subjected to column chromatography, using a gradient elution of EtOAc/petroleum spirits to afford an off-white solid. The solid was triturated with cold petroleum spirits to afford the product as a white solid. Yield: 0.30 g (56%). FT-IR (ATR) $\nu_{max}$/cm$^{-1}$: 3278 br w, 2917 m, 2850 m, 1648 s, 1615 w, 1558 m, 1516 m. $^{1}$H-NMR δ/ppm (d$_6$-DMSO): 9.59 (1H, s, NH), 9.14 (1H, s, OH), 7.38 (2H, d, $^{3}J_{H,H}$ = 8.9 Hz, Ar CH), 6.70 (2H, d, $^{3}J_{H,H}$

= 8.9 Hz, Ar CH), 5.31 – 5.41 (2 × 1H, 2 × m, 2 × olefinic CH), 2.25 (2H, m, NHCOC*H*$_2$), 1.95 – 2.08 (2 × 2H, 2 × m, 2 × $CH_2$), 1.59 (2H, m, $CH_2$), 1.21 – 1.39 (10 × 2H, 10 × m, 10 × $CH_2$), 0.88 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 170.4 (CONH), 153.0 (Ar C), 131.0 (Ar C), 129.64 (olefinic CH), 129.61 (olefinic CH), 120.8 (Ar CH), 114.9 (Ar CH), 36.3 (NHCO*C*$H_2$), 31.3 ($CH_2$), 29.1 ($CH_2$), 29.08 ($CH_2$), 29.0 ($CH_2$), 28.81 ($CH_2$), 28.68 ($CH_2$), 28.66 ($CH_2$), 28.60 ($CH_2$), 28.5 ($CH_2$), 26.6 ($CH_2$), 26.5 ($CH_2$), 25.2 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).



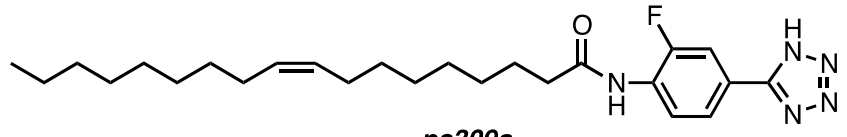

***ps301a***

**Synthesis of ps301a.** Methyl iodide (20 μL, 0.29 mmol) was added to a suspension of **ps300b** (100 mg, 0.27 mmol) and $K_2CO_3$ (74 mg, 0.54 mmol) in acetone (5 mL) and heated at reflux overnight. An additional portion of methyl iodide (20 μL, 0.29 mmol) was added and the mixture was heated at reflux overnight. The mixture was concentrated *in vacuo* then redissolved in EtOAc (10 mL) and washed with $H_2O$ (3 × 10 mL), brine (1 × 10 mL) and dried over $MgSO_4$. Petroleum spirits (4 mL) was added and the resulting solid was collected, washed with petroleum spirits (3 × 5 mL), and dried to afford the product as a white solid. Yield: 63.2 mg (60%). FT-IR (ATR) $\nu_{max}$/cm$^{-1}$: 3284 br w, 2917 m, 2849 m, 1649 s, 1605 w, 1549 m, 1514 m. $^1$H-NMR δ/ppm ($d_6$-DMSO): 9.71 (1H, s, NH), 7.51 (2H, d, $^3J_{H,H}$ = 9.0 Hz, Ar CH), 6.89 (2H, d, $^3J_{H,H}$ = 9.0 Hz, Ar CH), 5.31 – 5.42 (2 × 1H, 2 × m, 2 × olefinic CH), 3.74 (1H, s, $OCH_3$), 2.28 (2H, m, NHCOC*H*$_2$), 1.97 – 2.07 (2 × 2H, 2 × m, 2 × $CH_2$), 1.60 (2H, m, $CH_2$), 1.21 – 1.39 (10 × 2H, 10 × m, 10 × $CH_2$), 0.88 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 170.7 (CONH), 155.0 (Ar C), 132.5 (Ar C), 129.65 (olefinic CH), 129.61 (olefinic CH), 120.5 (Ar CH), 113.7 (Ar CH), 55.1 ($OCH_3$), 36.3 (NHCO*C*$H_2$), 31.3 ($CH_2$), 29.1 ($CH_2$), 29.01 ($CH_2$), 29.0 ($CH_2$), 28.8 ($CH_2$), 28.67 ($CH_2$), 28.66 ($CH_2$), 28.60 ($CH_2$), 28.5 ($CH_2$), 26.57 ($CH_2$), 26.55 ($CH_2$), 25.2 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).




***ps306a***

**Synthesis of ps306a.** Oxalyl chloride (0.71 mL, 8.24 mmol) was added over 10 min to a solution of oleic acid (2 mL, 6.34 mmol) and DMF (3 drops) in DCM (10 mL) at 0 °C, and the solution stirred at room temperature overnight. The solvent and excess oxalyl chloride was removed *in vacuo* and the residue redissolved in DCM (10 mL). This solution was added dropwise to a solution of 3-fluoro-4-aminobenzonitrile (0.70 g, 5.14 mmol) in DCM (10 mL) and $Et_3N$ (1 mL) that had been cooled in an ice bath. Upon complete addition, the solution was stirred at room temperature overnight. The solution was washed with saturated $Na_2CO_3$ solution (3 × 20 mL), $H_2O$ (20 mL), and brine (20 mL), and dried over $MgSO_4$. The resulting residue was subjected to column chromatography, using a gradient elution of EtOAc/petroleum spirits to afford the product as a white. Yield: 1.59 g (61%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3319 br w, 2919 m, 2850 m, 2236 w, 1681 s, 1616 w, 1589 m, 1519 s. $^1$H-NMR δ/ppm ($d_6$-DMSO): 10.07 (1H, s, NH), 8.33 (1H, dd, $^3J_{H,H}$ = 8.5 Hz, $^4J_{H,F}$ = 7.9 Hz, Ar CH), 7.89 (1H, dd, $^3J_{H,F}$ = 11.0 Hz, $^4J_{H,H}$ = 2.0 Hz, Ar CH), 7.66 (1H, ddd, $^3J_{H,H}$ = 8.5 Hz, $^4J_{H,H}$ = 2.0 Hz, $^5J_{H,F}$ = 0.9 Hz, Ar CH), 5.29 – 5.40 (2 × 1H, 2 × m, 2 × olefinic CH), 2.47 (2H, m, NHCOC$H_2$), 1.96 – 2.04 (2 × 2H, 2 × m, 2 × $CH_2$), 1.61 (2H, m, $CH_2$), 1.23 – 1.37 (10 × 2H, 10 × m, 10 × $CH_2$), 0.87 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 172.4 (CONH), 151.6 (d, $^1J_{C,F}$ = 247 Hz, Ar CF), 131.7 (d, $^2J_{C,F}$ = 11.1 Hz, Ar C), 129.6 (olefinic CH), 129.5 (olefinic CH), 129.3 (d, $^3J_{C,F}$ = 3.4 Hz, Ar CH), 122.9 (d, $^4J_{C,F}$ = 2.7 Hz, Ar CH), 119.2 (d, $^2J_{C,F}$ = 23.3 Hz, Ar CH), 117.9 (d, $^4J_{C,F}$ = 2.7 Hz, CN), 105.7 (d, $^3J_{C,F}$ = 9.4 Hz, Ar C), 35.9 (NHCO*C*$H_2$), 31.3 ($CH_2$), 29.09 ($CH_2$), 29.08 ($CH_2$), 28.8 ($CH_2$), 28.7 ($CH_2$), 28.62 ($CH_2$), 28.59 ($CH_2$), 28.55 ($CH_2$), 28.5 ($CH_2$), 26.6 ($CH_2$), 24.9 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).

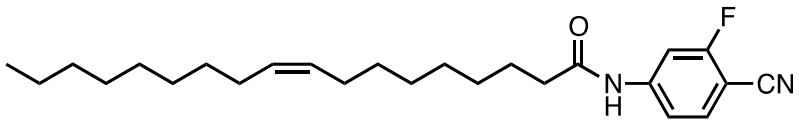



***ps309a***

**Synthesis of ps309a.** Concentrated HCl (0.65 mL, 6.61 mmol, 32%) was added to a solution of $Et_3N$ (0.92 mL, 6.61 mmol) in toluene (20 mL) that had been cooled in an

ice bath, and the mixture stirred until no more fumes were observed. $NaN_3$ (0.45 g, 6.61 mmol) and **ps306a** (0.53 g, 1.32 mmol) were added and the mixture heated at reflux for 2 d. The toluene was removed and the resulting residue suspended in $H_2O$ and acidified with concentrated HCl (~1 mL). The resulting solid was stirred for 5 min and then collected, washed with $H_2O$ (5 × 10 mL), dried, then washed with DCM (3 × 10 mL) and dried, affording the product as a white solid. Yield: 379 mg (65%).

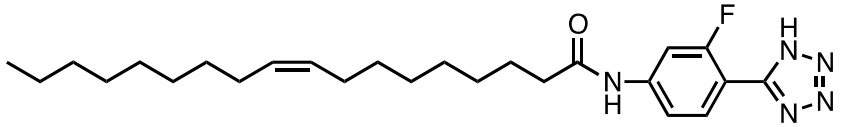
FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3271 br w, 2921 m, 2851 m, 1669 m, 1650 m, 1602 m, 1582 m, 1527 s. $^1$H-NMR δ/ppm ($d_6$-DMSO): 9.95 (1H, s, NH), 8.27 (1H, t, $^3J_{H,H}$ = 8.2 Hz, Ar CH), 7.83 – 7.93 (2 × 1H, 2 × m, 2 × Ar CH), 5.29 – 5.41 (2 × 1H, 2 × m, 2 × olefinic CH), 2.46 (2H, m, NHCOC*H*$_2$), 1.96 – 2.06 (2 × 2H, 2 × m, 2 × $CH_2$), 1.63 (2H, m, $CH_2$), 1.20 – 1.39 (10 × 2H, 10 × m, 10 × $CH_2$), 0.87 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 172.0 (CONH), 154.5 (tetrazolyl C), 152.8 (d, $^1J_{C,F}$ = 246 Hz, Ar CF), 129.57 (olefinic CH), 129.62 (olefinic CH), 129.1 (d, $^2J_{C,F}$ = 11.4 Hz, Ar C), 123.9 (Ar CH), 123.2 (d, $^3J_{C,F}$ = 3.4 Hz, Ar CH), 120.2 (d, $^3J_{C,F}$ = 8.4 Hz, Ar C), 113.9 (d, $^2J_{C,F}$ = 22.3 Hz, Ar CH), 35.9 (NHCO*C*H$_2$), 31.3 ($CH_2$), 29.1 ($CH_2$), 28.8 ($CH_2$), 28.7 ($CH_2$), 28.62 ($CH_2$), 28.57 ($CH_2$), 28.49 ($CH_2$), 26.6 ($CH_2$), 25.0 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).




***ps308c***

**Synthesis of ps308c.** Oxalyl chloride (0.71 mL, 8.24 mmol) was added over 10 min to a solution of oleic acid (2 mL, 6.34 mmol) and DMF (3 drops) in DCM (10 mL) at 0 °C, and the solution stirred at room temperature overnight. The solvent and excess oxalyl chloride was removed *in vacuo* and the residue redissolved in DCM (10 mL). This solution was added dropwise to a solution of 2-fluoro-4-aminobenzonitrile (0.70 g, 5.14 mmol) in DCM (10 mL) and $Et_3N$ (1 mL) that had been cooled in an ice bath. Upon complete addition, the solution was stirred at room temperature overnight. The solution was washed with saturated $Na_2CO_3$ solution (3 × 20 mL), $H_2O$ (20 mL), and brine (20 mL), and dried over $MgSO_4$. The resulting residue was subjected to column chromatography, using a gradient elution of EtOAc/petroleum spirits to afford the product as a white. Yield: 1.03 g (50%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3350 m, 2920 m,

2850 m, 2229 m, 1700 s, 1620 s, 1593 m, 1529 s. $^{1}$H-NMR δ/ppm (d$_6$-DMSO): 10.55 (1H, s, NH), 7.89 (1H, dd, $^{3}J_{H,F}$ = 12.6 Hz, $^{4}J_{H,H}$ = 2.0 Hz, Ar CH), 7.85 (1H, dd, $^{3}J_{H,H}$ = 8.6 Hz, $^{4}J_{H,F}$ = 7.9 Hz, Ar CH), 7.46 (1H, dd, $^{3}J_{H,H}$ = 8.6 Hz, $^{4}J_{H,H}$ = 2.0 Hz, Ar CH), 5.29 – 5.40 (2 × 1H, 2 × m, 2 × olefinic CH), 2.39 (2H, m, NHCOC*H*$_2$), 1.96 – 2.05 (2 × 2H, 2 × m, 2 × CH$_2$), 1.61 (2H, m, CH$_2$), 1.21 – 1.38 (10 × 2H, 10 × m, 10 × CH$_2$), 0.87 (3H, m, CH$_3$). $^{13}$C-NMR δ/ppm (d$_6$-DMSO): 172.4 (CONH), 163.0 (d, $^{1}J_{C,F}$ = 252 Hz, Ar CF), 145.6 (d, $^{3}J_{C,F}$ = 11.7 Hz, CN), 134.2 (d, $^{3}J_{C,F}$ = 3.4 Hz, Ar CH), 129.6 (olefinic CH), 129.5 (olefinic CH), 115.1 (d, $^{4}J_{C,F}$ = 2.7 Hz, Ar CH), 114.3 (Ar C), 105.5 (d, $^{2}J_{C,F}$ = 24.7 Hz, Ar CH), 93.1 (d, $^{2}J_{C,F}$ = 15.4 Hz, Ar C), 36.5 (NHCO*C*H$_2$), 31.3 (CH$_2$), 29.07 (CH$_2$), 29.04 (CH$_2$), 28.8 (CH$_2$), 28.7 (CH$_2$), 28.59 (CH$_2$), 28.57 (CH$_2$), 28.5 (CH$_2$), 28.4 (CH$_2$), 26.6 (CH$_2$), 24.7 (CH$_2$), 22.1 (CH$_2$), 13.9 (CH$_3$).

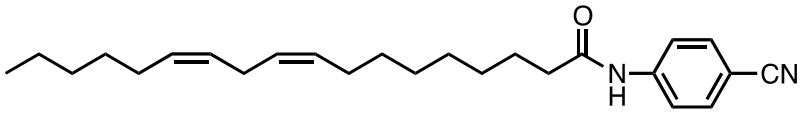



***ps312a***

**Synthesis of ps312a.** Concentrated HCl (0.60 mL, 6.12 mmol, 32%) was added to a solution of Et$_3$N (0.85 mL, 6.12 mmol) in toluene (20 mL) that had been cooled in an ice bath, and the mixture stirred until no more fumes were observed. NaN$_3$ (0.42 g, 6.12 mmol) and **ps308c** (0.49 g, 1.22 mmol) were added and the mixture heated at reflux for 2 d. The toluene was removed and the resulting residue suspended in H$_2$O and acidified with concentrated HCl (~1 mL). The resulting solid was stirred for 5 min and then collected, washed with H$_2$O (5 × 10 mL), dried, then washed with DCM (3 × 10 mL) and dried, affording the product as a white solid. Yield: 276 mg (51%).

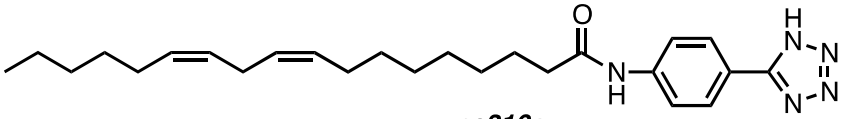
FT-IR (ATR) $\nu_{max}$/cm$^{-1}$: 3267 br w, 2951 m, 2852 m, 1669 s, 1630 w, 1606 s, 1540 s. $^{1}$H-NMR δ/ppm (d$_6$-DMSO): 10.43 (1H, s, NH), 8.02 (1H, m, Ar CH), 7.92 (1H, dd, $^{3}J_{H,F}$ = 13.5 Hz, $^{4}J_{H,H}$ = 2.0 Hz, Ar CH), 7.51 (1H, dd, $^{3}J_{H,H}$ = 8.6 Hz, $^{4}J_{H,H}$ = 2.0 Hz, Ar CH), 5.30 – 5.41 (2 × 1H, 2 × m, 2 × olefinic CH), 2.39 (2H, m, NHCOC*H*$_2$), 1.96 – 2.06 (2 × 2H, 2 × m, 2 × CH$_2$), 1.63 (2H, m, CH$_2$), 1.19 – 1.39 (10 × 2H, 10 × m, 10 × CH$_2$), 0.87 (3H, m, CH$_3$). $^{13}$C-NMR δ/ppm (d$_6$-DMSO): 172.2 (CONH), 159.2 (d, $^{1}J_{C,F}$ = 249 Hz, Ar CF), 143.6 (d, $^{2}J_{C,F}$ = 11.7 Hz, Ar C), 130.1 (d, $^{3}J_{C,F}$ = 3.6 Hz, Ar CH), 129.7 (olefinic CH), 129.6 (olefinic CH), 127.8 (Ar C), 115.2 (d, $^{4}J_{C,F}$ = 2.8 Hz, Ar CH), 106.0 (d, $^{2}J_{C,F}$ = 26.1 Hz, Ar CH), 36.5 (NHCO*C*H$_2$), 31.3 (CH$_2$), 29.09 (CH$_2$),

29.07 ($CH_2$), 28.8 ($CH_2$), 28.7 ($CH_2$), 28.63 ($CH_2$), 28.58 ($CH_2$), 28.5 ($CH_2$), 26.6 ($CH_2$), 24.8 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).




***ps314b***

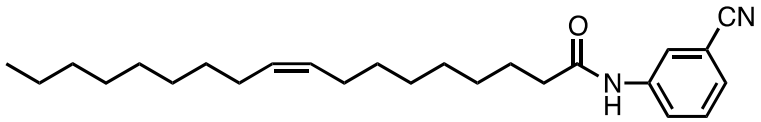
**Synthesis of ps314b.** Oxalyl chloride (0.72 mL, 8.36 mmol) was added over 10 min to a solution of linoleic acid (2 mL, 6.43 mmol) and DMF (3 drops) in DCM (10 mL) at 0 °C, and the solution stirred at room temperature overnight. The solvent and excess oxalyl chloride was removed *in vacuo* and the residue redissolved in DCM (10 mL). This solution was added dropwise to a solution of 4-aminobenzonitrile (0.68 g, 5.79 mmol) in DCM (10 mL) and $Et_3N$ (1 mL) that had been cooled in an ice bath. Upon complete addition, the solution was stirred at room temperature overnight. The solution was washed with saturated $Na_2CO_3$ solution (3 × 20 mL), $H_2O$ (20 mL), and brine (20 mL), and dried over $MgSO_4$. The resulting residue was subjected to column chromatography, using a gradient elution of EtOAc/petroleum spirits to afford the product as a pale yellow oil. Yield: 1.72 g (70%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3329 br w, 2926 m, 2855 m, 2226 m, 1730 w, 1705 w, 1676 m, 1593 s, 1512 s. $^1$H-NMR δ/ppm ($d_6$-DMSO): 10.33 (1H, s, NH), 7.74 – 7.83 (2 × 2H, 2 × m, 2 × Ar CH), 5.26 – 5.45 (4 × 1H, 4 × m, 4 × olefinic CH), 2.78 (2H, m, $CH_2$), 2.38 (2H, m, NHCOC*H*$_2$), 2.01 – 2.08 (2 × 2H, 2 × m, 2 × $CH_2$), 1.62 (2H, m, $CH_2$), 1.23 – 1.41 (7 × 2H, 7 × m, 7 × $CH_2$), 0.88 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 172.1 (CONH), 143.5 (Ar C), 133.2 (Ar CH), 129.69 (olefinic CH), 129.67 (olefinic CH), 127.73 (olefinic CH), 129.7 (olefinic CH), 119.1 (Ar C), 118.9 (Ar CH), 104.6 (CN), 36.5 (NHCO*C*$H_2$), 30.9 ($CH_2$), 29.0 ($CH_2$), 28.7 ($CH_2$), 28.6 ($CH_2$), 28.56 ($CH_2$), 28.5 ($CH_2$), 26.58 ($CH_2$), 26.57 ($CH_2$), 25.2 ($CH_2$), 24.8 ($CH_2$), 21.9 ($CH_2$), 13.9 ($CH_3$).




***ps316a***

**Synthesis of ps316a.** Concentrated HCl (0.78 mL, 7.93 mmol, 32%) was added to a solution of $Et_3N$ (1.11 mL, 7.93 mmol) in toluene (20 mL) that had been cooled in an ice bath, and the mixture stirred until no more fumes were observed. $NaN_3$ (0.54 g, 7.93 mmol) and **ps314b** (0.60 g, 1.59 mmol) were added and the mixture heated at

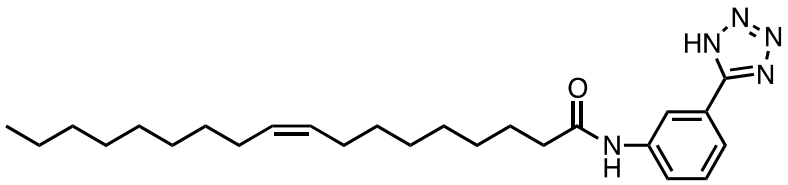
reflux for 2 days. The toluene was removed and the resulting residue suspended in $H_2O$ and acidified with concentrated HCl (~1 mL). The resulting solid was stirred for 5 min and then collected, washed with $H_2O$ (5 × 10 mL), dried, then washed with DCM (3 × 10 mL) and dried, affording the product as a white solid. Yield: 0.45 g (66%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3306 br w, 2926 m, 2855 m, 1668 s, 1600 s, 1537 s, 1500 s. $^1$H-NMR δ/ppm ($d_6$-DMSO): 10.20 (1H, s, NH), 8.00 (2H, d, $^3J_{H,H}$ = 8.7 Hz, Ar CH), 7.84 (2H, d, $^3J_{H,H}$ = 8.7 Hz, Ar CH), 5.28 – 5.42 (4 × 1H, 4 × m, 4 × olefinic CH), 2.77 (2H, m, $CH_2$), 2.38 (2H, m, NHCOC$H_2$), 1.99 – 2.09 (2 × 2H, 2 × m, 2 × $CH_2$), 1.63 (2H, m, $CH_2$), 1.22 – 1.40 (7 × 2H, 7 × m, 7 × $CH_2$), 0.88 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 171.7 (CONH), 154.9 (tetrazolyl C), 141.9 (Ar C), 129.71 (olefinic CH), 129.69 (olefinic CH), 127.74 (olefinic CH), 129.71 (olefinic CH), 127.7 (Ar CH), 119.2 (Ar CH), 118.4 (Ar C), 36.5 (NHCO*C*$H_2$), 30.9 ($CH_2$), 29.0 ($CH_2$), 28.7 ($CH_2$), 28.66 ($CH_2$), 28.63 ($CH_2$), 28.5 ($CH_2$), 26.61 ($CH_2$), 26.59 ($CH_2$), 25.2 ($CH_2$), 25.0 ($CH_2$), 21.9 ($CH_2$), 13.9 ($CH_3$).




***ps315a***

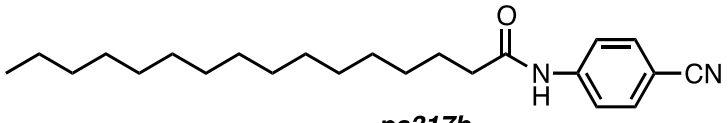
**Synthesis of ps315a.** Oxalyl chloride (0.71 mL, 8.24 mmol) was added over 10 min to a solution of oleic acid (2 mL, 6.34 mmol) and DMF (3 drops) in DCM (10 mL) at 0 °C, and the solution stirred at room temperature overnight. The solvent and excess oxalyl chloride was removed *in vacuo* and the residue redissolved in DCM (10 mL). This solution was added dropwise to a solution of 3-aminobenzonitrile (0.61 g, 5.14 mmol) in DCM (10 mL) and $Et_3N$ (1 mL) that had been cooled in an ice bath. Upon complete addition, the solution was stirred at room temperature overnight. The solution was washed with saturated $Na_2CO_3$ solution (3 × 20 mL), $H_2O$ (20 mL), and brine (20 mL), and dried over $MgSO_4$. The resulting residue was subjected to column chromatography, using a gradient elution of EtOAc/petroleum spirits to afford the product as a pale orange oil that solidified upon cooling. Yield: 1.89 g (96%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3314 br w, 2920 m, 2852 m, 2230 w, 1740 w, 1704 w, 1671 s, 1586 s, 1545 m. $^1$H-NMR δ/ppm ($d_6$-DMSO): 10.23 (1H, s, NH), 8.14 (1H, m, Ar CH), 7.82 (1H, m, Ar CH), 7.49 – 7.57 (2 × 1H, 2 × m, 2 × Ar CH), 5.29 – 5.41 (2 × 1H, 2 × m, 2 × olefinic CH), 2.35 (2H, m, NHCOC$H_2$), 1.96 – 2.08 (2 × 2H, 2 × m, 2 × $CH_2$), 1.62 (2H, m, $CH_2$), 1.21 – 1.39 (10 × 2H, 10 × m, 10 × $CH_2$), 0.87 (3H, m, $CH_3$). $^{13}$C-NMR

δ/ppm ($d_6$-DMSO): 171.8 (CONH), 140.1 (Ar C), 130.1 (Ar CH), 129.6 (olefinic CH), 129.5 (olefinic CH), 126.4 (Ar CH), 123.4 (Ar CH), 121.5 (Ar CH), 118.7 (Ar C), 111.5 (CN), 36.4 (NHCO*C*$H_2$), 31.2 ($CH_2$), 29.08 ($CH_2$), 29.06 ($CH_2$), 28.8 ($CH_2$), 28.7 ($CH_2$), 28.63 ($CH_2$), 28.58 ($CH_2$), 28.5 ($CH_2$), 26.6 ($CH_2$), 24.9 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).



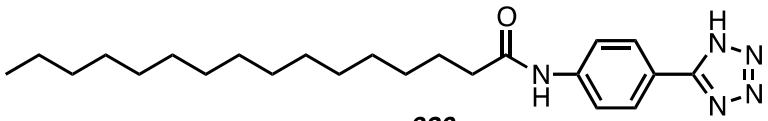

***ps318a***

**Synthesis of ps318a.** Concentrated HCl (0.73 mL, 7.48 mmol, 32%) was added to a solution of $Et_3N$ (1.04 mL, 7.48 mmol) in toluene (20 mL) that had been cooled in an ice bath, and the mixture stirred until no more fumes were observed. $NaN_3$ (0.51 g, 7.48 mmol) and **ps315a** (0.57 g, 1.50 mmol) were added and the mixture heated at reflux for 2 d. The toluene was removed and the resulting residue suspended in $H_2O$ and acidified with concentrated HCl (~1 mL). The resulting solid was stirred for 5 min and then collected, washed with $H_2O$ (5 × 10 mL), dried, then washed with DCM (3 × 10 mL) and dried, affording the product as a white solid. Yield: 0.48 g (76%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3545 br w, 3278 br w, 2920 m, 2851 m, 1657 s, 1576 m, 1535 m. $^1$H-NMR δ/ppm ($d_6$-DMSO): 10.20 (1H, s, NH), 8.44 (1H, dd, $^3J_{H,H}$ = 7.2 Hz, $^4J_{H,H}$ = 1.6 Hz, Ar CH), 7.77 (1H, ddd, $^3J_{H,H}$ = 8.2 Hz, $^4J_{H,H}$ = 2.0 Hz, $^4J_{H,H}$ = 1.0 Hz, Ar CH), 7.70 (1H, ddd, $^3J_{H,H}$ = 7.7 Hz, $^4J_{H,H}$ = 1.6 Hz, $^4J_{H,H}$ = 1.0 Hz, Ar CH), 7.55 (1H, dd, $^3J_{H,H}$ = 8.2 Hz, $^3J_{H,H}$ = 7.7 Hz, Ar CH), 5.31 – 5.41 (2 × 1H, 2 × m, 2 × olefinic CH), 2.37 (2H, m, NHCOC*H*$_2$), 1.95 – 2.08 (2 × 2H, 2 × m, 2 × $CH_2$), 1.64 (2H, m, $CH_2$), 1.21 – 1.39 (10 × 2H, 10 × m, 10 × $CH_2$), 0.87 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 171.6 (CONH), 155.6 (tetrazolyl C), 140.2 (Ar C), 129.8 (Ar CH), 129.64 (olefinic CH), 129.59 (olefinic CH), 124.8 (Ar C), 121.4 (Ar CH), 121.3 (Ar CH), 117.3 (Ar CH), 36.4 (NHCO*C*$H_2$), 31.2 ($CH_2$), 29.1 ($CH_2$), 28.8 ($CH_2$), 28.65 ($CH_2$), 28.63 ($CH_2$), 28.56 ($CH_2$), 28.49 ($CH_2$), 26.56 ($CH_2$), 26.55 ($CH_2$), 25.0 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).




***ps317b***

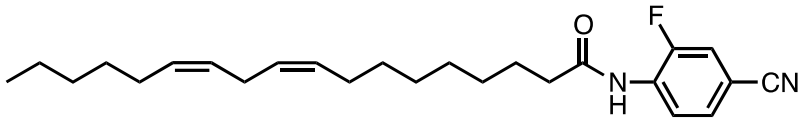
**Synthesis of ps317b.** Oxalyl chloride (0.87 mL, 10.14 mmol) was added over 10 min to a suspension of palmitic acid (2 g, 7.80 mmol) and DMF (3 drops) in DCM (10 mL) at room temperature, and the suspension stirred at room temperature overnight. The solvent and excess oxalyl chloride was removed *in vacuo* and the residue redissolved in DCM (10 mL). This solution was added dropwise to a solution of 4-aminobenzonitrile (0.83 g, 7.02 mmol) in DCM (10 mL) and $Et_3N$ (1 mL) that had been cooled in an ice bath. Upon complete addition, the solution was stirred at room temperature overnight. The solution was washed with saturated $Na_2CO_3$ solution (3 × 20 mL), $H_2O$ (20 mL), and brine (20 mL), and dried over $MgSO_4$. The resulting residue was suspended in methanol (20 mL) and stirred for 5 min. The solid was collected, washed with methanol (5 × 10 mL), and dried to afford the product as a white solid. Yield: 1.71 g (61%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3310 br w, 2915 s, 2849 m, 2224 w, 1674 m, 1605 m, 1528 m. $^1$H-NMR δ/ppm ($d_6$-DMSO): 10.30 (1H, s, NH), 7.81 (2H, d, $^3J_{H,H}$ = 8.8 Hz, Ar CH), 7.78 (2H, d, $^3J_{H,H}$ = 8.8 Hz, Ar CH), 2.38 (2H, m, NHCOC$H_2$), 1.62 (2H, m, $CH_2$), 1.21 – 1.37 (12 × 2H, 12 × m, 12 × $CH_2$), 0.89 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 172.1 (CONH), 143.5 (Ar C), 133.2 (Ar CH), 119.1 (Ar C), 118.9 (Ar CH), 104.6 (CN), 36.5 (NHCO*C*$H_2$), 31.3 ($CH_2$), 29.02 ($CH_2$), 29.00 ($CH_2$), 28.98 ($CH_2$), 28.94 ($CH_2$), 28.8 ($CH_2$), 28.71 ($CH_2$), 28.68 ($CH_2$), 28.5 ($CH_2$), 24.8 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).




***ps320c***

**Synthesis of ps320c.** Concentrated HCl (0.78 mL, 7.99 mmol, 32%) was added to a solution of $Et_3N$ (1.11 mL, 7.99 mmol) in toluene (20 mL) that had been cooled in an ice bath, and the mixture stirred until no more fumes were observed. $NaN_3$ (0.54 g, 7.99 mmol) and **ps317b** (0.57 g, 1.60 mmol) were added and the mixture heated at reflux for 2 d. The toluene was removed and the resulting residue suspended in $H_2O$ and acidified with concentrated HCl (~1 mL). The resulting solid was stirred for 5 min and then collected, washed with $H_2O$ (5 × 10 mL), dried, then washed with DCM (10 × 10 mL) and dried, affording the product as a white solid. Yield: 0.41 g (65%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3558 br w, 3290 br w, 2917 s, 2847 m, 1663 s, 1618 w, 1597 w, 1534 s. $^1$H-NMR δ/ppm ($d_6$-DMSO): 10.23 (1H, s, NH), 8.01 (2H, d, $^3J_{H,H}$ = 8.6 Hz,

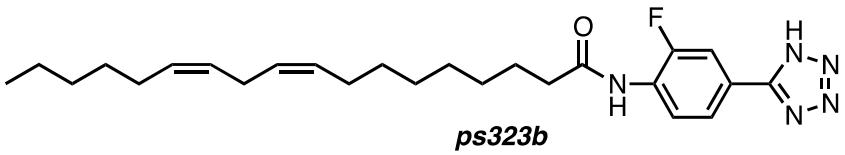
Ar CH), 7.84 (2H, d, $^{3}J_{H,H}$ = 8.6 Hz, Ar CH), 2.38 (2H, m, NHCOC*H*$_2$), 1.63 (2H, m, $CH_2$), 1.20 – 1.38 (12 × 2H, 12 × m, 12 × $CH_2$), 0.88 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 171.7 (CONH), 155.1 (tetrazolyl C), 141.7 (Ar C), 127.6 (Ar CH), 119.2 (Ar CH), 118.8 (Ar C), 36.4 (NHCO*C*H$_2$), 31.3 ($CH_2$), 29.0 ($CH_2$), 28.98 ($CH_2$), 28.96 ($CH_2$), 28.9 ($CH_2$), 28.74 ($CH_2$), 28.7 ($CH_2$), 28.6 ($CH_2$), 25.0 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).




***ps319***

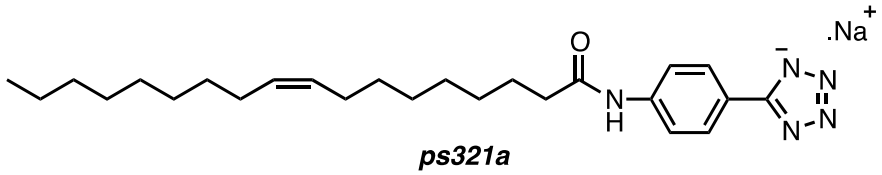
**Synthesis of ps319.** Oxalyl chloride (0.72 mL, 8.36 mmol) was added over 10 min to a solution of linoleic acid (2 mL, 6.43 mmol) and DMF (3 drops) in DCM (10 mL) at 0 °C, and the solution stirred at room temperature overnight. The solvent and excess oxalyl chloride was removed *in vacuo* and the residue redissolved in DCM (10 mL). This solution was added dropwise to a solution of 3-fluoro-4-aminobenzonitrile (0.79 g, 5.79 mmol) in DCM (10 mL) and $Et_3N$ (1 mL) that had been cooled in an ice bath. Upon complete addition, the solution was stirred at room temperature overnight. The solution was washed with saturated $Na_2CO_3$ solution (3 × 20 mL), $H_2O$ (20 mL), and brine (20 mL), and dried over $MgSO_4$. The resulting residue was subjected to column chromatography, using a gradient elution of EtOAc/petroleum spirits to afford the product as a pale yellow oil that solidified upon cooling. Yield: 0.98 g (39%). FT-IR (ATR) $\nu_{max}$/cm$^{-1}$: 3328 br w, 2925 m, 2855 m, 2232 w, 1709 m, 1616 m, 1594 m, 1517 s. $^{1}$H-NMR δ/ppm ($d_6$-DMSO): 10.07 (1H, s, NH), 8.32 (1H, m, Ar CH), 7.91 (1H, dd, $^{3}J_{H,F}$ = 11.1 Hz, $^{4}J_{H,H}$ = 1.9 Hz, Ar CH), 7.68 (1H, ddd, $^{3}J_{H,H}$ = 8.6 Hz, $^{4}J_{H,H}$ = 1.9 Hz, $^{5}J_{H,F}$ = 0.9 Hz Ar CH), 5.28 – 5.43 (4 × 1H, 4 × m, 4 × olefinic CH), 2.77 (2H, m, $CH_2$), 2.48 (2H, m, NHCOC*H*$_2$), 2.00 – 2.10 (2 × 2H, 2 × m, 2 × $CH_2$), 1.61 (2H, m, $CH_2$), 1.24 – 1.40 (7 × 2H, 7 × m, 7 × $CH_2$), 0.88 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 172.4 (CONH), 151.7 (d, $^{1}J_{C,F}$ = 247 Hz, Ar CF), 131.6 (d, $^{2}J_{C,F}$ = 11.2 Hz, Ar C), 129.70 (olefinic CH), 129.68 (olefinic CH), 129.3 (d, $^{3}J_{C,F}$ = 3.5 Hz, Ar CH), 127.74 (olefinic CH), 127.71 (olefinic CH), 122.9 (d, $^{4}J_{C,F}$ = 2.9 Hz, Ar CH), 119.3 (d, $^{2}J_{C,F}$ = 23.4 Hz, Ar CH), 105.7 (d, $^{3}J_{C,F}$ = 9.3 Hz, Ar C), 117.9 (d, $^{3}J_{C,F}$ = 2.7 Hz, CN),

35.9 (NHCO*C*$H_2$), 30.9 ($CH_2$), 29.0 ($CH_2$), 28.7 ($CH_2$), 28.6 ($CH_2$), 28.52 ($CH_2$), 28.5 ($CH_2$), 26.6 ($CH_2$), 25.2 ($CH_2$), 24.9 ($CH_2$), 21.9 ($CH_2$), 13.9 ($CH_3$).



***ps323b***

**Synthesis of 323b.** Concentrated HCl (0.76 mL, 7.78 mmol, 32%) was added to a solution of $Et_3N$ (1.08 mL, 7.78 mmol) in toluene (20 mL) that had been cooled in an ice bath, and the mixture stirred until no more fumes were observed. $NaN_3$ (0.53 g, 7.78 mmol) and **ps319** (0.61 g, 1.56 mmol) were added and the mixture heated at reflux for 2 d. The toluene was removed and the resulting residue suspended in $H_2O$ and acidified with concentrated HCl (~1 mL). The resulting solid was stirred for 5 min and then collected and washed with $H_2O$ (5 × 10 mL). The solid was dissolved in DCM (30 mL) and washed with $H_2O$ (3 × 30 mL), and dried over $MgSO_4$. The resulting residue was triturated with DCM (2 × 3 mL), petroleum spirits (3 × 10 mL), and then dried to afford the product as a white solid. Yield: 0.39 g (58%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 2922 w, 2854 w, 1734 s, 1667 w, 1524 w, 1444 w. $^1$H-NMR δ/ppm ($d_6$-DMSO): 9.92 (1H, s, NH), 8.20 (1H, t, $^3J_{H,H}$ = 8.2 Hz, Ar CH), 7.86 – 7.95 (2 × 1H, 2 × m, 2 × Ar CH), 5.28 – 5.44 (4 × 1H, 4 × m, 4 × olefinic CH), 2.77 (2H, m, $CH_2$), 2.46 (2H, m, NHCOC*H*$_2$), 2.00 – 2.09 (2 × 2H, 2 × m, 2 × $CH_2$), 1.63 (2H, m, $CH_2$), 1.23 – 1.40 (7 × 2H, 7 × m, 7 × $CH_2$), 0.88 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 172.0 (CONH), 155.2 (tetrazolyl C), 153.0 (d, $^1J_{C,F}$ = 245.5 Hz, Ar CF), 129.7 (2 × olefinic CH), 128.4 (d, $^2J_{C,F}$ = 11.5 Hz, Ar C), 127.75 (olefinic CH), 127.72 (olefinic CH), 124.0 (Ar CH), 122.9 (d, $^3J_{C,F}$ = 3.2 Hz, Ar CH), 121.6 (d, $^3J_{C,F}$ = 8.0 Hz, Ar C), 113.7 (d, $^2J_{C,F}$ = 22.2 Hz, Ar CH), 35.8 (NHCO*C*$H_2$), 30.9 ($CH_2$), 29.0 ($CH_2$), 28.7 ($CH_2$), 28.63 ($CH_2$), 28.57 ($CH_2$), 28.53 ($CH_2$), 26.61 ($CH_2$), 26.59 ($CH_2$), 25.2 ($CH_2$), 25.0 ($CH_2$), 21.9 ($CH_2$), 13.9 ($CH_3$).



***ps321a***

**Synthesis of ps321a.** An aqueous solution of NaOH (11.95 μL, 6.33 M, 0.076 mmol), was added to a solution of **ps297b** (32.2 mg, 0.076 mmol) in ethanol (2 mL) and the solution stirred at room temperature for 30 min. The solution was dried and the resulting residue was azeotropically dried by repeated dissolution in ethanol and evaporation steps. DCM (2 mL) and $Et_2O$ (5 mL) were added to the dried residue and the resulting solid was collected, washed with $Et_2O$ (2 × 5 mL), and dried to afford the product as a white solid. Yield: 17.3 mg (51%). FT-IR (ATR) $\nu_{max}/cm^{-1}$: 3316 br w, 2922 m, 2852 m, 1662 s, 1603 s, 1541 m. $^1$H-NMR δ/ppm ($d_6$-DMSO): 9.87 (1H, s, NH), 7.90 (2H, d, $^3J_{H,H}$ = 8.8 Hz, Ar CH), 7.62 (2H, d, $^3J_{H,H}$ = 8.8 Hz, Ar CH), 5.31 – 5.42 (2 × 1H, 2 × m, 2 × olefinic CH), 2.33 (2H, m, NHCOC$H_2$), 1.97 – 2.09 (2 × 2H, 2 × m, 2 × $CH_2$), 1.63 (2H, m, $CH_2$), 1.22 – 1.40 (10 × 2H, 10 × m, 10 × $CH_2$), 0.88 (3H, m, $CH_3$). $^{13}$C-NMR δ/ppm ($d_6$-DMSO): 171.0 (CONH), 160.3 (tetrazolate C), 138.1 (Ar C), 129.64 (olefinic CH), 129.62 (olefinic CH), 127.6 (Ar C), 126.0 (Ar CH), 118.9 (Ar CH), 36.4 (NHCO$CH_2$), 31.2 ($CH_2$), 29.09 ($CH_2$), 29.07 ($CH_2$), 28.8 ($CH_2$), 28.68 ($CH_2$), 28.67 ($CH_2$), 28.66 ($CH_2$), 28.56 ($CH_2$), 28.5 ($CH_2$), 26.57 ($CH_2$), 26.55 ($CH_2$), 25.1 ($CH_2$), 22.1 ($CH_2$), 13.9 ($CH_3$).

### 3.2.2 Cell culture and cell viability assessment

NCI-H716 cells (ATCC® CCL-251™) were grown in RPMI cell culture media, GLUTag cells generously gifted from Prof. Daniel J Drucker (University of Toronto), were kept in DMEM media low glucose (1 g/L), while STC-1 purchased from ATCC® (CRL-3254™), were cultured in DMEM high glucose media (4.5 g/L). All media was supplemented with a final concentration of 10% Fetal Bovine Serum (FBS), glutamine 2 mM, HEPES 20mM, and Penicillin 100U/ml Streptomycin 100 μg/ml.

### 3.2.3 Downregulating GPR119 expression

#### 3.2.3.1 siRNA

Study of the GPR119 knockdown with RNA interference was performed by transfection of commercial oligonucleotides purchased from the indicated providers with Dharmacon™ DharmaFECT™ Transfection Reagent Formulation 1, following the manufacturer's protocols.

The following siRNAs, hereafter referred to as sequences 1, 2, 3, 4 (From QIAGEN) 5, 6, 7 and 8 (From Dharmacon), were used for downregulation of the human GPR119 expressed in NCI-H716

| QIAGEN | siRNA name | Lot number | FlexiTube GeneSolution siRNA Gene ID:139760 Cat #1027416 |
|---|---|---|---|
| SI00161161 | Hs_Gpr119_4 | 201703160112 | CAGGAGTGTCACCTCTACCTA |
| SI00161154 | Hs_Gpr119_3 | 20110420 | CAGTCTCTGCTTCACCTTGAA |
| SI00161147 | Hs_Gpr119_2 | 20130516 | CGGGCTGTGGTTAGTGTCTTA |
| SI00161140 | Hs_Gpr119_1 | 20130516 | CTCCCTCATCATTGCTACTAA |

| Dharmacon™ | ON-Target plus Human GPR119 (139760) siRNA |
|---|---|
| J-005521-05 | GCUCAGAGUUUGAUGGCUA |
| J-005521-06 | ACCUAGUGCUGGAACGGUA |
| J-005521-07 | CUUCAAAGCUCUCCGUACU |
| J-005521-08 | CCACAAGAAUGAUGGUGUC |

GLUTag and STC-1 cells were transfected with the following siRNA sequences referred to sequences 4, 5, 6 and 7 targeting murine GPR119

| QIAGEN | siRNA name | Lot number | FlexiTube GeneSolution siRNA Gene ID:236781 Cat #1027416 |
|---|---|---|---|
| SI04928056 | Mm_Gpr119_7 | 20130102 | TTCTCCCTAGATGAAGTATTA |
| SI04928049 | Mm_Gpr119_6 | 20130102 | TGGCCTTTGCTTCACCTTGAA |
| SI04928042 | Mm_Gpr119_5 | 20130102 | CTGAGCCTATAGCACATCTAA |
| SI01056020 | Mm_Gpr119_4 | 20130921 | CCGTTACTTCCAGATCATGAA |

All knockdown experiments were compared with cells transfected with equivalent amounts of negative control siRNA AATTCTCCGAACGTGTCACGT cat. #1027310 from QIAGEN. Intact cells, or whole protein lysates of transfected cells were analysed after 48 or 72 hours via immunofluorescence, or immunoblotting respectively.

### 3.2.3.2 esiRNA

Endoribonuclease-prepared short interfering RNAs (esiRNA) were developed as previously described (Heninger & Buchholz, 2007). Briefly, an efficiently targetable region of less than 600 base pairs, with low off-target cross-silencing potential was isolated. In particular to obtain an efficient RNA interference of the human GPR119 transcript (ENSEMBL ID: ENSG00000147262) a publicly available target region from Eupheria Biotech was chosen, while for the murine GPR119 (ENSEMBL ID: ENSMUSG00000051209) it was manually selected with the DEQOR web server (Henschel, Buchholz, & Habermann, 2004) from its consensus coding sequence (CCDS). Primers targeting the region of interest, were generated with the Primer3 software, and appended with the T7 promoter sequences (Forward: *TCACTATAGGGAGAG* and Reverse: *TCACTATAGGGAGAC*)

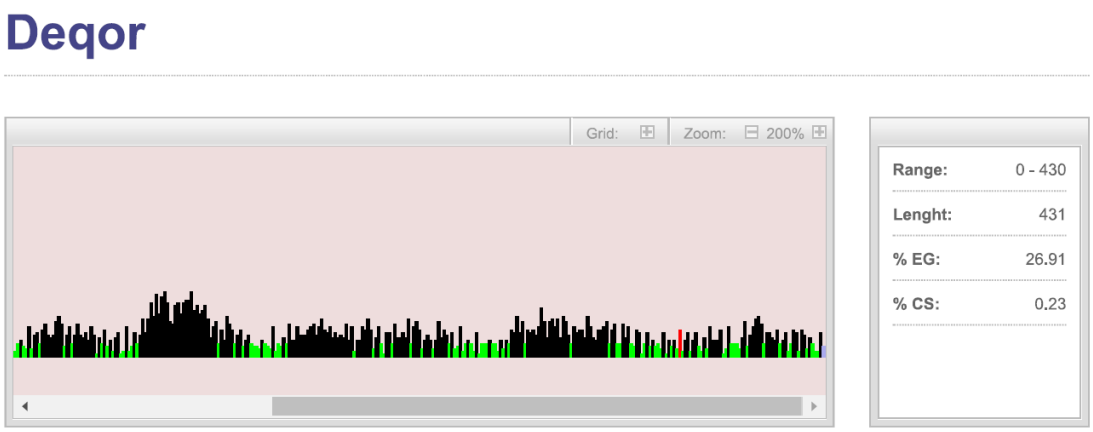
| | |
|---|---|
| Human GPR119 Forward primer | *TCACTATAGGGAGAG*CTCACAGACCAGCTCTCCAG |
| Human GPR119 Reverse primer | *TCACTATAGGGAGAC*TTTCGAATCTGCTGGCTGTG |
| Murine GPR119 Forward primer | *TCACTATAGGGAGAG*TGCTGTCCTAACCATCCTCA |
| Murine GPR119 Reverse primer | *TCACTATAGGGAGAC*AGGCTACACAAGGTCTTCTGT |

Human GPR119 CCDS targetable region chrX:130384846-130385264 underlined, with the primers highlighted in bold:

ATGGAATCATCTTTCTCATTTGGAGTGATCCTTGCTGTCCTGGCCTCCCTC
ATCATTGCTACTAACACACTAGTGGCTGTGGCTGTGCTGCTGTTGATCCA
CAAGAATGATGGTGTCAGTCTCTGCTTCACCTTGAATCTGGCTGTGGCTG
ACACCTTGATTGGTGTG<u>GCCATCTCTGGCCTA**CTCACAGACCAGCTCTC**
**CAG**CCCTTCTCGGCCCACACAGAAGACCCTGTGCAGCCTGCGGATGGCA
TTTGTCACTTCCTCCGCAGCTGCCTCTGTCCTCACGGTCATGCTGATCACC
TTTGACAGGTACCTTGCCATCAAGCAGCCCTTCCGCTACTTGAAGATCAT
GAGTGGGTTCGTGGCCGGGGCCTGCATTGCCGGGCTGTGGTTAGTGTCTT
ACCTCATTGGCTTCCTCCCACTCGGAATCCCCATGTTCCAGCAGACTGCCT
ACAAAGGGCAGTGCAGCTTCTTTGCTGTATTTCACCCTCACTTCGTGCTG
ACCCTCTCCTGCGTTGGCTTCTTCCCAGCCATGCTCCTCTTTGTCTTCTTCT
ACTGCGACATGCTCAAGATTGCCTCCATG**CACAGCCAGCAGATTCGAAA**
GATGGAACATGCAGGAGC</u>CATGGCTGGAGGTTATCGATCCCCACGGACT

CCCAGCGACTTCAAAGCTCTCCGTACTGTGTCTGTTCTCATTGGGAGCTTT
GCTCTATCCTGGACCCCCTTCCTTATCACTGGCATTGTGCAGGTGGCCTGC
CAGGAGTGTCACCTCTACCTAGTGCTGGAACGGTACCTGTGGCTGCTCGG
CGTGGGCAACTCCCTGCTCAACCCACTCATCTATGCCTATTGGCAGAAGG
AGGTGCGACTGCAGCTCTACCACATGGCCCTAGGAGTGAAGAAGGTGCT
CACCTCATTCCTCCTCTTTCTCTCGGCCAGGAATTGTGGCCCAGAGAGGC
CCAGGGAAAGTTCCTGTCACATCGTCACTATCTCCAGCTCAGAGTTTGAT
GGCTAA

Detail of Deqor-guided selection of human GPR119 targetable region

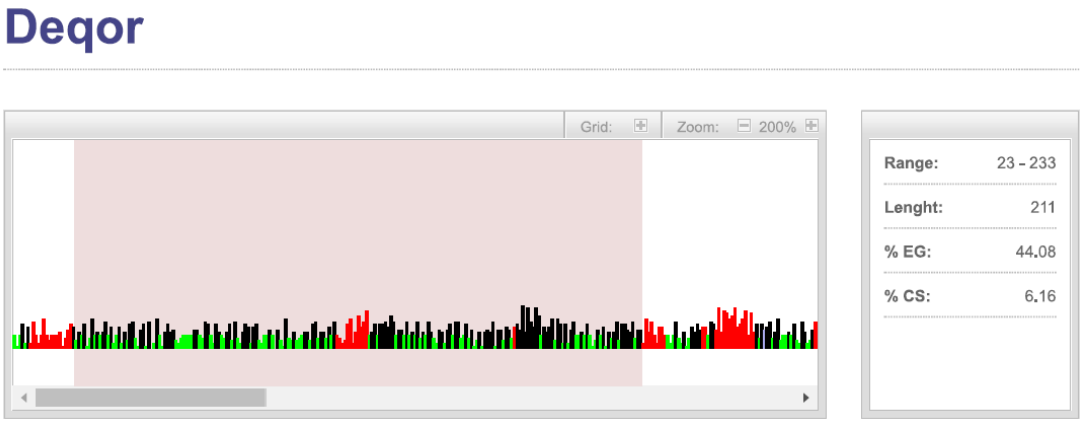



Murine GPR119 CCDS targetable region chrX:48674020-48674226 underlined, with the primers highlighted in bold:

ATGGAGTCATCCTTCTCATTTGGAGTGATCCT**TGCTGTCCTAACCATCCT**
**C**ATCATTGCTGTTAATGCACTGGTAGTTGTGGCTATGCTGCTATCAATCTA
CAAGAATGATGGTGTTGGCCTTTGCTTCACCTTGAATCTGGCCGTGGCTG
ATACCTTGATTGGCGTGGCTATTTCTGGTCTAGTTACAGACCAGCTCTCCA
GCTCTGCTCAGCATAC**ACAGAAGACCTTGTGTAGCCT**TCGGATGGCATT
TGTCACTTCTTCTGCAGCTGCCTCTGTCCTCACCGTCATGCTGATTGCCTT
TGACAGATACCTTGCCATTAAGCAGCCCCTCCGTTACTTCCAGATCATGA
ATGGGCTTGTGGCTGGAGCATGCATTGCAGGACTGTGGTTGGTATCTTAC
CTTATCGGCTTCCTCCCACTCGGAGTCTCCATATTCCAGCAGACCACCTAC
CATGGACCCTGCAGCTTCTTTGCTGTGTTTCACCCAAGGTTTGTGCTGACC
CTCTCCTGTGCTGGCTTCTTCCCAGCTGTGCTCCTCTTTGTCTTCTTCTACT
GTGACATGCTCAAGATTGCCTCTGTGCACAGCCAGCAGATCCGGAAGAT
GGAACATGCAGGAGCCATGGCCGGAGCTTATCGGCCCCCACGGTCTGTC
AATGACTTCAAGGCTGTTCGTACTATAGCTGTTCTTATTGGGAGCTTCACT

CTGTCCTGGTCTCCCTTTCTCATAACTAGCATTGTGCAGGTGGCCTGCCAC
AAATGCTGCCTTTACCAAGTGCTGGAAAAGTACCTGTGGCTCCTTGGAGT
TGGCAACTCCCTACTCAACCCACTCATCTATGCCTATTGGCAGAGGGAGG
TTCGGCAGCAGCTCTACCACATGGCCCTGGGAGTGAAAAAGTTCTTCACT
TCAATCCTCCTCCTTCTCCCAGCCAGGAATCGTGGTCCAGAGAGGACCAG
AGAAAGCGCCTATCACATCGTCACTATCAGCCATCCGGAGCTCGATGGCT
AA

Detail of Deqor-guided selection of murine GPR119 targetable region



Whole-cell RNA was extracted from GLUTag, NCI-H716 and STC-1 cells while growing in log-phase, with an ISOLATE II RNA Mini Kit (Cat # BIO-52072, Bioline). RNA was quantified by absorption at 260 nm, and 1 µg was then reverse transcribed into DNA with oligo dT primers to enrich the preparations with the cellular transcriptomes. Human and murine GPR119 target sequences were then amplified in separate reactions, with a MyTaq$^{TM}$ HS Red Mix kit (Cat # BIO-25047, Bioline).
The PCR reaction was assembled in 25 µl following manufacturer's instructions, and 2 µl of cDNA template were amplified with the following protocol:

| | | |
|---|---|---|
| 94°C | 3' | |
| 94°C | 30" | x 6 cycles |
| 60°C | 30" | |
| 72°C | 30" | |
| 94°C | 30" | x 6 cycles |
| 62°C | 30" | |
| 72°C | 30" | |
| 94°C | 30" | x 22 cycles |
| 65°C | 30" | |
| 72°C | 30" | |
| 72°C | 5' | |
| 12°C | ∞ | |

2 µl of this first PCR reaction were used as template for a second polymerization carried out with communal primers appended with an extended T7 promoter
Forward GCTAATACGACTCACTATAGGGAGAG
Reverse GCTAATACGACTCACTATAGGGAGAC

| | | |
|---|---|---|
| 94°C | 3’ | |
| 94°C | 30” | x 42 cycles |
| 60°C | 30” | |
| 72°C | 30” | |
| 72°C | 5’ | |
| 12°C | ∞ | |

4 µl of both PCR reactions were resolved on an agarose gel additioned with GelGreen® (Biotium) and imaged under UV light to validate correct template amplification.

An *in vitro* transcription reaction was assembled as follows:

| | |
|---|---|
| 10x reaction buffer | 1 µl |
| 2nd PCR Template | 4.5 µl |
| 25 mM NTPs | 3 µl |
| RNaseOUT™ | 0.5 µl |
| Pyrophosphatase (PPase) | 0.25 µl (0.025 U) |
| T7 Polymerase | 0.85 µl |

Pyrophosphatase (Cat #EF0221) was purchased from ThermoFisher Scientific, RNaseOUT™ (Cat #10777019) from Invitrogen ThermoFisher Scientific, T7 RNA Polymerase and supplied 10x reaction buffer (Cat # 10881775001) from Roche.
Reverse transcription was achieved by incubation at 37°C for 12 hours, and annealing of the final target dsRNA by melting at 90°C and slow cooling by 0.1°C per second down to 25°C. After 3 minutes the temperature was then lowered at 20°C until digestion of 10 µl with 1 µl (1U/µl) of Ambion™ RNaseIII Short cut enzyme (EC. 3.1.2.4; cat #AN2290, ThermoFisher Scientific) for up to 30 minutes at 37°C in a final volume of 100 µl in digestion buffer (140 mM NaCl, 2.7 mM KCl, 20 mM Tris, 0.5 mM EDTA, 5 mM $MgCl_2$, 5% Glycerol, 1 mM DTT diluted in ultrapure water).

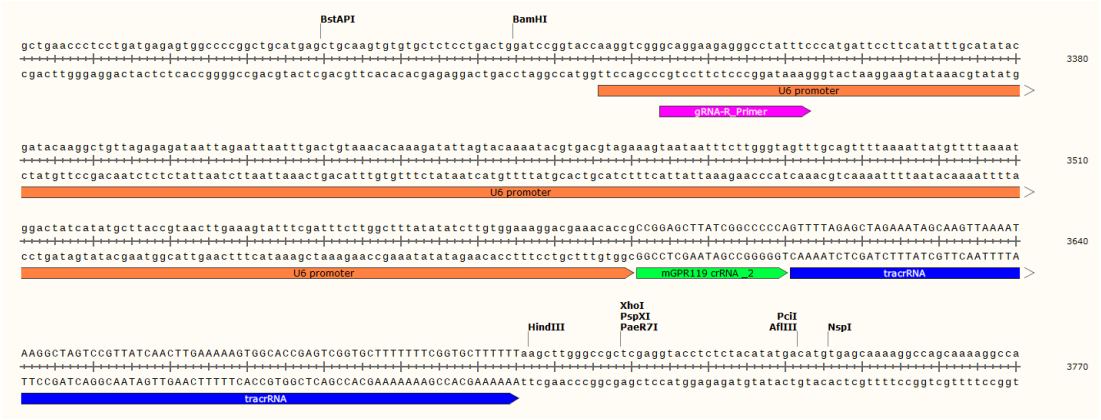
3 µl of the digestion reaction were separated in a 4% agarose gel to assess correct dicing into 18-22 nt long dsRNA fragments, which were then purified with Q Sepharose® Fast Flow (Cat #17-0510-01, GE Healthcare) in Micro Bio-Spin™ Chromatography columns (Bio-Rad Laboratories).

### 3.2.3.3 CRISPR-Cas9 Knockout of GPR119

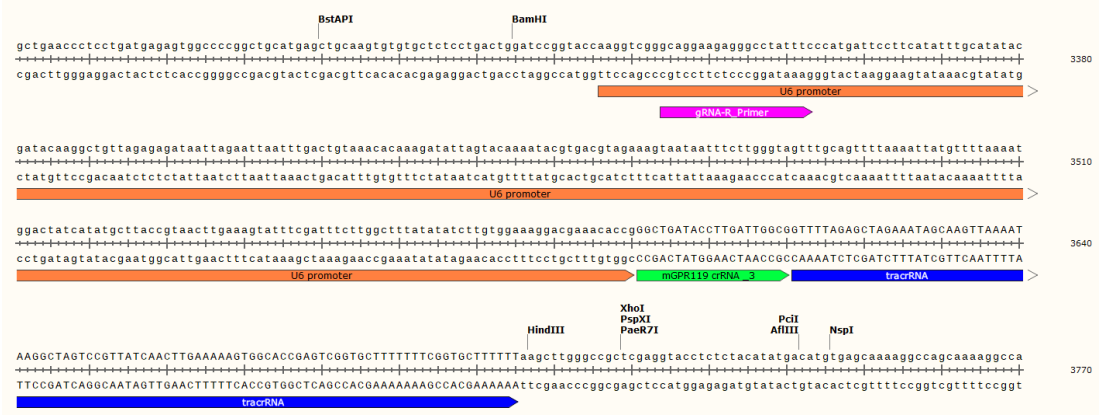
Two guide RNA sequences targeting the murine GPR119 gene, namely

GPR119 CRISPR Guide crRNA 2: CCGGAGCTTATCGGCCCCCA and

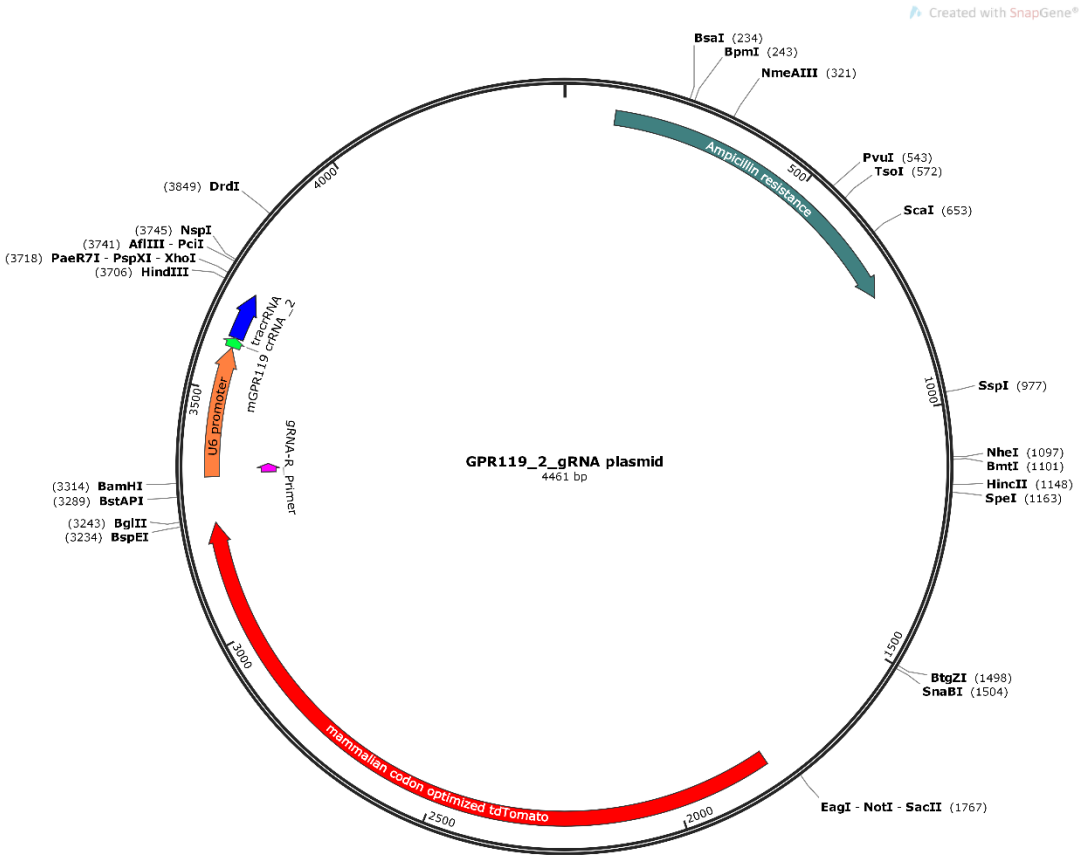
GPR119 CRISPR Guide crRNA 3: GGCTGATACCTTGATTGGCG were obtained from GeneScript ® CRISPR sgRNA Database. These sequences were appended in frame with a tracrRNA, and purchased from Integrated DNA Technologies Inc., and then cloned within the BamHI (Cat #R3136, NEB) and HindIII (Cat #R31204, NEB) restriction sites of a dTomato expressing vector, following manufacturers indications.




Detail of murine GPR119 targeting crRNA-tracrRNA (sgRNA) sequence 2

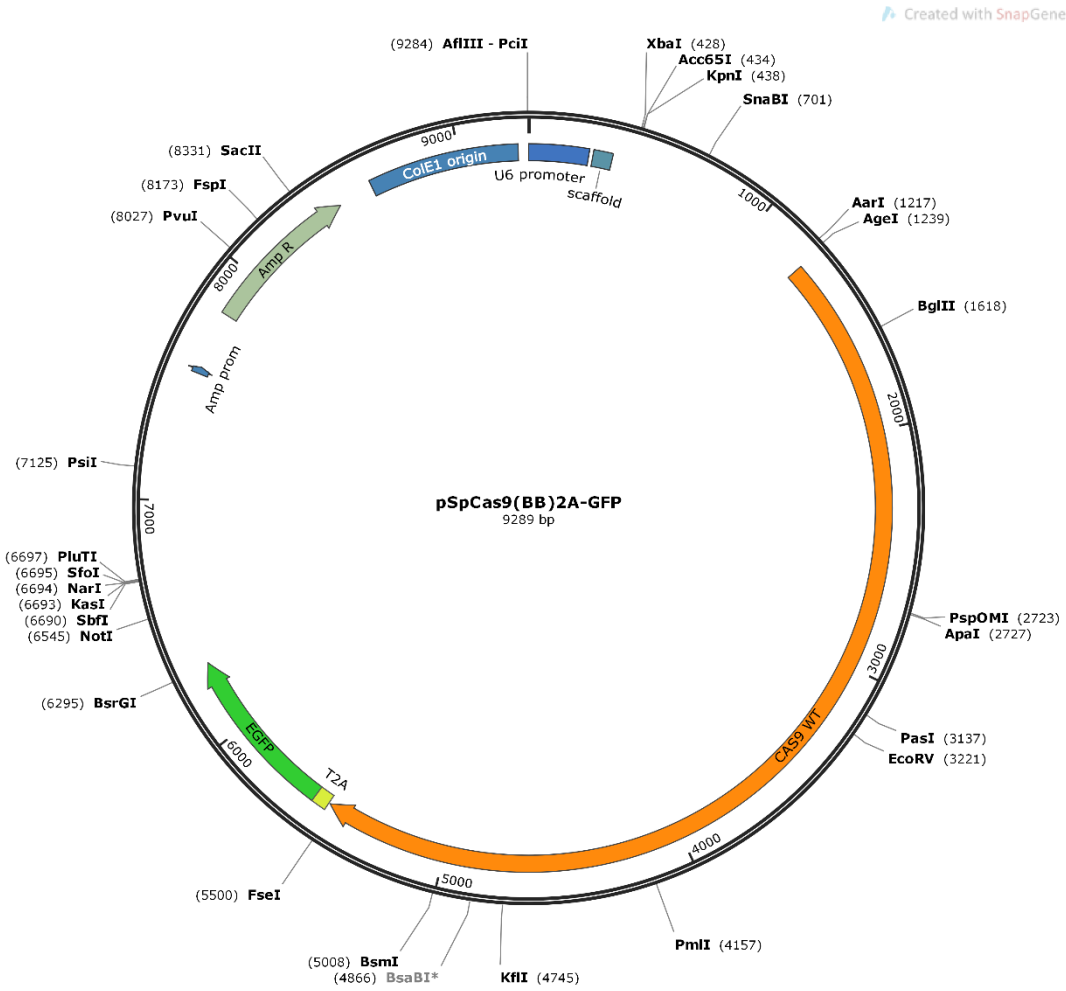



Detail of murine GPR119 targeting crRNA-tracrRNA (sgRNA) sequence 3




Representative map of gRNA-expressing vector used for the individual expression of either crRNA2 or crRNA3. Correct cloning of all plasmids was validated via sequencing [Australian Genome Research Facility].

To generate a GPR119 deficient cellular model, 400,000 STC-1 cells were seeded per well of a 6-well plate one day before co-transfection with 700 ng/well of pSpCas9(BB)2A-GFP, and 700 ng/well of each gRNA expressing vector with Lipofectamine® 3,000 Reagent (ThermoFisher Scientific) according to manufacturer's recommendations. In two independent experiments, one or two days after transfection, cells from two duplicate wells were detached with trypsin, pooled and processed to isolate respectively ca. 3,000 or ca. 6,000 GFP expressing cells via Fluorescence-activated cell sorting (FACS). A pool of $GFP^-$ cells was also collected to obtain negative controls not expressing Cas9. Cells were collected into a well of a 96 well plate to recover. After 3 days, cells were detached and seeded into three 96-well plates for the isolation and expansion of individual clones. After few weeks, wells containing single growing colonies were passaged and expanded to collect whole-cell protein lysates which were then probed for GPR119 protein expression by Western Blotting.




Streptococcus pyogenes SpCas9 expressing vector.

### 3.2.4 Immunoblotting

Whole cell lysates were collected by scraping 60 to 80% confluent cells in RIPA buffer (Tris-HCl 25 mM, NaCl 75 mM, NP-40 0.5%, Sodium Deoxycholate 0.025%, SDS 0.05%, EDTA 2.5mM) added with a broad-spectrum Protease inhibitor cocktail (Cat#P8340, Sigma Aldrich) and a broad Phosphatase Inhibitor cocktail (Cat#P5726, Sigma Aldrich) when studying phosphorylated targets. Membranes were disrupted, and protein solubilization was incentivized by sonication at 4°C. Insoluble pellets were spun down at 20,000g for 15 minutes at 4°C, and total protein content was quantified from the cleared supernatants with a Micro BCA™ Protein Assay kit [ThermoFisher Scientific] according to the manufacturer instructions. An equal amount of protein was then diluted with Laemmli Buffer added with a final concentration of 2.5% β-Mercaptoethanol before freezing at -20°C until further analysis.

Equal amounts of protein lysates were loaded and separated by electrophoresis onto a discontinuous HCl-Tris-Glycine based 6% Stacking and 10% Running polyacrylamide gel. Separated proteins were then transferred by semi-dry blotting onto 0.45 µm nitrocellulose membranes [Biorad] in a pre-chilled buffer of 20% Methanol, 1x Trans-Blot® Turbo™ [BioRad] diluted in ultrapure water [electrical resistance superior to 18.8 megaΩ]. To assess effective and homogeneous transfer of all proteins, detergents were firstly rinsed off the membranes with ultrapure water, then incubated with Red Ponceau solution [Sigma Aldrich] for 10 minutes at room temperature while maintaining agitation. Non-specific Red Ponceau staining was then washed off with ultrapure water until background signal was negligible.

Membranes were then cut into individual strips according to the molecular weight of the target protein and blocked for 1 hours at room temperature with either BSA 3% or fat-free Milk 3% dissolved in TBS-Tween20 0.05% at pH 7.6 (TBS-T).

Blocking buffer was then washed off twice with TBS-T before incubating the membranes with antibodies solution overnight (16-20 Hours) at 4°C.

α-Actinin was probed with the monoclonal Rabbit-hosted antibody D6F6 XP® cat #6487 (Cell Signaling Technology ®) dissolved 1/1,000 in BSA 3% in TBS-T additioned with Sodium Azide ($NaN_3$) 0.01% (w/v).

All the following GPR119-tropic antibodies were dissolved in Milk 3% TBS-T at the specified dilutions. Five different immunoglobulins were tested: the Rabbit polyclonal

1:1,000 Lot #GR540010-19 and lot #GR3245318-8 cat #ab75312 (Abcam); Goat polyclonal 1:1,000 lot #P1E270217 cat #NB100-94884 (Novus Biologicals); Rabbit polyclonal 1:1,000 lot #16L1 cat #NBP2-47661 (Novus Biologicals); Rabbit polyclonal 1:1,000 cat #NLS548 (Novus Biologicals); Goat polyclonal 1:500 lot #D1013 cat #sc-48195 (Santa Cruz Biotechnology, Inc).

Phosphorylated proteins, and respective total levels were studied with the following antibodies purchased from Cell Signaling Technologies® diluted 1:1,000 in BSA 3% TBS-T with $NaN_3$.

Phosphorylated Threonine 202 and Tyrosine 204 p42/44 MAPK (pERK1/2), monoclonal rabbit antibody D13.14.4E XP® cat #4370; total p42/44 MAPK (tERK1/2), monoclonal mouse L34F12 cat #4696; phospho Serine 133 CREB, monoclonal rabbit (87G3) cat #9198; total CREB monoclonal mouse (86B10) cat #9104.

Unbound primary antibodies were washed off 3 times with TBS-T, and incubated with the host-specific secondary antibodies, namely: anti Rabbit-HRP cat #7074 (Cell signalling Technologies ®), diluted 1:40,000 in BSA 3% TBS-T; anti-Goat-HRP cat #A5420 (Sigma Aldrich), diluted 1:20,000 in Milk 3% TBS-T; anti-Mouse-HRP cat #7076 (Cell signalling Technologies ®), diluted 1:10,000 in Milk 3% TBS-T. After 1 hour of incubation at room temperature with secondary antibodies, membranes were again washed thrice with TBS-T, and twice with TBS. HRP-mediated Electrochemiluminescence (ECL) was then achieved by soaking membranes with Amersham™ ECL™ Prime Western Blotting Reagent according to the manufacturer instructions, and signal was acquired with ChemiDoc™ XRS+ (Biorad). Densitometric analysis of bands was carried out with Image Lab™ v 6.0.1 build 34 software from non-saturated unprocessed images. After imaging phospho-proteins, the membranes' immunocomplexes were stripped at 50°C for 10 minutes in stripping buffer (Tris-HCl 62.5 mM pH 6.8, SDS 2%, β-Mercaptoethanol 100 mM), blocked again for 30 minutes in BSA TBS-T, and re-probed overnight at 4°C with murine-raised antibodies for detection of total levels of either ERK1/2 or CREB, to minimize any non-specific signal from residual rabbit-raised anti-phospho antibodies.

### 3.2.5 Immunofluorescence

To study the expression of GPR119, NCI-H716 cells were seeded on glass coverslips coated with a thin layer of Cultrex® Reduced Growth Factor Basement Membrane extract, PathClear® (Cat #3433-005-01, Trevigen®). Cells were transfected with siRNAs oligonucleotide sequences 1, 2, 3 and 4 (QIAGEN) diluted at a final concentration of 300 nM. After 48, or 72 hours media was discarded, cells were washed 3 times with HBSS, and fixed for 10 minutes at room temperature with formaldehyde 4% in PBS. Cells were then washed again 3 times for 5 minutes, and permeabilized with a solution of Triton X-100 0.1% in HBSS for 3 minutes at room temperature. After a double wash with HBSS for 5 minutes, cells were blocked with a solution of 1% BSA, 300 mM Glycine, 10% Goat serum (Cat #16210-064, ThermoFisher Scientific) dissolved in HBSS and incubated at 37°C for 1 hour. Cells were then incubated overnight at 4°C with the primary antibody anti-GPR119 (Cat #NLS-548, Novus Biologicals) diluted 1/75 in HBSS additioned with 1% BSA, 3% Goat serum, Triton X-100 0.02%. The next day unbound antibody was washed off 3 times for 5 minutes with HBSS, followed by incubation with a secondary anti-Rabbit-$F(ab')_2$ Alexa Fluor® 488 Conjugate (Cat #4412, Cell Signaling Technologies) diluted 1/750 in HBSS with 1% BSA, 3% Goat serum, for 1 hour at room temperature. The coverslips were then washed again 3 times for 5 minutes with HBSS, and counter stained with Hoechst33258 for 3 minutes followed by a final rinse in ultrapure water before mounting on a glass slide with a drop of ProLong® Gold Antifade (Cat #9071, Cell signalling Technology®). Images were acquired on a Confocal microscope Nikon A1+.

### 3.2.6 Secretion assay studies

#### 3.2.6.1 In vitro secretion study

For the GLP-1, and PYY secretion assay studies, 50,000 NCI-H716 cells, or 70,000 of either GLUTag or STC-1 cells were seeded in duplicate per each well of a 24-well plate pre-coated with a thin layer of 0.15 mg/ml Matrigel® Matrix (Cat #354234, Corning) in complete media, and incubated at 37°C in humidified 5% $CO_2$ atmosphere for 3 days before the secretion experiment. After the incubation period, media was

withdrawn, and cells washed twice with 0.5 ml of pre-warmed Hank's Balanced Salt Solution (HBSS). Cells were then treated for 2 hours at 37°C in a humidified 5% $CO_2$ atmosphere with different drugs dissolved in 250 µl of either RPMI serum free media (NCI-H716) DMEM low glucose (1g/L, GLUTag) or DMEM high glucose (4.5 g/L, STC-1). All chemicals were dissolved in DMSO as stock solutions of 10 mM and stored in nitrogen atmosphere at -20°C. Oleoyl-LPI (Cat # 850100P) was purchased from Avanti Polar Lipids Inc. Oleoylethanolamide (Cat # O0383), Phorbolmyristateacetate (PMA, cat # P1585), Forskolin (Cat # F3917) and Isobutyl-methylxanthine (IBMX, cat #I5879) from Sigma Aldrich. Supernatants were collected, cellular debris spun down at 1,000g for 5 minutes, and then stored at -20°C before quantification of total secreted GLP-1 with a commercial ELISA kit (EZGLP1T-36K, Merck, Millipore) specific for both GLP-1 (7-36)amide and GLP-1(9-36)amide species. Data are expressed as percentage of secretion against vehicle treated cells. Statistical significance was calculated using the GraphPad Prism 6.01 software (La Jolla California, USA).

#### 3.2.6.2 Ex-vivo secretion study

Primary cultures of colon-derived L-cells were prepared as described by Reimann and colleagues (Psichas, Tolhurst, Brighton, Gribble, & Reimann, 2017). Colon and rectum were quickly harvested from sacrificed mice, or guinea pigs, and intraluminal content was washed out with ice-cold L-15 medium. The tissue was then minced into 3 to 5-millimetre pieces and washed twice in 50 ml of ice cold L-15 medium. Supernatant was carefully removed, and the cleaned intestinal fragments were then sequentially incubated up to 6 times for 10 minutes at 37°C into fresh 7 ml solution of 0.3 mg/ml Collagenase-P (Lot #10674927, Cat #11213857001, Roche) in serum free DMEM medium. Each digestion batch was inspected under an inverted microscope to assess the presence of colonic crypts, which were then pooled together and resuspended in fresh warm complete DMEM medium with the ROCK-1, and anoikis inhibitor Y-27632 at 10 µM (Cat #S1049, Selleckchem). Crypts were further dissociated into individual cells by pipetting and sieved through a 100 µM nylon strainer before being plated onto a 24-well plate coated with a 0.15 mg/ml thin layer of Matrigel® Matrix (Lot #7002568, and Lot #7051011, Cat #354234, Corning). After one day of incubation at 37°C 5% $CO_2$ in a humidified incubator, cells were washed

with 500 µl of warm DMEM, and treated with different drugs dissolved in 250 µl of serum free DMEM. Oleoyl-LPI and mimetics were all tested in quadruplicate at 20 µM, DMSO at 0.2% was used as the equivalent vehicle control. After a 2-hour long incubation at 37°C 5% $CO_2$, the supernatants were collected on ice, and quickly stored at -20°C until total GLP-1 levels (7-36 amide and 9-36 amide) were quantified with an ELISA commercial kit (EZGLP1T-36K Merck, Millipore).

### 3.2.7 In vivo studies

#### 3.2.7.1 In vivo Oral Glucose Tolerance Test (OGTT)

Healthy 9-week-old C57bl/6J mice, were acclimatised to the environment for 1 week before proceeding. The day of the experiment, the animals were moved into a new cage with clean beddings without access to food for 6 to 8 hours and were then given an oral dose of 20 mg/kg of drugs dissolved in PEG400 80%, Tween80 10%, Ethanol 10% (PET) as described in (Panaro et al., 2017). The GPR119 agonist AR231453 (Cat #BML-GP-100-0025, Enzo Life Sciences) was used as a known GLP-1 secretagogue control. After 30 minutes a second bolus of 2 g/kg of glucose was administered by oral gavage. Blood was drawn from the tail artery by nicking with a sterile scalpel, at time 0' just prior to the glucose administration, then after 7, 15, 30, and 60 minutes, and glycaemia measured with a hand-held Accu-Chek Guide® glucometer at each time point. The same animals were left to recover for a period of at least 3 weeks before reutilizing them for an acute hormone secretion study.

#### 3.2.7.2 In vivo acute hormone secretion study

Animals were purchased from the Animal Resource Centre (ARC, Perth, WA), and housed in ventilated cages with free access to food and water, under a 12 hours light/dark cycle. Healthy 9-week old C57bl/6J mice or diabetic LeprDB+/+ (db/db) on the same strain background, were accustomed to the environment for 1 week before proceeding. As a model of diabetes, we used the db/db mouse as an animal faithfully replicating the human pathology. This is a widely utilized animal model, genetically deficient for the leptin receptor, that presents hyperphagia-driven obesity and type 2 diabetes with hyperglycaemia and hyperinsulinemia at 10 weeks of age (Bogdanov et al., 2014; Chen et al., 2007; Guilbaud et al., 2019; Hinder et al., 2018; K. Kobayashi

et al., 2000; Noratto, Murphy, & Chew, 2019; Panagia, Schneider, Brown, Cole, & Clarke, 2007; Sullivan et al., 2004; Wua, Wang, Li, & Men, 2013). The day of the experiment, animals were fasted for 6 to 8 hours, then gavaged with the respective drug dissolved in PET. After 30 minutes, each animal was gavaged a second time with 2g/kg of glucose, after further 5 minutes blood sample was drawn from the submandibular vein with a 5 mm long sterile lancet as previously described (Golde, Gollobin, & Rodriguez, 2005). A sample of 100 µl to 300 µl of total blood was drawn into a pre-chilled tube pre-coated with EDTA 0.5 M pH = 8.0 containing 5 µl of DMSO-free Protease inhibitor cocktail 100x (Cat # S8830, Sigma Aldrich). Plasma was separated by centrifugation at 1,500g for 15 minutes at 4°C, and aliquots were quickly frozen on dry ice and stored at -20°C until the day of analysis. Total GLP-1 content was quantified with the commercial Millipore ELISA kit EZGLP1T-36K, total PYY with the Crystal Chem ELISA kit #81501, and Insulin with the Mercodia Ultrasensitive Mouse Insulin ELISA #10-1249-01.

## 3.3 Results and discussion

### 3.3.1 Structure Activity Relationship study of Oleoyl-LPI mimetics in vitro

As described in the manuscript presented in the previous chapter § 2, Oleoyl-LPI is a potent GLP-1 secreting food-derived molecule exerting its pharmacology via the activation of GPR119. Nonetheless the direct therapeutic use of Oleoyl-LPI in humans is hampered by its financially prohibitive use at scale, and its non-quantitative delivery to distal GLP-1 secreting cells. To overcome both these issues, in collaboration with A/ Prof. Max Massi, and Dr. Peter, Simpson (Curtin University), we developed a library of 20, easy to synthesize, stable Oleoyl-LPI related mimics with a PLA resistant amidic bond in place of the ester connecting the lipidic chain to the hydrophilic head. The chemical synthesis of the myo-inositol pharmacophore of Oleoyl-LPI, is not a simple, nor economically sustainable process, therefore we generated a library of easy to synthesize compounds primarily iterating on the nature of their hydrophilic head. Of these drugs, ps317b, and ps320c, due to their saturated Stearic chain could not be dissolved in DMSO as 5 mM stock solutions despite heavy sonication and heating, they were therefore excluded from further analysis foreseeing major problems of solubility in future studies. To evaluate if the remaining 18 compounds maintained

their GLP-1 secreting capabilities *in vitro*, the human colorectal adenocarcinoma-derived cell line NCI-H716, the murine colorectal GLUTag, as well as the duodenal STC-1 cell lines were used as cellular models of intestinal L-cells. As shown in figure 1, in a first explorative experiment with the human NCI-H716 cells, Oleoyl-LPI still appears to be the best GLP-1 secreting agent, however we obtained proof of concept evidence that the synthetic tetrazole-based heads of compounds ps297b, ps309a, ps312a, 316a, ps318a, ps321a and ps323b can functionally mimic the myo-inositol structure, which indeed is a known pharmacophore described previously for other synthetic GPR119 agonists (Ritter, Buning, Halland, Pöverlein, & Schwink, 2016).

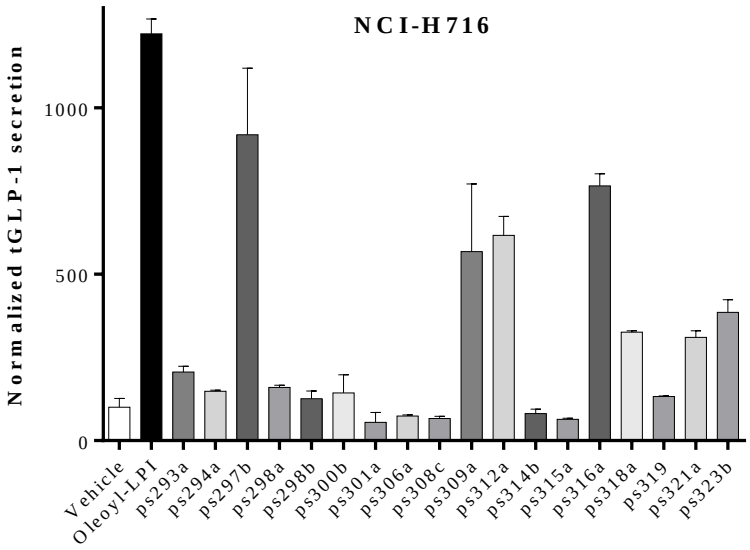



**Figure 1:** Panel of 20 µM Oleoyl-LPI and derivatives-mediated total GLP-1 secretion in NCI-H716 cells. Averages ± Standard Deviation (SD) of a single experiment are shown.

Compounds ps297b and ps316a, sharing an identical hydrophilic head, were also studied in a dose-response study in NCI-H716 (Figure 2) to dissect possible pharmacological differences in terms of potency and efficacy of either Oleoyl-bearing (ps297b) or linoleoyl-bearing (ps316a) mimetics. All tested compounds induced a powerful dose-dependent GLP-1 secretion with similar potency (EC50s ≈ 30 µM), but ps297b, ps316a and Oleoyl-LPI had much stronger efficacy than the lipid OEA.

As shown in Figure 2, both of our synthetic drugs induce an efficient secretion of GLP-1. However, the Linoleoyl chain of ps316a appears to confer an equal or better bioactivity when compared to either Oleoyl-LPI or ps297b, possibly to be rationalized

by the conferral of different pharmacology. Nonetheless, upon further analysis via $^{1}$H-NMR by Dr. Peter Simpson, the Linoleoyl- chain appeared extremely unstable, and was shown to rearrange even in dark conditions when stored in solid form, the study of ps316a, as well as of the other Linoleoyl-based mimetics ps319 and 323b was therefore terminated.

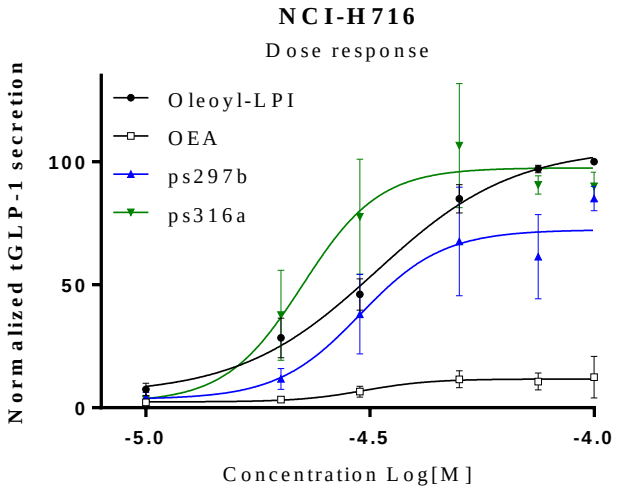



**Figure 2:** tGLP-1 secretion in NCI-H716 treated with different drugs concentrations ranging from 10, to 100 µM. Data are presented as averages of 2 to 4 independent experiments ± standard error of the mean (SEM) normalized on Oleoyl-LPI activity at 100 µM.

Given this extraordinary activity of some of the Oleoyl-based mimetic drugs, we sought to validate their Structure activity relationship (SAR) in two other murine cell line models of L-cells, GLUTag and STC-1. With further experiments also in the human colon-like cell model NCI-H716, we confirmed our previous data, showing robust and statistically significant GLP-1 secretion across all cell lines, especially with compounds ps318a and its regio-isomer ps297b, which differ only by the orientation of their hydrophilic tetrazole pharmacophore [Figure 3].

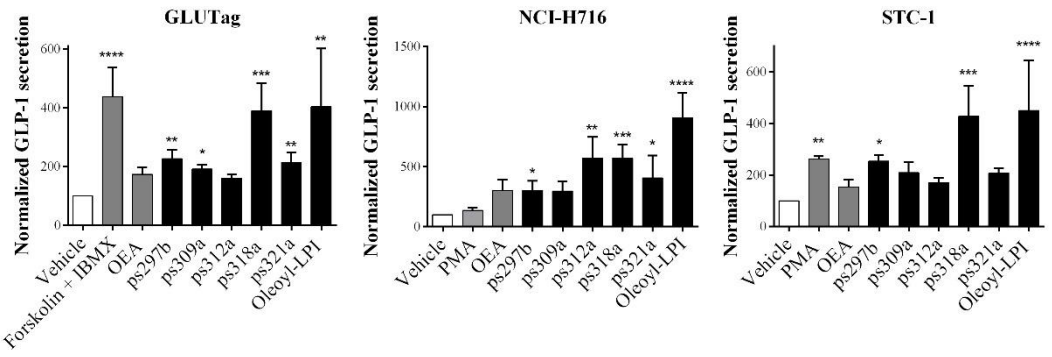



**Figure 3**: Structural activity relationship study of Oleoyl-based compounds screened at 20 µM in GLUTag (A), NCI-H716 (B), and STC-1 (C) cells. Oleoyl-LPI 20 µM, OEA 20 µM, PMA 3 µM and Forskolin 10 µM with IBMX 10 µM were used as positive controls of GLP-1 secretion. Vehicle-normalized secreted total GLP-1 levels are shown with error bars indicating the SEM of at least 3 independent experiments. Statistical significance was calculated with One-way ANOVA followed by Kruskal-Wallis and Dunn's multiple comparisons tests, and is presented as follows, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001

### 3.3.2 Ex vivo GLP-1 secretion

Primary cell preparations derived from the intestines of sacrificed animals, are a recognized *ex-vivo* cellular model vastly used in literature for the preliminary study of GLP-1 secreting drugs. This technique was described more than a decade ago by Reimann and colleagues, (Reimann et al., 2008) and more recently detailed in a more technical manuscript aimed at the processing of mouse small intestines (Psichas et al., 2017), but applicable also to the colon. Indeed, in the manuscript I co-authored and presented in chapter §2 (Arifin et al., 2018) we used mouse derived colonic preparations to study the pharmacology, and demonstrate the robust GLP-1 secreting capabilities of Oleoyl-LPI beyond our *in vitro* model. Nonetheless despite these experiments were successfully carried out in a different laboratory by my co-first author Dr. Syamsul Ahmad Arifin, and notwithstanding the detailed protocols available in literature, we encountered technical difficulties, and could not robustly establish viable preparations necessary for the screening of multiple drugs. As shown in figure 4, a typical preparation of murine large intestines according to the protocol Psichas et al. (Psichas et al., 2017) could not consistently generate healthy cells one

day after seeding. Only a fraction of the cells, less than 10% attached to the plates displaying epithelial like morphology (Panel C), while most cells remained detached and were removed upon gentle washing (Most cells visible in panels A and B). However, the majority of these epithelioid cells also detached upon treatment in serum-free DMEM medium.

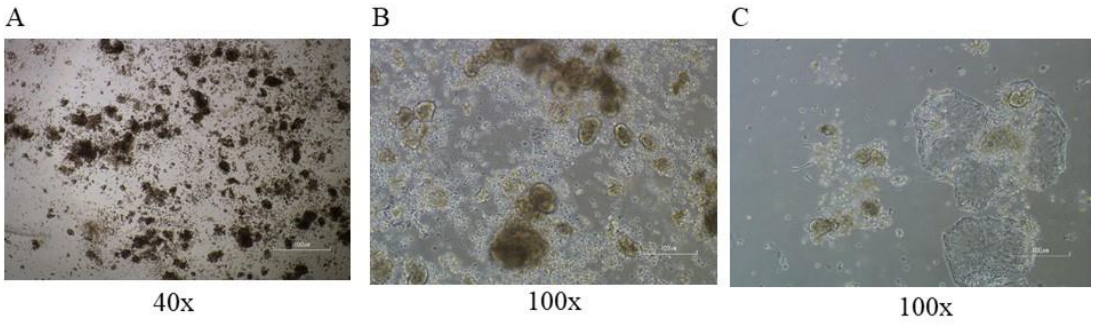



**Figure 4:** Typical morphology of primary cultures of murine large intestines one day after seeding, before (A, and B) and after (C) washing with DMEM prior to treatment with drugs. Images were acquired from different fields of view of the same well at the indicated magnification factor, with an inverted light-microscope.

In figure 5 are shown the normalized GLP-1 levels measured in the supernatants of the few viable and responsive primary cell preparations we obtained. In panel A of figure 5, two different mice colons, processed, and treated in parallel displayed encouraging, albeit non-consistent GLP-1 secreting properties for the Oleoyl-LPI mimetics ps297b and ps318a. Reimann and colleagues report that with the protocol they describe (Psichas et al., 2017), mouse-derived colonic preparations should yield a monolayer of adherent cells the day after seeding, nonetheless in tens of different attempted colonic-preparations, only isolated patches of cells could be obtained across the whole plate, with a confluency of less than 20%. Considering that only 1% of the cells of an intact intestinal mucosa are GLP-1 secreting cells, often the levels of total GLP-1 that we detected in supernatants were well below the sensitivity of 1.5 pM of the ELISA kit we utilized (EZGLP1T-36K, Merck Millipore). In order to maximize the number of cells in each preparation, given the conserved peptidic sequence of total GLP-1 across multiple species, and given also the availability, we studied the GLP-1 secreting pharmacology also in *ex vivo* colonic tissues of guinea pigs prepared following the same procedure (Figure 5 panel B).

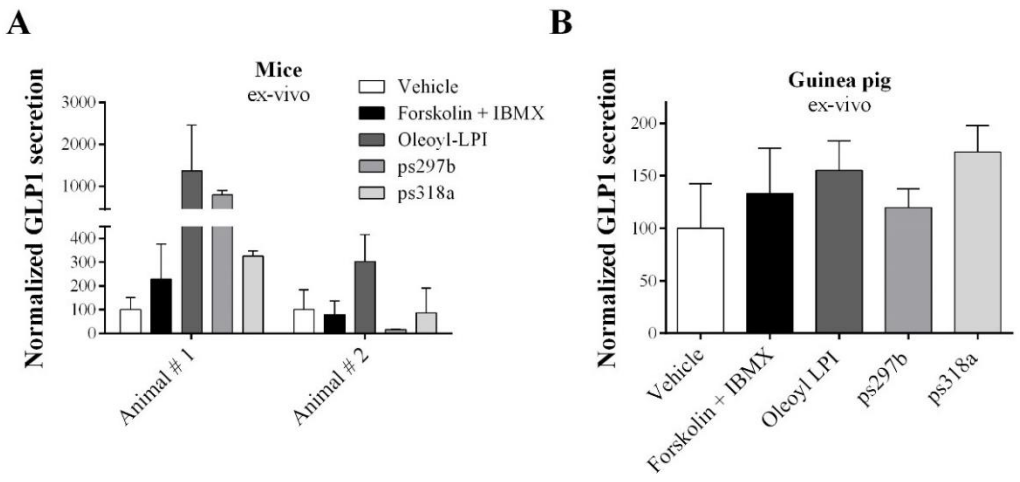



**Figure 5: Ex-vivo primary colon preparations of mice (A) and guinea pig (B) treated with different drugs at 20 µM indicate an increase in GLP-1 secretion**. Oleoyl-LPI 20 µM and Forskolin 10 µM with IBMX 10 µM were used as positive controls as known GLP-1 secretagogues. (A) Two different mice were sacrificed, tissues processed and treated in parallel. (B Guinea pig colonic preparation. All cells were treated for 2 hours with the indicated drugs. Each individual panel indicates averages of a single experiment performed in quadruplicate with error bars displaying standard deviations.

Nonetheless, we yet encountered similar problems and could not obtain a satisfactorily robust *ex vivo* platform where to statistically evaluate the pharmacological properties of our drugs. In particular, it is likely that these difficulties stem from the requirement of these sensitive cells to be plated on coatings of validated batches of Matrigel®, a known chemically non-defined heterogeneous preparation of extracellular matrix (ECM) proteins with important batch-to-batch variability well recognized across the field (Hughes, Postovit, & Lajoie, 2010). Anyhow, we did not see any noticeable difference with the use of two different batches of Matrigel, nor with the use of Growth Factor Reduced Cultrex® (Lot #39795F17, Cat# 3433-005-01), an equivalent solution of ECM-like proteome commercialized under a different name. These observations that we provide, offer a platform for future studies that will need to optimize the procedure to achieve sufficient robustness for our applications, by validating individual batched of reagents and media. For logistical and financial reasons, we decided to continue the study of our drugs directly *in vivo* as a more translationally relevant model.

### 3.3.3 Oleoyl-LPI mimetics activate ERK1/2 and CREB intracellular cascades

Having demonstrated that compounds ps297b and ps318a consistently induce the release of GLP-1 across 3 cell line models of L-cells, we wanted to dissect the intracellular pathways that these compounds might be stimulating. From our previous work, described in chapter §2, we know that Oleoyl-LPI stimulates the phosphorylation of ERK1/2 and cAMP mediated activation of CREB, we wanted then to study whether ps297b and ps318a share this pharmacology, or if they phenocopy more closely the response elicited by the Oleoyl-bearing OEA.

As shown in figure 6 both ps297b and ps318a consistently activated MAPK-ERK1/2, although to a lower degree than Oleoyl-LPI, while only ps318a showed a consistent activation of CREB, phenocopying more closely the pharmacology of Oleoyl-LPI. Other GPR119 agonists have also shown to activate ERK1/2 in murine colonic crypts (Peiris et al., 2018). On the other hand, OEA did not elicit neither signalling cascade, indicating a condition of biased signalling, where GPR119 agonism clearly elicits two different intracellular responses. This pharmacological behaviour where the same ligands stimulate different intracellular cascades, is known as biased signalling, and as described before (Hassing, Fares, et al., 2016). In this specific scenario, it can be rationalized by assuming that Oleoyl-LPI and its analogues, as well as other agonists of GPR119, in contrast to OEA, stabilize the same receptor in a conformation that exposes a set of intracellular residues sufficient to trigger different degrees of activation of downstream proteins, including different Gα, GRKs mediated Arrestin signalling, as well as Gβγ signalling that then mediate ERK1/2 activation.

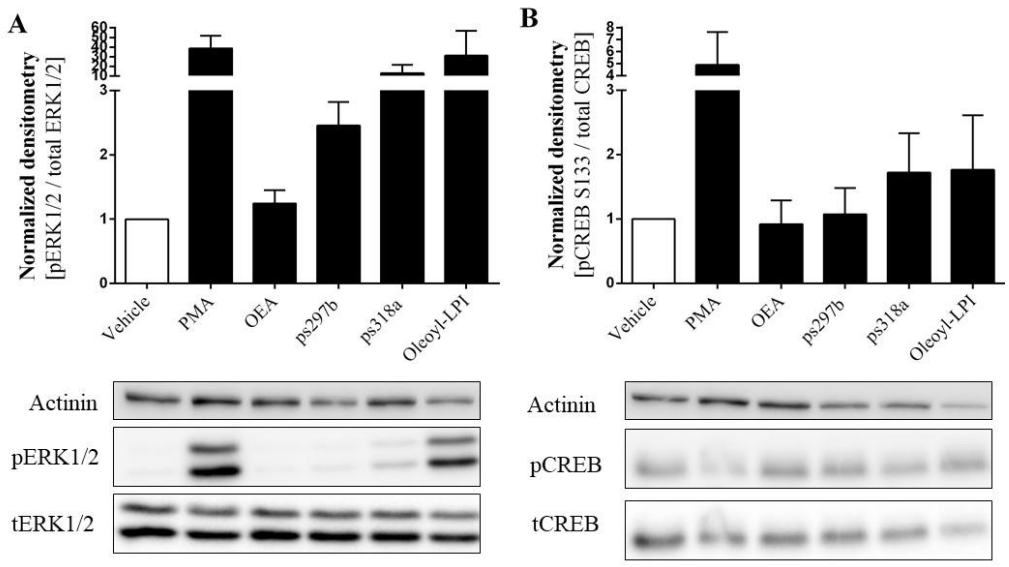



**Figure 6: ps318a activates ERK and CREB signalling cascade similarly to Oleoyl-LPI.** Lysates of STC-1 cells treated for 10 minutes with 20 µM of each drug, or PMA 3 µM, were immunoblotted for active phosphorylated and total ERK1/2 (pERK1/2, tERK1/2), as well as phospho and total CREB (pCREB, tCREB). The normalized densitometric analysis of 3 independent experiments is shows as averages ± SEM. A representative western blot is shown at the bottom.

### 3.3.4 Oleoyl-LPI mimetics induce the release of GLP-1 in vivo

Considering the powerful GLP-1 properties of Oleoyl-LPI *in vitro* (Figures 1, 2 and 3), and *ex vivo* from mice tissue as we published earlier (Arifin et al., 2018), as well as from human colonic biopsies (unpublished data), we sought to validate if this activity was maintained *in vivo* [Figure 7]. Healthy mice orally gavaged with 10 mg/kg, a therapeutically relevant dose for other synthetic GPR119 agonists (S. Patel et al., 2014), showed a minor trend, of not statistically significant increase in the acute glucose-stimulated levels of plasmatic total GLP-1 levels. However, we could study the dose-dependency of this pharmacology given the impractical high costs of the pure commercial lipid. On the other hand this represents an important proof of concept, especially in light of the chosen dose of 10 mg/kg, which is far lower than a reported 1,000 mg/kg for a structurally related GPR119 agonist 2-Oleoyl-Glycerol (2-OG) (Hassing, Engelstoft, et al., 2016), a dose at which it was yet uncapable to affect plasmatic GLP-1 levels.

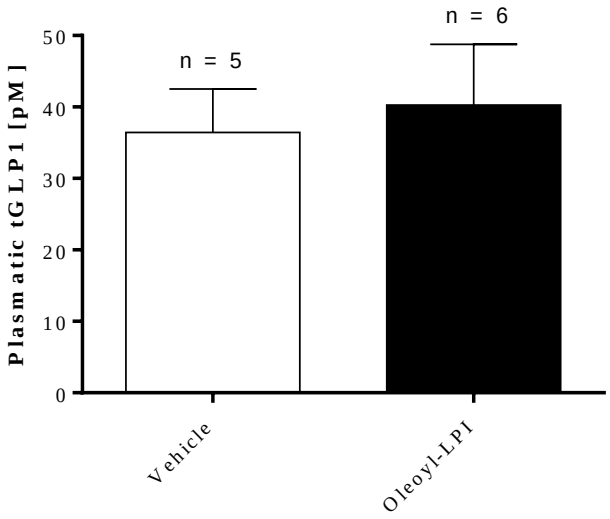



**Figure 7: Oleoyl-LPI administered orally does not affect significantly the peripheral levels of total GLP-1**. Plasmatic levels of glucose-stimulated total GLP-1 were quantified from healthy mice orally gavaged with 10 mg/kg of Oleoyl-LPI. Vehicle matched controls are shown. Average values are shown ± SEM of indicated number of animals per group. P-value calculated as a one-sided T test equal to 0.7156.

The limited effect size increase in peripheral GLP-1 levels elicited by pure Oleoyl-LPI could indeed be rationalized by its metabolization in the first part of the intestine, preventing it to quantitatively reach more distal GLP-1 secreting cells. We then set out to relate this activity to the more resilient analogues ps297b, ps318a and ps321a. These three compounds were chosen not only for their robust activity *in vitro*, but also to dissect possible structural-specific pharmacology dictated by the orientation of the tetrazole pharmacophore in their hydrophilic head (See section §3.2.1 for reference), as well as the possible pharmacokinetic benefits of the salt of ps297b, namely ps321a. Not being bound by financial constraints as for pure Oleoyl-LPI, two doses were given to healthy animals by oral gavage, 20 and 50 mg/kg to dissect possible dose-specific pharmacology. As shown in figure 8, even though the animals did not show any statistically significant increase in their peripheral levels of GLP-1, all of them displayed a clear, dose-independent trend with a small effect size in this acute setting.

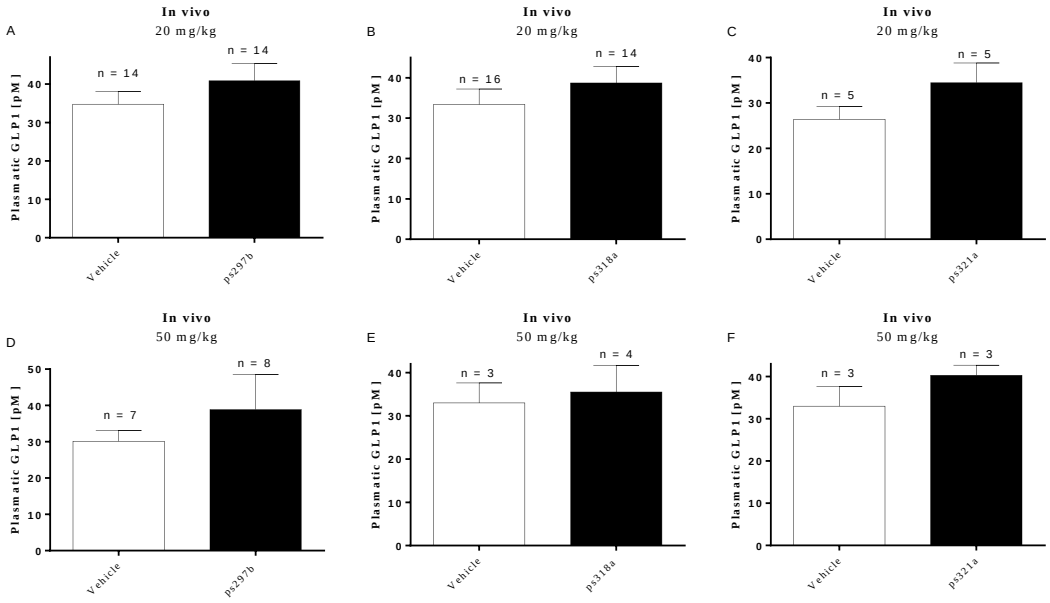



**Figure 8: Oleoyl-LPI derived mimetics do not significantly improve the acute glucose-stimulated secretion of GLP-1 in healthy mice**. Compounds ps297b (A, D), ps318a (B, E) and ps321a (C, F) were administered by oral gavage at either 20 mg/kg (A, B, C), or 50 mg/kg (D, E, F) to healthy fasted mice 30 minutes prior to a bolus of glucose. Plasmatic levels of total GLP-1 of vehicle-matched controls are displayed for each drug as averages ± SEM of the indicated number of animals per each cohort.

Another gut hormone, namely Peptide-YY (PYY), is often co-secreted with GLP-1 by intestinal L-cells, and many GLP-1 secreting agents, including GPR119 agonists, are known to induce the release of PYY too (as summarized in Table 1 of Review 1, chapter §1.1), even from more proximal small intestinal tracts (Billing et al., 2018; S. Patel et al., 2014), albeit GPR119 activity is known to be more sensitive in the distal intestine (Moss et al., 2016). Considering the possible presence of biased signalling of GPR119, we cannot yet exclude the differential modulation and excretion of different hormones. Surprisingly, despite being co-secreted with GLP-1 by the same endocrinal cells, PYY is known to be differentially modulated in response to bariatric surgeries (Guida et al., 2019), as well with the use of anti-diabetic DPP-4 inhibitors (Guida et al., 2018) proving that its secretion can be differentially modulated, although the signalling pathways behind this phenomenon have not yet been understood. Indeed a useful pharmacological property of PYY, similarly to GLP-1, is its capacity to elicit satiety in humans (Behary et al., 2019; Tan et al., 2017) and so its therapeutic secretion

would yet represent an important therapeutical potential for western type diet-driven diseases like obesity and type 2 diabetes. Consequently, we investigated whether Oleoyl-LPI and its structural lead analogues could modulate the plasmatic levels PYY. However, as shown in figure 9, the glucose-stimulated peripheral levels of PYY did not differ significantly upon treatment with either drug.

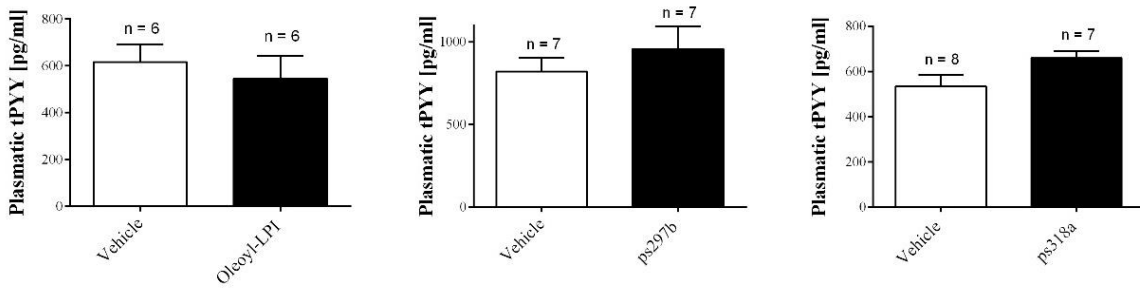



**Figure 9: Oleoyl-LPI, ps297b and ps318a do not significantly affect the plasmatic levels of total PYY in healthy mice.** Healthy animals were gavaged with either 10 mg/kg of Oleoyl-LPI, or 20 mg/kg of each mimetic. ps297b induces a promising increase in the plasmatic glucose-stimulated total PYY levels. Averages are shown ± SEM of the indicated number of animals in each group.

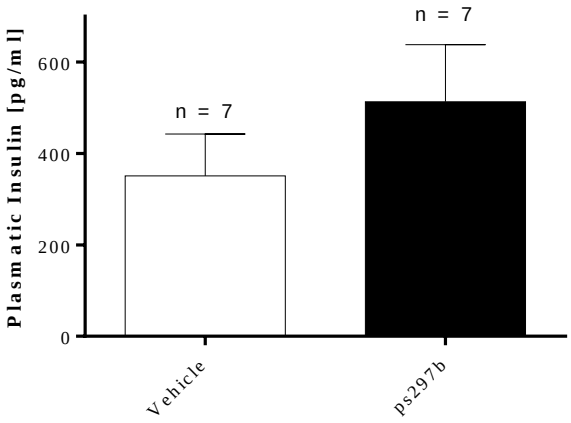



**Figure 10: Compound ps297b is an insulinotropic drug.** Healthy mice were gavaged with 20 mg/kg of ps297b. Plasmatic insulin levels were measured from blood samples drawn after an oral bolus of glucose 2 g/kg. Vehicle matched controls are shown with averaged ± SEM of the indicated number of animals per each group. P-value calculated as one-sided T-student with a post-hoc Wilcoxon matched pairs rank test is equal to 0.0781.

ps297b elicits a clear trend of increased peripheral levels of total GLP-1 and total PYY, to validate whether this is sufficient to increase the incretin effect, we also assessed the peripheral levels of insulin. As shown in figure 10, similarly to the incretin hormones, glucose-stimulated peripheral insulin levels showed only a minor and non-significant, yet consistent increase. We then evaluated the acute impact that this pharmacology might have on oral glucose tolerance.

To this end, different Oleoyl-LPI mimetics were administered at either 20 or 50 mg/kg 30 minutes before a bolus of glucose, and glycaemia was measured over a period of 1 hour as shown in figure 11. No compound, including the most promising ps297b, was capable to impact glucose management, indicating that the impact on peripheral hormones is not effective in this acute healthy mouse model. For comparison the synthetic GPR119 agonist AR231453 given at 20 mg/kg, does ameliorate the glucose management in this animal model, corroborating the significance of this animal model.

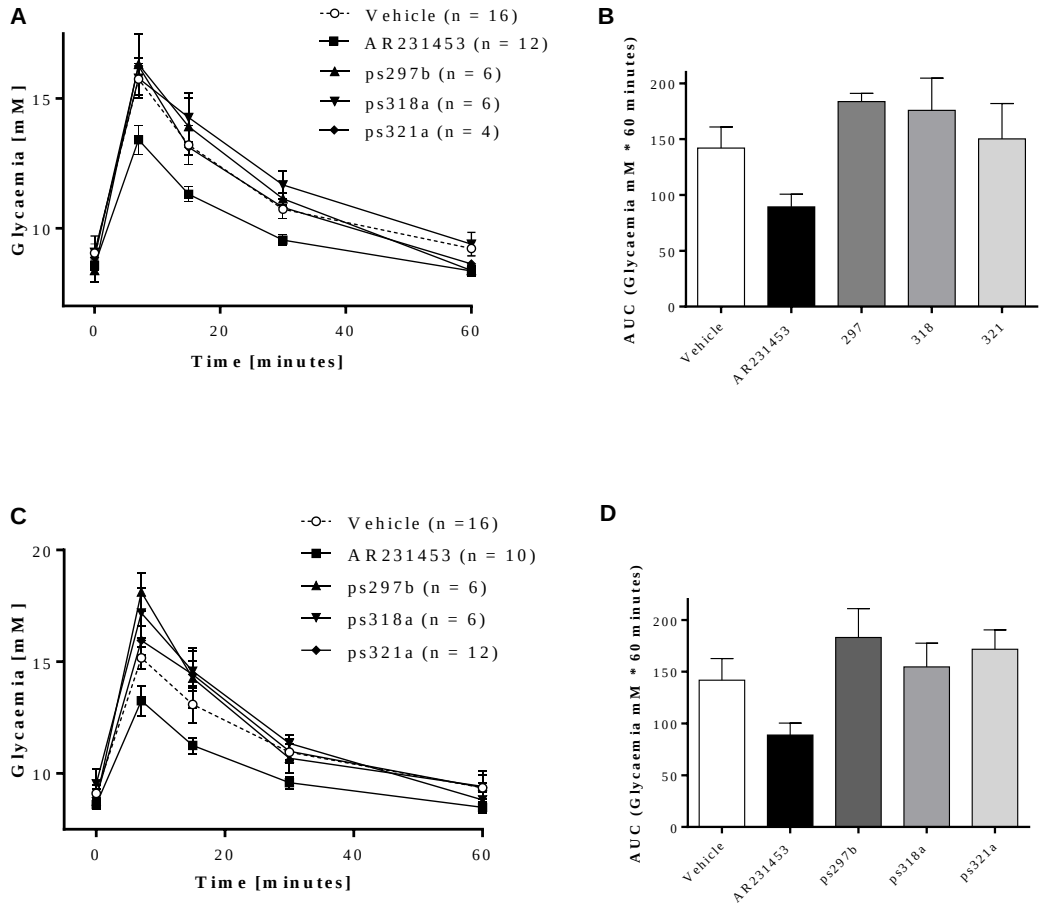



**Figure 11: Compounds ps297b, ps318a and ps321a do not acutely improve the glucose tolerance in healthy mice.** Compounds were given at either 20 mg/kg (A, B) or 50 mg/kg (C, D) orally 30 minutes before a bolus of glucose given at time 0. Glycaemia was measured over 60 minutes, and the Area Under the Curve (AUC) calculated (B, D). Averages are shown with error bars representing SEM of indicated number of animals per cohort.

The use of healthy mouse models is indeed a valid platform useful to obtain proof of concept pharmacological insight in novel drugs. But in our particular case, our target population of type 2 diabetic and obese patients, present a peculiar and yet still debated dysregulation in their gut hormone secretions (Hira, Pinyo, & Hara, 2020). Our hypothesis that a metabolically challenged mouse model responds differently to the same drugs is indeed grounded on important metabolic adaptations seen in these pathologies. In particular mice fed an obesogenic diet have been shown to have increased levels of multiple receptors including GPR119 in their intestines, as well as

the total number of L-cells (Peiris et al., 2018). Similarly, expression levels of GPR119 are known to be not only elevated in the pancreatic islets of db/db mice (Chu et al., 2007; Sakamoto et al., 2006), but it is also reported that the sensitivity of GPR119-mediated insulin secretion is increased in diet induced obese mice (Ahlkvist, Brown, & Ahrén, 2013). One possible explanation is that in metabolic diseases GPR119 activity is increased as a reactive response to the metabolic stressors, such as insulin resistance and plasmatic glucose elevation. With this importance notion, and assuming that the most likely target of our drugs is indeed GPR119, we sought to validate their activity in a leptin receptor deficient (db/db) mouse, a dietary-driven model of type 2 diabetes and obesity. Indeed, in accordance to our hypothesis, ps297b did induce a statistically significant increase in plasmatic total GLP-1 levels as shown in figure 12. Curiously, the regio-isomer ps318a did not elicit any significant increase, indicating that despite its closer pharmacological resemblance to O-LPI in terms of downstream signalling, other factors must come into play in our animal models. A possible explanation is that the two analogues have different pharmacokinetics, due to different solubilities, that will need to be further dissected in future studies. Due to the non-practicality of Oleoyl-LPI use *in vivo*, we did not yet study whether its activity might also be accentuated in diabetic mice as for ps297b.

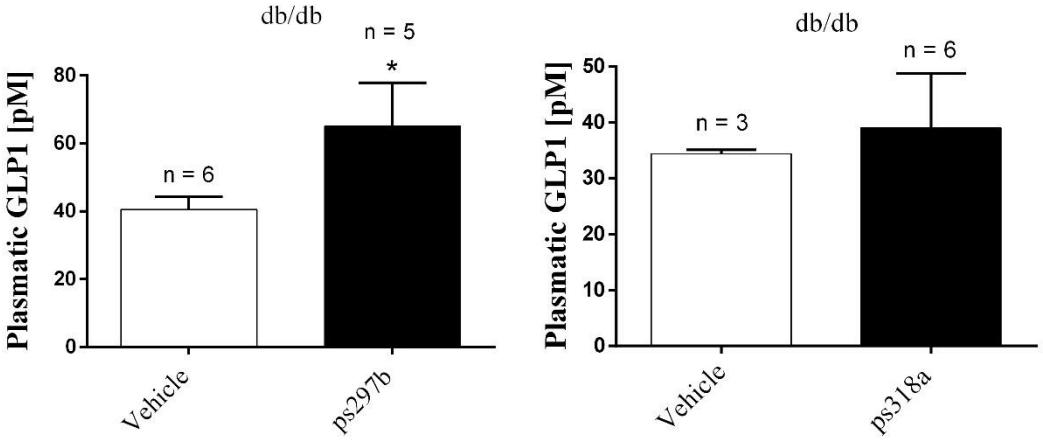



**Figure 12: ps297b increases the glucose-stimulated circulating GLP-1 levels in diabetic mice.** Plasmatic total GLP-1 levels are shown for vehicle-matched controls of ps297b and ps318a delivered orally at 20 mg/kg. Data are shown as averages ± SEM of the indicated number of animals per cohort, with statistical significance representing p-value inferior to 0.05 in a T-test.

### 3.3.5 Study of GPR119 tropism

The structural similarities, and bioactivity of ps297b, provide the rationale for its tropism for GPR119. The most potent endogenous agonists of this receptor, are Oleoyl-based molecules, i.e. Oleoyl-LPI (Manuscript 1, Chapter §2), but also OEA (Overton et al., 2006), 2-OG (K. B. Hansen et al., 2011), and Oleoyl-LPC (Soga et al., 2005), supporting that also ps297b can signal through the same receptor.

To study this tropism, we sought to validate in silico the interaction of Oleoyl-LPI, and relate it to ps297b. The crystallographic structure of GPR119 has not yet been resolved, nonetheless multiple researchers have studies its biology modelling its tertiary architecture by homology with similar receptors of known structure (Drzazga et al., 2018; Engelstoft et al., 2014).

On this premise, our collaborators from Curtin University, Ms. Elena Kokh, and Prof. Ricardo Mancera, generated a 3D homology model of GPR119 complexed with Oleoyl-LPI and ps297b. Both compounds are shown to interact with a deep orthosteric site with their Oleoyl-chains (Figure 13), engaging with the side chains of the aminoacid residues L61, C78, V85, A89, F157, L169, V160, F165, W282.

The phenylalanine 157 in particular, has been highlighted as a highly conserved residue across species, lying within the ECL-2b extracellular loop of GPR119, and appears to be critical for the constitutive activity of GPR119, as well as its responsiveness to the synthetic agonist AR231453 (Engelstoft et al., 2014). On the other hand, while F157 is not as critical, the adjacent F158 appears more important to maintain the responsiveness to OEA, likely indicating that the same orthosteric site allows for a leeway in the structure of the pharmacophore. This indicates that the orientation of the oleoyl-based chain, is dictated by the interaction of the hydrophilic head of the same molecule, that by hydrogen-bonding with residues of the extracellular portion of the receptor can differentially shape the engagement of the orthosteric site. Indeed this is key evidence that not only provides further support to the tropism of ps297b for this receptor, but it also adds granularity to the now accepted concept that the orthosteric site of a receptor such as GPR119, can be ligated in different ways, leading to the stabilization of different arrangements of the receptor, ultimately activating different downstream signalling pathways that lead to different pharmacological readouts.

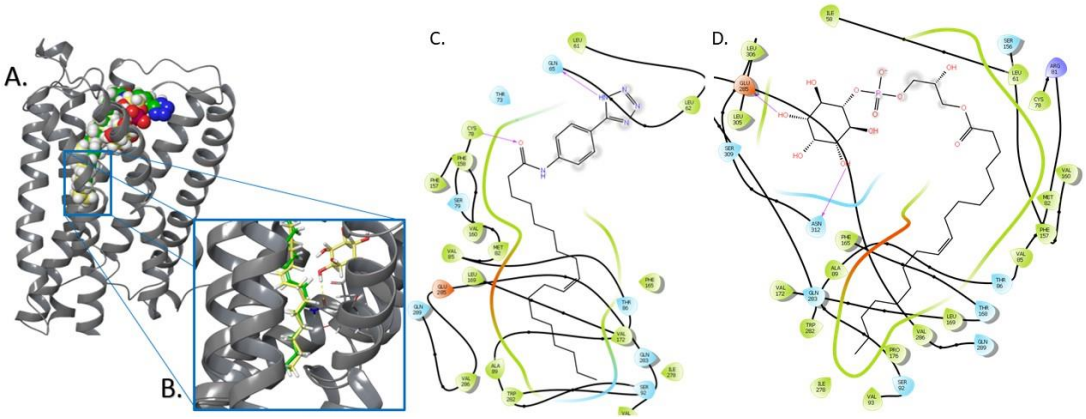



**Figure 13: Oleoyl-LPI, and ps297b are predicted to interact with the orthosteric site of GPR119**. 3D model highlighting the thermodynamically stable predicted positions of Oleoyl-LPI (yellow) and ps297b compound (green) interacting with the GPR119 orthosteric site. (A) General view showing overlapping ligands demonstrating fitting into the same binding pocket. (B) Closer view on lipophilic tails of Oleoyl-LPI (yellow) and ps297b (green) docked into GPR119. Detail of GPR119 side chains engaging with ps297b (C) and Oleoyl-LPI (D) are indicated by purple arrows.

Having demonstrated *in silico* the similar interaction of ps297b and Oleoyl-LPI with GPR119, we sought to validate this tropism with experimental evidence. Our *in vitro* cellular models of GLP-1 secretion, NCI-H716 and GLUTag, are known to express GPR119, as we evidenced in the manuscript presented under chapter §2 (Figures 3B, and supplementary 2B), at the protein level via immunoblotting with a polyclonal goat-derived antibody from Santa Cruz Biotechnology (Cat # sc48195). STC-1 cells are also reported in literature to express a functional GPR119 (Chepurny, Holz, Roe, & Leech, 2016) and were therefore used, together with NCI-H716 and GLUTag, to analyse the responsiveness to our drugs in loss of function studies.

RNA interference is a recognized approach for downregulating the expression of a target protein such as GPR119. In our manuscript (chapter §2), our collaborator and co-first author Dr. Syamsul Ahmad Arifin downregulated GPR119 at the transcript level with a proprietary pool of a total of 4 siRNA sequences from Dharmacon which were transfected at a concentration of 300 nM with the Lipofectamine 2000® reagent (Thermo Fisher Scientific). Cell lysates from both NCI-H716 and GLUTag cell lines

were analysed after 48 hours, demonstrating downregulation of GPR119 both at the transcript and protein level. We wanted to replicate this downregulation with the independent use of single validate siRNA sequences to minimize possible sequence-specific off-target RNA interference.

Nonetheless, we could not obtain robust, and reproducible immunodetection of GPR119 with the polyclonal antibody sc48195, which in our hands generated non-specific bands, of which none situated at the expected theoretical weight of 37 kDa for GPR119. Indeed as mentioned earlier, this same antibody was used successfully, and proven to be specifically and sensibly detecting GPR119 in both GLUTag and NCI-H716 (Arifin et al., 2018), but being a polyclonal preparation, a different batch is likely to blame.

We then sought to validate the GPR119 downregulation in NCI-H716 transfected with GPR119-targeting siRNA, via Immunofluorescence with a different antibody from Novus, (NLS54). As shown in figure 14, at either 48 or 72 hours, we could not detect any consistent reduction in GPR119 staining. These unexpected results can indeed be explained by the difficulty in transfecting L-cells, coupled with rebound overexpression of the targeted protein in addition, or simply due to the lack of a commercially available genetically validated antibody.

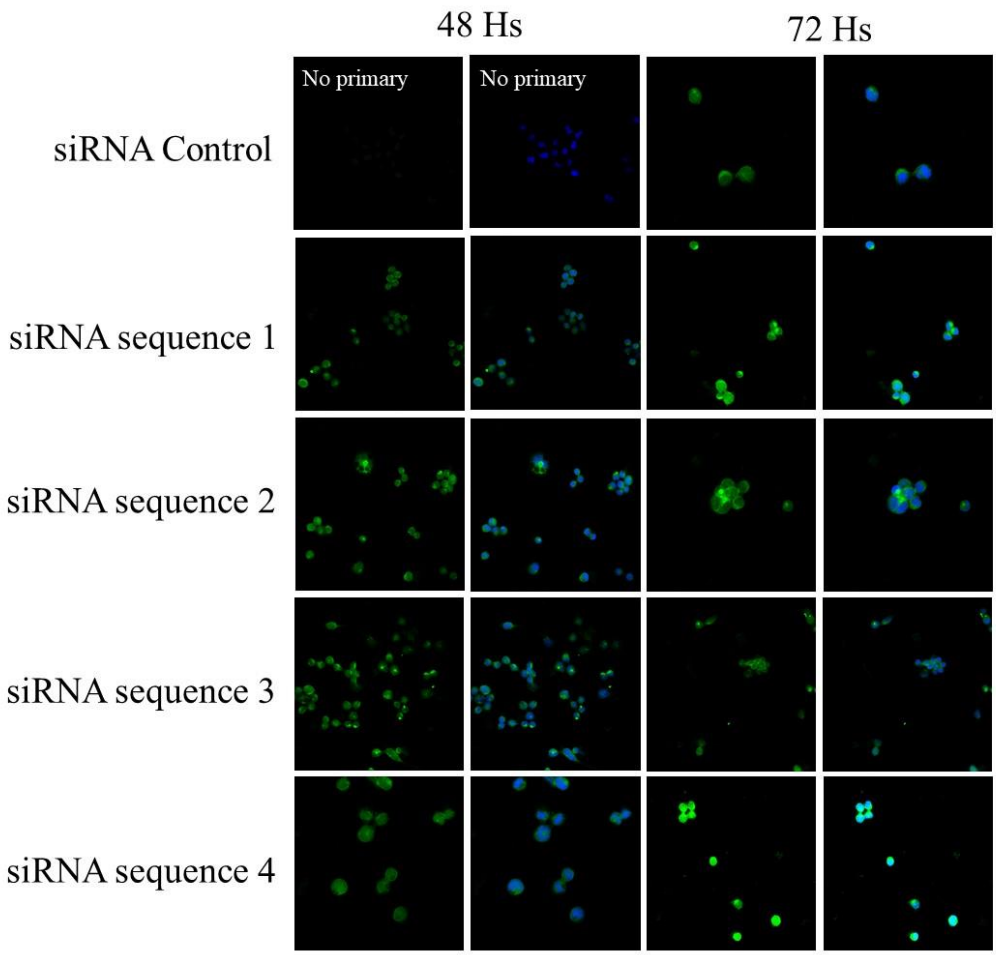



**Figure 14: Immunofluorescence of GPR119 in NCI-H716 cells transfected with panel of interfering RNAs.** Representative fields of view of NCI-716 cells transfected with the indicated panel of GPR119-targeting siRNA from QIAGEN, and analysed after either 48, or 72 hours for GPR119 expression (green) via immunofluorescence with the Novus antibody NLS548. Co-staining of nuclei with Hoechst33258 (blue) is shown on the right-side panels. Cells sham stained without the primary antibody do not show any appreciable background signal. Equal exposure times were used across the panel.

Considering the inefficient downregulation of GPR119 with the QIAGEN panel of siRNA sequences, we set out to validate a new panel of RNAs from Dharmacon in our human cell line model. Lysates of NCI-H716 transfected with either siRNA, were analysed after 48 or 72 hours with Western Blotting for GPR119 expression, using another polyclonal antibody raised against an internal sequence of human GPR119 (ab75312, Abcam), nonetheless we could not obtain any meaningful reduction in immunodetection indicating the technical difficulty at transfecting these cells.

We also attempted to validate the GPR119 downregulation in the murine cell lines GLUTag and STC-1, and we analysed by western blot the expression of GPR119 with two different polyclonal antibodies raised against the human (NB100-94884, Novus) or murine (NBP2-47661) C-term of GPR119.

Nonetheless, also these cell lines proved to be difficult to transfect, and we could not achieve any consistent and robust downregulation necessary to validate multiple drugs.

Assuming that the commercial siRNAs were not efficiently delivered, nor that they were targeting appropriately the mRNA of GPR119, we sought to generate a pool of endonuclease prepared siRNA targeting multiple regions of the same transcript, with reported increased efficiency and minimal off-targets effects (Heninger & Buchholz, 2007). As shown in figure 15, the targetable region of all 3 cell lines, could be readily amplified by PCR (panels A and B), however after generating dsRNA copies of our targetable region *in vitro*, we could not obtain any dicing into short 20-22 base pair long oligonucleotides, necessary to deliver any RNA interference in mammalian cells (Panel C, figure 15). Unfortunately, particularly the transcription *in vitro* requires further optimization, and is yet an ongoing work in progress. To overcome these technical problems, we generated a more robust and stable genetically deficient cellular model with CRISPR-Cas9 technology. However, also this procedure is a technically challenging methodology, and knockout clones of STC-1 cells are currently under investigation for their genomic analysis of the GPR119 locus. Due to these logistical difficulties, we are also in the process of re-establishing a colony of GPR119 deficient mice. Nonetheless, this study provides foundational pharmacological evidence that underlines the therapeutical potential of ps297b as a novel GLP-1 secreting agent in metabolic diseases.

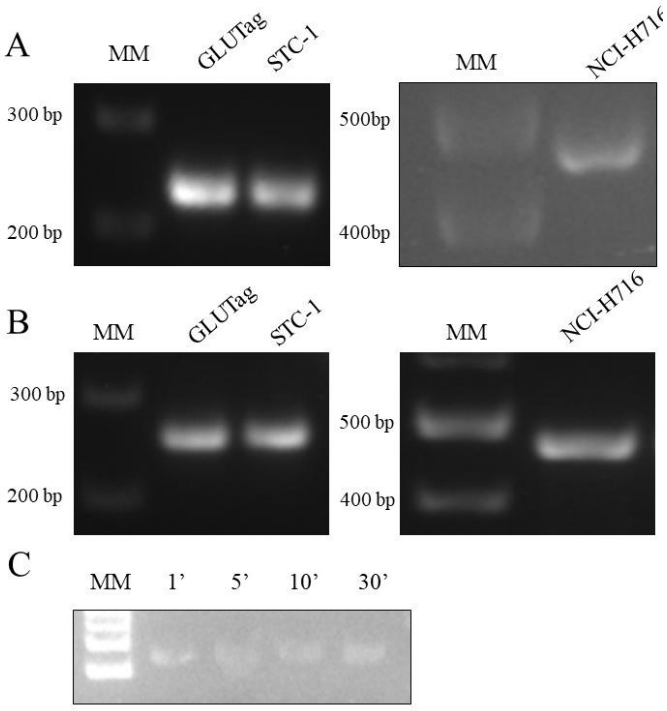



**Figure 15: Production of endonuclease-prepared siRNA. (A)** GPR119 targeting regions were obtained by amplifying cDNA from GLUTag, STC-1 or NCI-H716. Resolved bands of expected size, GLUTag and STC-1 207 bp, or NCI-H716 419 bp are shown. (B) PCR products from reaction (A) reamplified in a second PCR with common primers extending the T7 promotor, resulting in amplicons of 229 bp and 441 bp for either murine, or human GPR119. (C) murine GPR119 amplicon dsRNA obtained by transcription in vitro of PCR products shown in B, and diced with RNaseIII for either 1, 5, 10 or 30 minutes as indicated. MM, molecular marker.

### 3.4 Conclusion, significance and future directions

Targeting the EECs to induce the release of GLP-1, is a novel therapeutical approach with vast potential and applicability to multiple metabolic diseases where drugs agonising GLP-1R are already on the market with proven efficacy. The ability to physically contain the drugs within the gastrointestinal lumen, by minimizing their absorption, minimizes on-target and off-target toxicity, offering a broad therapeutical window and ease of administration via the oral route.

Oleoyl-LPI is a potent and effective GLP-1 secretion agent *in vitro*, and *ex vivo*, but as shown in figures 8 and 10, it does significantly increase the glucose-stimulated release of either GLP-1 and PYY in healthy mice at the low dose of 10 mg/kg, although a

small effect size can be appreciated in the small number of animal used. Assuming instability upon digestion, in addition to the exposure to acidic stomach environment, we cannot exclude efficacy *in vivo* at equal or higher doses, with targeted delivery to the ileum with enteric coated pills. Similarly, as shown in figure 2, 3 and 12, the synthetic Oleoyl-LPI mimetics and structurally related ps297b, ps318a, and ps321a display minor yet consistent GLP-1 secreting capabilities in healthy animals, resulting in not significant improvement in glucose tolerance up to a dose of 50 mg/kg (figure 11). On the other hand, ps297b, does increase acutely the glucose-stimulated GLP-1 levels in the diabetic db/db mouse model, reinstating its therapeutical potential.

Whether this increase in GLP-1 is sufficient to elicit the clinical benefits seen with commercial GLP-1RAs remains to be validated with a more translatable, diet-induced mouse model of diabesity. We did not assess the glucose-tolerance in our db/db model to validate the impact of ps297b bioactivity due to the glycaemic fasting levels already superior to 33 mM. A chronic non-genetic mouse model of obesity and early stage diabetes would represent a better platform where to assess the metabolic impact of ps297b. Delivery to the ileum and beyond, where most GLP-1 secreting cells lie, yet represents a critical pharmacokinetic issue that will need to be resolved with technologies such as nanoparticles, or other excipients already on the market for the intestinal delivery of drugs.

This study provides initial evidence indicating that ps297b signals through GPR119. However, more robust experimental evidence is required to validate whether the molecular target of our LPI analogues is indeed GPR119. A simple strategy could be the use of pharmacological antagonism, an easy and elegant approach to validate drugs targets. Unfortunately, there are no commercially available GPR119-specific antagonists such as the recently described AR436352 (Tough et al., 2018).

Furthermore, it is yet unclear whether GLP-1 secretion is purely under CREB and ERK- mediated transcriptional control. This hypothesis can be validated in a future study pre-treating cells with transcriptional inhibitors such as Actinomicin D.

It has been known for a few decades already that the upstream PKA and PKC stimulation induces the release of GLP-1 in the majority of L-cell models (Brubaker, Schloos, & Drucker, 1998). Interestingly, in contrast to GPR119-mediated insulin release in β-cells, GPR119-mediated GLP-1 release in L-cells is glucose-independent

while being Calcium dependent (Lan et al., 2012). To further understand the pharmacology of Oleoyl-LPI and our synthetic mimetics, a future study will need to analyse the intracellular electrochemical cascade elicited by our drugs, to better quantify the transcriptional control elicited by ERK1/2 and CREB activity.

The *in silico* evidence we provide for ps297b as well as Oleoyl-LPI interacting with GPR119, will need to be validated experimentally by use of cellular systems expressing heterologous GPR119 mutated in key residues that are shown to interact with either drug. Agonism of intestinal GPR119 only, represents an unprecedented approach that would avoid all the toxicity problems affecting most of the systemically-acting GPR119 agonists (Ritter et al., 2016).

# Chapter 4

# Chapter 4: Investigating the GLP-1 secreting properties of cannabinoids

## 4.1 Introduction

Metabolic diseases like obesity and type 2 diabetes, are complex pathologies characterized by homeostatic imbalances at the level of multiple systems. Maintaining blood glucose levels within physiological levels requires the coordination of multiple organs beyond the pancreatic islets. As described in the previous 3 chapters of this thesis, the EECs communicates with the pancreas by secretion of incretin hormones such as GLP-1, directly and indirectly modulating blood glucose levels among other physiological functions. GPR119 is a key receptor underpinning the intestinal release of incretins that drive the release of insulin, it is also expressed by pancreatic β-cells, and its activation causes the release of insulin, ultimately driving the clearance of glucose from the bloodstream (chapter §2). But the pharmacology of GPR119 is glucose-dependent, and indeed in conditions of hypoglycaemia, its activation leads to the release of Glucagon (Li et al., 2018). These are the main pharmacological reasons as to why activation of GPR119 holds a superior therapeutical potential when compared to insulin, especially in metabolic diseases such as type 2 diabetes and obesity (Al-Barazanji et al., 2015; Mo, Yang, & Tao, 2014). LPI lipids, as we described in chapter §2, are bio-active molecules where the nature of the acyl-tail defines their pharmacology. We demonstrated that the Oleoyl-LPI subspecies, is the only one to induce GPR119-mediated GLP-1 secretion. The endocannabinoid system (ECS) together with the EEC, and the pancreatic islets, plays a key role in maintaining a robust homeostatic balance of energy storage and utilization. All LPI species are indeed elements of the endocannabinoid system, not only for their pharmacologic tropism for GPR55, that we can herein refer to as cannabinoid receptor 3 (CB3), but also because of their structural and pathophysiological similarities to other endocannabinoids, especially in inflammatory driven diseases. Indeed, a recent study reports that in ulcerative colitis (UC) or Chron disease (CD) PLA1 activity is increased, and elevated levels of different endocannabinoids including anandamide (AEA), and OEA are reported. On the other hand, elevated levels of the structurally related endocannabinoid 2-arachidonoyl-glycerol (2-AG) were shown only in CD and colon-rectal cancer (CRC) but not UC. Stearoyl-LPI (S-LPI) and A-LPI were shown to be elevated in inflammatory bowel disease (IBD), with the latter also raised in CRC

(Grill et al., 2019; Raimondo et al., 2018). Similarly, multiple LPIs including O-LPI and A-LPI have also been reported to be raised in a mouse model of atherosclerosis (Yan et al., 2019). These studies clearly indicate that different endocannabinoids including LPIs, are modulated in multiple inflammatory diseases, nonetheless the clinical implications of this are not completely understood. The endocannabinoid system is a key element of our physiologies, that multiple civilizations for millennia have modulated with plant derived phytocannabinoids for medicinal purposes. Only three decades ago the endogenous receptors of the main psychoactive drug of plants of the genus Cannabis, tetrahydrocannabinol (THC) was first described in 1988 and given the eponymous name of Cannabinoid receptor 1 (CB1) (Devane, Dysarz, Johnson, Melvin, & Howlett, 1988; Matsuda, Lolait, Brownstein, Young, & Bonner, 1990). Soon afterwards an endogenous molecule, N-arachidonoylethanolamine (AEA), or Anandamide, was shown to be the physiological ligand of CB1 (Devane et al., 1992), a strong indication that this receptor had a physiological function. The next year, a second receptor, CB2, located primarily in immune cells was reported to be activated by phytocannabinoids (Munro, Thomas, & Abu-Shaar, 1993), for which a different endogenous ligand, 2-arachidonoylglycerol (2-AG) was unveiled in 1995 (Mechoulam et al., 1995; Stella, Schweitzer, & Piomelli, 1997). Curiously, AEA and 2-AG are also found in some types of food such as chocolate or milk (Di Marzo et al., 1998), together with other structurally related N-acylethanolamines (NAEs). Surprisingly, these food-derived, as well as the endogenous cannabinoids are metabolized by enzymes expressed in different tissues including the intestine, the liver and the brain (Desarnaud, Cadas, & Piomelli, 1995; Katayama et al., 1997), indicating that we evolved a tight relationship between the ECS and our diets. In the last two decades many other endocannabinoids have been described for their capacity to modulate positively or negatively the activity of CB1 and CB2, firstly N- arachidonoyl dopamine (NADA) (Bisogno et al., 2000), then 2-arachidonoyl glyceryl ether (Noladin ether) (Hanus et al., 2001), and O-arachidonoyl ethanolamine (Virodhamine, or OAE) (Porter et al., 2002). While these novel compounds were being described, other studies were unveiling a deeper layer of complexity in the ECS. In particular, Anandamide was shown to only partially exert its pharmacology through CB1 (Adams, Compton, & Martin, 1998), and as confirmed in knockout mice (Breivogel, Griffin, Di Marzo, & Martin, 2001; Di Marzo et al., 2000) it was clear that a third cannabinoid receptor was present in the brain.

Indeed, two candidates appeared to underpin this pharmacology. The phytocannabinoid Cannabidiol (CBD), as well as THC and the endocannabinoids AEA, noladin ether, virodhamine, and 2-AG, were all shown to bind and modulate by different degrees GPR55-mediated Gq signalling pathways (Lauckner et al., 2008; Ryberg et al., 2007), while both CBD and THC were reported to also modulate the activity of GPR18 (Console-Bram, Brailoiu, Brailoiu, Sharir, & Abood, 2014; McHugh, Page, Dunn, & Bradshaw, 2012). GPR55 in particular has low structural homology with either CB1 and CB2, and its main ligand is Arachidonoyl-LPI (Oka et al., 2009), which indeed as mentioned earlier, shares its arachidonoyl-chain with all the other endocannabinoids AEA, 2-AG, virodhamine and noladin ether. Nonetheless, GPR55 is not only the target of phyto and endo-cannabinoids, it also physically interacts with both CB1 and CB2, forming a functionally unique receptor heteromer with a unique pathophysiology (Kargl et al., 2012; Martínez-Pinilla et al., 2014; Moreno et al., 2014). Indeed in the last decade, a vast number of studies has started to unveil that both CB1 and CB2 receptors, functionally interact with multiple other apparently unrelated receptors, and surprisingly multiple drugs have shown to exert their therapeutical effects by modulating the physical structure of these unique signalling platforms (Galindo et al., 2018; Hudson, Hébert, & Kelly, 2010a, 2010b; Navarro, Borroto-Escuela, et al., 2018; Navarro, Varani, et al., 2018; Pinna et al., 2014; Reyes-Resina et al., 2018; Viñals et al., 2015). A further layer of pharmacological complexity that needs to be accounted for when studying the pharmacological modulation of endocannabinoid receptors, is the vastly reported phenomenon of biased agonism, where different ligands binding the same receptor, elicit different dose-dependent intracellular signalling cascades (Al-Zoubi, Morales, & Reggio, 2019; Hassing, Fares, et al., 2016; Henstridge et al., 2010; Hudson et al., 2010a; Navarro, Reyes-Resina, et al., 2018; Shoemaker, Ruckle, Mayeux, & Prather, 2005; M. Smith et al., 2015; Soethoudt et al., 2017; Sugiura, Kishimoto, Oka, Gokoh, & Waku, 2004; S. Wang et al., 2018; Wouters et al., 2020). Similarly to CB1, CB2 and GPR55, the activity of GPR119 can also be modulated by different endogenous molecules, beyond Oleoyl-LPI, N-Oleoylethanolamine (OEA) (Overton et al., 2006) and 2-Oleoyl-glycerol (2-OG) (K. B. Hansen et al., 2011), which indeed are Oleoyl-based structural cousins of all the GPR55 acting endocannabinoids mentioned earlier. Indeed, after the publication of the manuscript presented in chapter §2, knowing that GPR55 is expressed in GLUTag and NCI-H716 cells as we evidenced via transcript

amplification and protein immunodetection, we reported that Arachidonoyl-LPI could not elicit GLP-1 in these models. We sought then to also verify whether this peculiar pharmacology was maintained in STC-1 cells, a duodenal-like L-cell line model. Surprisingly, we observed that A-LPI could exert a GLP-1 stimulating activity similar to Oleoyl-LPI, indicating that beyond GPR119, other possible elements of the endocannabinoid system, such as GPR55 might come into play at defining the GLP-1 secreting pathways. Indeed, GPR119 shares much of its pharmacological impact on metabolic diseases with GPR55. Eating is a known hedonic activity where endocannabinoids play a key role, accumulating when fasting, and dropping soon after eating (DiPatrizio et al., 2015). Some types of food contain cannabinoids, but at negligible concentrations, however endocannabinoids are proven to be directly modulated when we eat, drink or even exercise for pleasure (DiPatrizio, Astarita, Schwartz, Li, & Piomelli, 2011; Feuerecker, Hauer, Gresset, et al., 2012; Feuerecker, Hauer, Toth, et al., 2012; P. Monteleone et al., 2012) acting at pharmacologically relevant concentrations as true psychotropic agents that can be dysregulated in pathologies like anorexia (A. M. Monteleone et al., 2015). Indeed, CB1 activation with drugs like THC, is known to increase appetite, making it a useful pharmacological target. On the other hand Rimonabant, a CB1 inverse-agonist, is known to not only induce satiety, but also ameliorate insulin sensitivity, and dyslipidaemia in obese individuals, indicating how the ECS plays a key role in metabolic pathologies (Christensen, Kristensen, Bartels, Bliddal, & Astrup, 2007; Van Gaal, Rissanen, Scheen, Ziegler, & Rössner, 2005). Curiously Rimonabant also induces the release of Glucagon which drives hyperglycaemia, and does not appear to affect the release of GLP-1 (Cani, Montoya, Neyrinck, Delzenne, & Lambert, 2004), but synergizes with the beneficial effects of the GLP-1R agonist exendin-4 (K. N. Patel et al., 2014). This phenotype is corroborated by mice genetically deficient for CB1, which display increased insulin and GLP-1 sensitivity, in addition to increased meal-stimulated GLP-1 levels (González-Mariscal, Krzysik-Walker, Kim, Rouse, & Egan, 2016). Further confirming the detrimental effects of CB1 activity, healthy men dosed with THC under the form of Nabilone, show an acutely inhibition in glucose-stimulated Glucagon and GLP-1 secretion, while surprisingly displaying at the same time increased GIP secretion (Chia et al., 2017). The pharmacology of CB1 contrasts in many ways with GPR55, which activation is known to improve the sensitivity of insulin as well as increase its secretion (Liu et al., 2016; Vong, Tseng, Kwan, Lee, & Hoi, 2019),

together with GIP and GLP-1 (McCloskey et al., 2020; McKillop, Moran, Abdel-Wahab, Gormley, & Flatt, 2016; X. F. Wang et al., 2020). This complex relationship is further corroborated by strong epidemiological evidence, as well as mechanistic animal data indicating that two CB1-antagonising phytocannabinoids CBD and Tetrahydrocannabivarin (THCV) reduce the risk of non-alcoholic fatty liver disease (NAFLD) (Adejumo et al., 2017; Silvestri et al., 2015), a pathology where GLP-1R activation is known to be beneficial (Bifari et al., 2018; He, Sha, Sun, Zhang, & Dong, 2016; L. Zhang et al., 2013).

Curiously, the expression of CB1 and GLP-1R have been reported to be lowered in the livers of diabetics rats (Coskun, Beydogan, & Bolkent, 2019), while the DPP-4 inhibitor Sitagliptin upregulates the expression of both receptors (Coskun, Koyuturk, Karabulut, & Bolkent, 2017). The phytocannabinoid THCV in particular, which has been reported to behave as a neutral antagonist of CB1 at low doses (Thomas et al., 2005), has been shown to have important anti-diabetogenic properties, with capacity to improve insulin sensitivity in obese mice (Wargent et al., 2013), and ameliorate glucose tolerance in healthy and diabetic humans treated daily with 5 mg THCV for 13 weeks (Jadoon et al., 2016). This pharmacology is similar but yet different to some degrees to the other phytocannabinoid CBD, likely due to their shared yet not unique tropism and allosteric modulation of CB1. All these studies paint a broad and complex picture, where the endocannabinoid system exerts a direct effect on GLP-1 secreting cells and impacts on the overall metabolism. Nonetheless, there is only a single study mentioned earlier (Chia et al., 2017), indicating that THC negatively modulates GLP-1 in men. No Structural activity relationship study of phytocannabinoids, such as THCV, or the CBD related Cannabidivarin (CBDV) and the abundant phytocannabinoid precursor Cannabigerol (CBG) has been carried out in terms of GLP-1 release. Indeed these compounds, together with THC, represent a valuable library of structurally-related drugs with a complex and only partially understood pharmacological profile toward CB1 and CB2 (Navarro et al., 2020). CB1 is recognized to be present along the whole intestine, in particular different studies reported its expression in smooth muscle cells, intraepithelial plasma cells, generally across the crypt epithelium, and broadly across the enteric nervous system (ENS) (Casu et al., 2003; Duncan, Davison, & Sharkey, 2005; K. Wright et al., 2005). More recent studies have provided clear evidence that indeed CB1 is also expressed by different enteroendocrine cells. In particular I cells, found in the duodenum, defined

by their secretion of cholecystokinin (CCK), were shown to co-express CB1 and GPR119 (Sykaras, Demenis, Case, McLaughlin, & Smith, 2012). In another study, GIP-secreting duodenal K-cells, GLUTag cells, as well as small intestinal, but not colonic GLP-1 secreting L-cells, were all shown to express the transcript for CB1 (Moss et al., 2012). In addition, cAMP driven GIP secretion from murine primary small intestinal preparations, but not GLP-1 secretion from either small or large intestinal preparations, showed a significant inhibition in presence of the CB1 specific agonist methanandamide (mAEA). GLUTag cells showed a partial inhibition in cAMP-driven GLP-1 secretion in presence of mAEA indicating that CB1 does play a role in incretin hormone secretion. The same study highlights the presence of the transcript of CB2 in both murine GIP and GLP-1 secreting K and L-cells.

CB2 is also being immunodetected more broadly across multiple submucosal immune cells including macrophages, as well as in epithelial cells, and the ENS, appearing upregulated in inflamed conditions (K. Wright et al., 2005; K. L. Wright, Duncan, & Sharkey, 2008). At the same time, endocannabinoids are broadly reported across the GI tract, especially in cancerous tissues (Ligresti et al., 2003).

In this study we show preliminary data indicating that the 3 most recognized L-cell line models, STC-1, GLUTag and NCI-H716, all express the prototypical cannabinoid receptors CB1, CB2 at the protein level, in addition to the unique GLP-1 secreting properties of the phytocannabinoids THC and THCV. Furthermore, we show that the bioactivity of THC and THCV can be increased more than additively with the endocannabinoid OEA. THC and THCV, but also the inactive CBD, all activate by different degrees ERK1/2, CREB and Akt pathways in an apparent CB1 and CB2 independent manner. This study lays the foundations for future investigative work aimed at understanding the pharmacological role of CB1 and CB2 in the promising GLP-1 secreting properties of some phytocannabinoids. Other receptors should also be assumed to be possibly interacting as functionally unique heteromer platforms, defining key cell specific pharmacology that need to be dissected in native enteroendocrine cell systems.

Future studies should validate the physiological expression of cannabinoid receptors in situ, to detect at the protein level both cannabinoid receptors in enteroendocrine cell, and ideally by co-staining highlighting the presence of functional heteromers such as the known GPR55-CB1/CB2. Further dose response analysis, in addition to tailored

delivery *in vivo*, would help us to characterize this mechanism of action, aiming to ultimately generate safer GLP-1 secreting drugs.

## 4.2 Materials and methods

### 4.2.1 Cell culture, secretion assay and drugs procurement

Cell lysates were obtained from NCI-H716, GLUTag, and STC-1 cells grown as described in the previous chapter §3.2.2. MIN-6 cell line, provided by Jun-Ichi Miyazaki (Osaka University, Graduate School of Medicine, Osaka, Japan) were grown in DMEM high glucose (4.5 g/L) supplemented with 15% FBS, glutamine 2 mM, HEPES 20 mM, β-mercaptoethanol 50 µM and Penicillin 100 U/ml Streptomycin 100 µg/ml. All cell lysates were dissolved in RIPA buffer added with protease inhibitor, and phosphatase inhibitor cocktails, as described in chapter §3.2.4. For the secretion assay studies, STC-1 cells were seeded in 24 well plates coated with a thin layer of Matrigel as described in chapter §3.2.6. Cells were treated with the indicated drugs dissolved in serum free DMEM medium. In pharmacological competition studies, the CB1 specific antagonist CE-178253 (PZ0205-5MG, Sigma Aldrich) and the CB2 specific antagonist, compound 27 described in (Shi et al., 2017) as a gift from Dr. Gunosewoyo, were used at a final concentration of 10 nM and 50 nM respectively. Cells were pre-treated with either antagonist for 1 hour, followed by treatment for 30 minutes in presence of the same amount of antagonist added with either phytocannabinoid. For immunoblot analysis of downstream pathways, cells were pre-treated with either antagonist for 30 minutes and then treated with either phytocannabinoid in fresh media for further 10 minutes always in presence of the antagonist drug. All pre-treatments and treatments were performed at 37°C. The vehicle treated cells were exposed to an equivalent volume of solvent, DMSO equal to 0.2%. Total GLP-1 content was quantified from cleared supernatants with an ELISA Kit (EZGLP1T-36K, Merck, Millipore).

Oleoylethanolamide (OEA) was purchased from Sigma Aldrich (Cat #O0383) dissolved in DMSO at 10 mM, and stored under nitrogen atmosphere at -20°C. Δ9-Tetrahydrocannabinol (THC), Cannabidiol (CBD), and Cannabigerol (CBG) were purchased from THCPharm (Frankfurt, Germany), while Δ9-Tetrahydrocannabivarin (THCV) and Cannabidivarin (CBDV) from Cayman Chemical (Cat # 18091 and 9001574 respectively). Arachidonoyl-LPI was obtained from Avanti Polar Lipids Inc.

(Cat # 850105P), dissolved in DMSO at 10 mM and stored under nitrogen atmosphere at -20°C. Statistical significance in all data was analysed with the software GraphPad Prism v6.01

### 4.2.2 Transcript amplification

CB1, CB2, GPR55 and GPR119 transcripts of human and mouse origin, were amplified with the following primers originally designed to generate Endoribonuclease-prepared short interfering RNAs (esiRNA) as described in the previous chapter §3.2.3.2. Primers were generated with the Primer3 software, and appended with the T7 promoter sequences (Forward: *TCACTATAGGGAGAG* and Reverse: *TCACTATAGGGAGAC*)

| | |
|---|---|
| Human GPR119 Forward primer | *TCACTATAGGGAGAG*CTCACAGACCAGCTCTCCAG |
| Human GPR119 Reverse primer | *TCACTATAGGGAGAC*TTTCGAATCTGCTGGCTGTG |
| Murine GPR119 Forward primer | *TCACTATAGGGAGAG*TGCTGTCCTAACCATCCTCA |
| Murine GPR119 Reverse primer | *TCACTATAGGGAGAC*AGGCTACACAAGGTCTTCTGT |
| Human GPR55 Forward primer | *TCACTATAGGGAGAG*TCGTAGAGTGCAGAGCCAAG |
| Human GPR55 Reverse primer | *TCACTATAGGGAGAC*ACCTGGTCTGTGGGTTCTTC |
| Murine GPR55 Forward primer | *TCACTATAGGGAGAG*CGTGACAACTGCTCATTCGA |
| Murine GPR55 Reverse primer | *TCACTATAGGGAGAC*AGACACTCCCATACATGCTGA |
| Human CB1 Forward primer | *TCACTATAGGGAGAG*GGATGGGAAGGTACAGGTGA |
| Human CB1 Reverse primer | *TCACTATAGGGAGAC*CTGTGAACACTGGCTGCATT |
| Murine CB1 Forward primer | *TCACTATAGGGAGAG*ATCTTAGACGGCCTTGCAGA |
| Murine CB1 Reverse primer | *TCACTATAGGGAGAC*GTGGAACACGTGGAAGTCAA |
| Human CB2 Forward primer | *TCACTATAGGGAGAG*GTGACAGAGATAGCCAATGG |
| Human CB2 Reverse primer | *TCACTATAGGGAGAC*TAGGAAGGTGGATAGCGCAG |
| Murine CB2 Forward primer | *TCACTATAGGGAGAG*TCCCACTGATCCCTAACGAC |
| Murine CB2 Reverse primer | *TCACTATAGGGAGAC*TAACAAGGCACAGCATGGAA |

Whole-cell RNA was extracted from GLUTag, NCI-H716 and STC-1 cells while growing in log-phase, with an ISOLATE II RNA Mini Kit (Cat # BIO-52072, Bioline). RNA was quantified by absorption at 260 nm, and 1 µg was then reverse transcribed into DNA with oligo dT primers to enrich the preparations with the cellular transcriptomes. Target sequences were then amplified in separate reactions, with a MyTaqTM HS Red Mix kit (Cat # BIO-25047, Bioline).

The PCR reaction was assembled in 25 µl following manufacturer's instructions, and 2 µl of cDNA template were amplified with the following cycle:

| | | |
|---|---|---|
| 94°C | 3' | |
| 94°C | 30" | x 6 cycles |
| 60°C | 30" | |
| 72°C | 30" | |
| 94°C | 30" | x 6 cycles |
| 62°C | 30" | |
| 72°C | 30" | |
| 94°C | 30" | x 22 cycles |
| 65°C | 30" | |
| 72°C | 30" | |
| 72°C | 5' | |
| 12°C | ∞ | |

2 µl of this first PCR reaction were used as template for a second polymerization carried out with communal primers appended with an extended T7 promoter

Forward GCTAATACGACTCACTATAGGGAGAG

Reverse GCTAATACGACTCACTATAGGGAGAC

| | | |
|---|---|---|
| 94°C | 3' | |
| 94°C | 30" | x 42 cycles |
| 60°C | 30" | |
| 72°C | 30" | |
| 72°C | 5' | |
| 12°C | ∞ | |

4 µl of the second PCR reaction were resolved on an agarose gel additioned with GelGreen® (Biotium), and imaged under UV light.

### 4.2.3 Western Blots

Cell lysates were separated by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and processed as described in chapter §3.2.4. Briefly, total protein transfer onto nitrocellulose membranes was assessed by Red ponceau staining before proceeding with immunodetection. For the detection of phosphorylated proteins, CB1 and CB2, as well as α-Actinin for loading control, membranes were blocked for 1 hours at room temperature in BSA3% diluted in TBS-T buffer. After a double wash in TBS-T, proteins were immunodetected with antibodies diluted in the same blocking buffer, overnight (16-20 hours) at 4°C. The next day, unbound primary antibodies were washed off three times with TBS-T buffer and incubated for a further 1 hour with secondary anti Rabbit antibody (Cat #7074, Cell Signaling Technology®) diluted 1/40,000 in BSA 3% TBS-T. ECL signal was acquired with emission windows tailored to avoid any signal saturation across each membrane and protein under analysis. After quantifying the signal of phospho-proteins, membranes were stripped at 50°C for 10 minutes in stripping buffer (Tris-HCl 62.5 mM pH 6.8, SDS 2%, β-Mercaptoethanol 100 mM), followed by blocking in BSA 3% in TBS-T for 30 minutes, and re-incubation with anti-total primary antibodies overnight at 4°C. The following day, membranes were washed two times with TBS-T and probed with secondary anti-mouse-HRP antibody (Cat #7076, Cell Signaling Technology®) diluted 1/10,000 in Milk 3% TBS-T for 1 hour at room temperature. Signal was acquired with electrochemiluminescence as before. Phospho ERK1/2, CREB and their respective total antibodies, are the same immunoglobulins described in chapter § 3.2.4. Phospho Serine 473 Akt was detected with a monoclonal, rabbit-hosted antibody (Clone D9E, Cat #4060, Cell Signaling Technology®) diluted 1/1,000 in BSA 3% TBS-T, and total Akt was quantified with a mouse-raised monoclonal antibody (Clone 40D4, Cat #2920, Cell Signaling Technology®). Cannabinoid Receptor 1 (CB1) and 2 (CB2) were detected with the polyclonal rabbit-based cat #ab23703 (Abcam) and rabbit polyclonal cat #ab45942 (Abcam) diluted at 1:500 and 1:1,000 respectively in BSA 3% TBS-T $NaN_3$.

## 4.3 Results and discussion

### 4.3.1 Phytocannabinoids induce the release of GLP-1 from STC-1 cells

As shown in the manuscript presented in chapter §2, GLUTag and NCI-H716 cell lines release GLP-1 in response to Oleoyl-LPI but not to other LPI species such as Arachidonoyl-LPI. Surprisingly, the duodenal L-cell line STC-1 did respond to Arachidonoyl-LPI (Figure 16). This LPI species, is indeed structurally more similar to many other endocannabinoids such as AEA, and 2-AG, making STC-1 a useful model to study cannabinoids-related GLP-1 secretion (Thompson & Tobin, 2020).

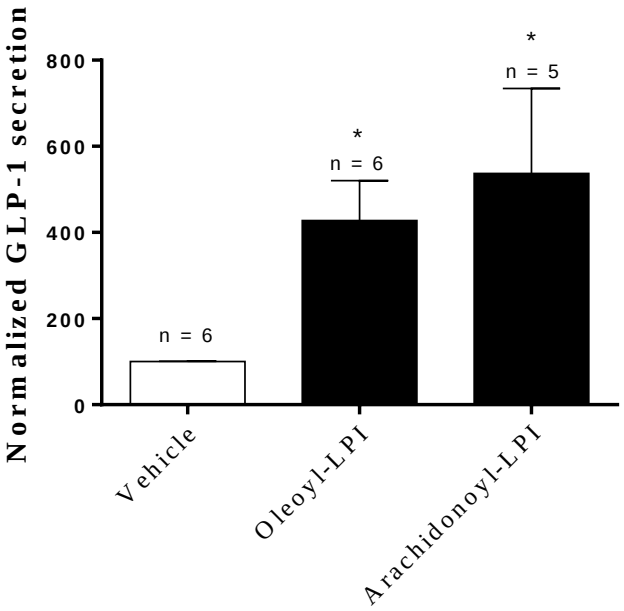



**Figure 16 Arachidonoyl-LPI is an efficient GLP-1 secreting agent in STC-1 cells.** Vehicle matched normalized GLP-1 secretion in STC-1 cells treated with 20 µM of either LPI species. Average data are shown ± SEM of the indicated number of independent experiments. Statistically significant increase compared to the vehicle is indicated for p-value < 0.05.

Given also the vast literature indicating a complex and yet far from being understood relationship between the ECS and the EEC, we wanted to evaluate whether true phytocannabinoids, could indeed modulate the secretion of GLP-1 in STC-1 cells.

To this end, we screened a small library of different cannabis-derived phytocannabinoids, including the most abundant and understood CBD, and THC, as well as the structurally related CBDV, and THCV, and the metabolic precursor CBG.

As shown in figure 17, at 5 µM THC and THCV are potent GLP-1 secreting agents in STC-1 cells, with CBDV also exhibiting some activity, while CBD and CBG were completely inactive.

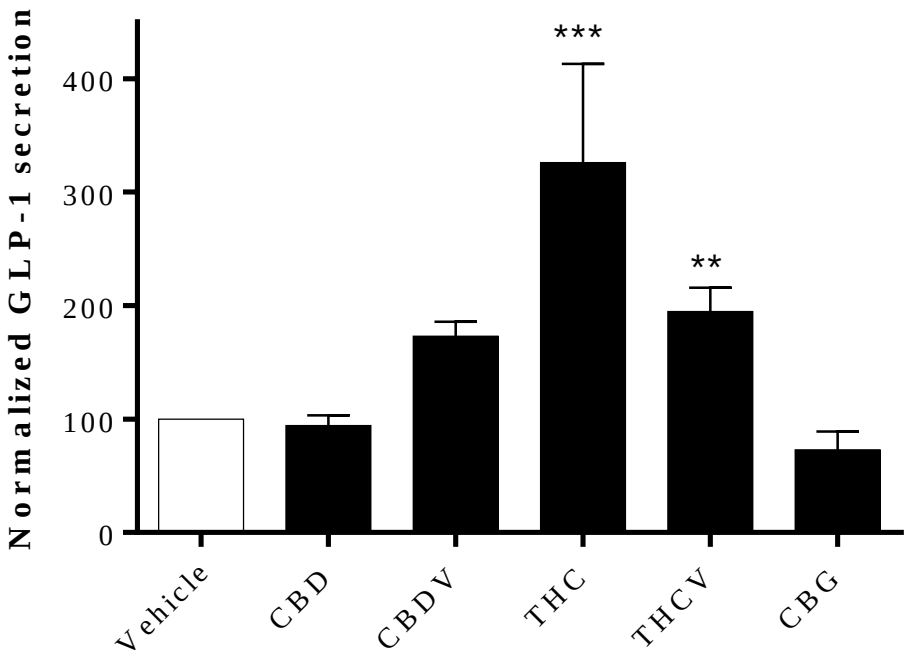



**Figure 17: Phytocannabinoids induce the secretion of GLP-1.** Total GLP-1 levels in supernatants of STC-1 cells treated with the indicated phytocannabinoids diluted at 5 µM for 30 minutes. Average normalized values are shown, with error bars indicating the standard error of the mean of at least 3 independent experiment. Statistically significant secretion was assessed with non-parametric One-way ANOVA followed by Kruskal-Wallis, and Dunn's multiple comparisons test with the software GraphPad Prism v6.01. ** indicated p-value < 0.01, and *** < 0.001.

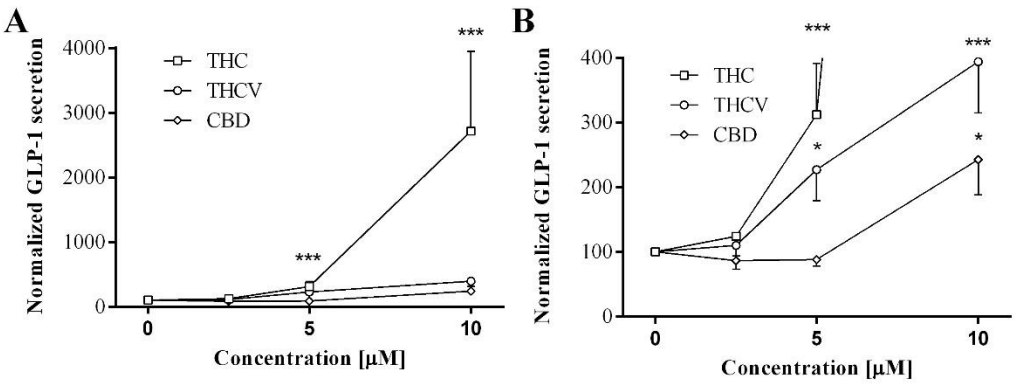



**Figure 18: Phytocannabinoids induce a dose-dependent GLP-1 secretion**. Vehicle-controlled normalized GLP-1 secretion in STC-1 cells treated with either 2.5, 5 or 10 µM of THC, THCV or CBD. Statistical significance was assessed by One-way ANOVA analysis followed by Dunnett's multiple comparison test of each drug in relation to the vehicle control and is indicated as *** p-value < 0.001, * p-value < 0.05, (c) p-value < 0.05. A and B depict the same data as averages of at least 4 independent experiments ± SEM, with B highlighting GLP-1 secretion up to 400%.

In a dose-response analysis, both THC and THCV did not show any significant activity at 2.5 µM (figure 18), while at 10 µM, THC showed a more than additive increase in GLP-1 secretion. CBD for comparison, did not show any activity at either 2.5 or 5 µM, but at the highest concentration did indeed display a statistically significant increase in GLP-1, possibly to be ascribed to its weak agonism of CB1 at higher doses as reported previously (McPartland, Duncan, Di Marzo, & Pertwee, 2015). Given this evidence, we considered 5 µM as an appropriate dose to use for further analysis, to pharmacologically characterize both the lack of activity of CBD, and the yet not so dissimilar effect of both THC and THCV.

THC and THCV are known to primarily modulate the activity of CB1 and CB2, and so we sought to verify whether either receptor was responsible for GLP-1 secretion. To this end, THC or THCV treatment at 5 µM, was repeated in presence of CB1 or CB2 specific antagonism. As shown in figure 19, THC-mediated GLP-1 secretion was ablated by antagonising either receptor, while THCV did not show any significant difference in GLP-1 secretion, indicating a clear distinction in pharmacology.

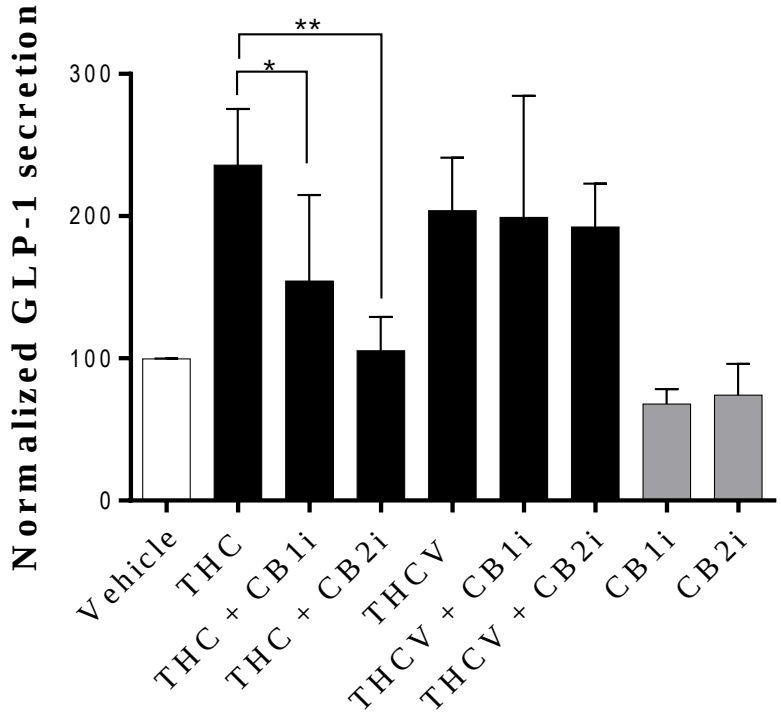



**Figure 19: THC induces a CB1-mediated GLP-1 release.** Normalized levels of GLP-1 secretion in STC-1 cells treated with either THC or THCV 5 µM in presence of CB1 or CB2 specific antagonists (CB1i and CB2i). Average values of 3 (THC) and 4 (THCV) independent experiments are shown ± SEM. Statistically significant inhibition of THC secretion was assessed by Two-way ANOVA, with Dunnett's post hoc correction. * p-value < 0.05, ** p-value < 0.01.

### 4.3.2 Phytocannabinoids activate ERK1/2, Akt and CREB cascades.

Having demonstrated that THC and THCV could efficiently trigger the release of GLP-1, with the former but not the latter depending on both CB1 and CB2, we sought to investigate which intracellular signalling pathways are they activating. From our previous study, presented in chapter §2, we know that ERK1/2 and the cAMP/PKA/CREB pathways are necessary for Oleoyl-LPI mediated GLP-1 secretion. Cannabinoid receptors CB1 and CB2, are known to signal through inhibitory type G proteins, leading to an inhibition of cAMP cascade, while at the same time activating MAPK cascades, particularly ERK1/2, and Akt as well. As shown in figure 20, THC, THCV and CBD as well, could all activate ERK1/2, CREB and Akt. Antagonism with CB1 or CB2-specific drugs, although not sufficient to statistically significantly inhibit either pathway, it does appear to halve the activation of ERK1/2. This effect cannot be seen in the other two cascades, furthermore while THCV appears as the most efficient

Akt activator, THC and CBD activate ERK1/2 more than THCV, but at the same time THC and THCV activate CREB the most. This data indicates that the activation of these signalling pathways, particularly ERK1/2, is at least partially independent from either CB1 or CB2 receptor in this cell line.

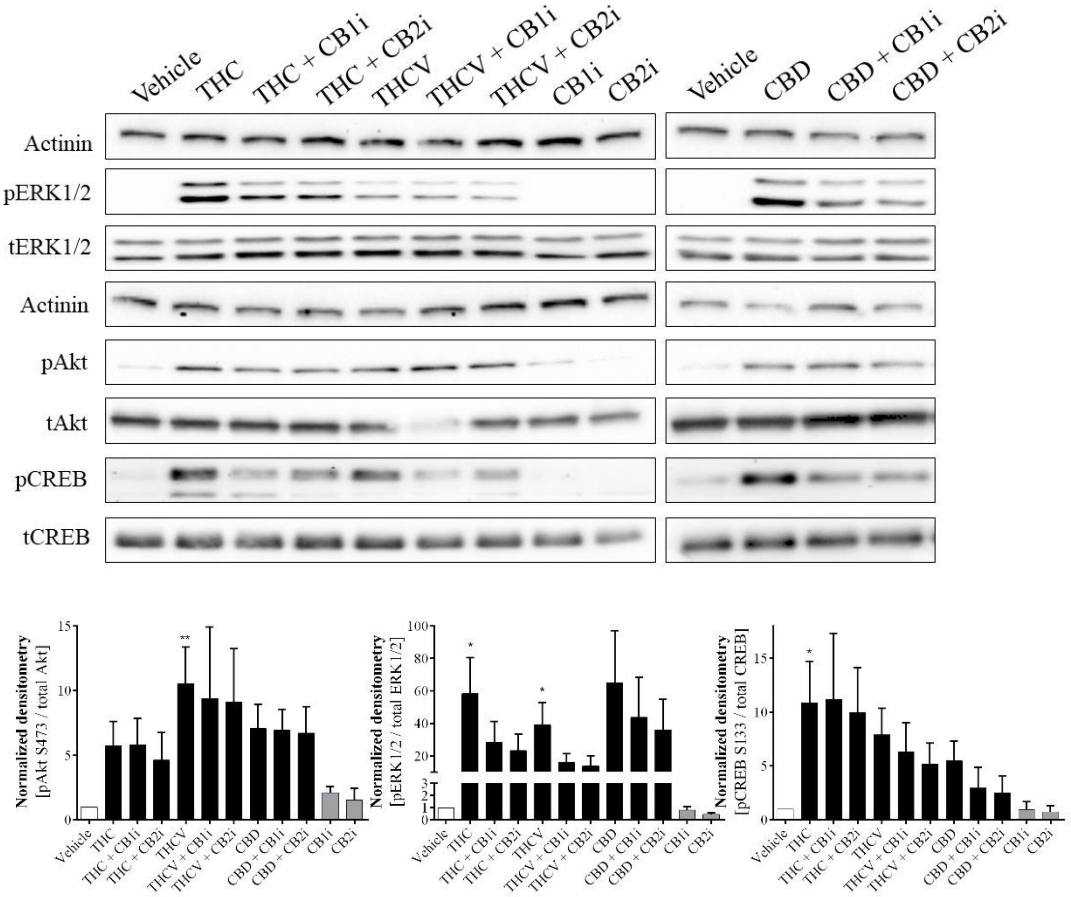



**Figure 20: Phytocannabinoids activate Akt, ERK1/2 and CREB in enteroendocrine cells**. (Top panel) Representative immunoblots of STC-1 cell lysates treated with the indicated phytocannabinoids at 5 µM, either alone or in presence of CB1 or CB2 specific antagonists (CB1i, and CB2i). Actinin is shown at the top of each independent membrane. (Bottom panel) Densitometric analysis of normalized protein signal baselined on vehicle treated lysates. (left panel) phospho Serine 473 and total Akt, (middle panel) phospho Threonine 202 / Tyrosine 204 and total p42/p44 ERK1/2, (right panel) phospho Serine 133 and total CREB. Average values ± SEM of 4 independent experiments are shown. Statistical significance was assessed by One-way ANOVA followed by Kruskal-Wallis and Dunn's multiple correction. * p-value < 0.05, ** p-value < 0.01. CBD modulates phospho S473 Akt, and THCV activates CREB, with α-values of respectively 0.0746 and 0.0613.

### 4.3.3 OEA potentiates phytocannabinoids-induced GLP-1 release

The CB1 and CB2-independent modulation of downstream signalling pathways shown in figure 20, indicate the involvement of other cannabinoid receptors, perhaps in the form of functional heteromers as introduced earlier (chapter §4.1).

Indeed, STC-1 express GPR55, CB1 and CB2, and these three receptors are known to physiologically modulate each other. In particular, GPR55 activity is known to inhibit THC mediated CB2 activation (Moreno et al., 2014), while at the same time GPR55-CB1 heteromers are more sensitive to anandamide than CB1 alone (Kargl et al., 2012). Assuming the presence of functional GPR55-CB1 and GPR55-CB2 heteromers in STC-1, we therefore hypothesized that OEA, acting as a dual GPR119 (Lauffer et al., 2009) and GPR55 agonist (Drzazga et al., 2017), could influence the responsiveness of phytocannabinoids and ideally ameliorate CB1 driven THC-mediated GLP-1 secretion. Indeed, OEA is widespread across the human body, although at the very low physiological plasmatic concentrations of 6-8 nM (Little et al., 2018), however given its localized synthesis within the intestinal mucosa, a much higher concentration gradient can be presumed. We therefore treated STC-1 cells with a concentration of 20 µM, known to induce GLP-1 secretion, and studied its impact on THC and THCV driven GLP-1 secretion. As shown in figure 21 (Panels A and B), OEA does exert a significant, more than additive increase in GLP-1 secretion especially in combination with THC (panel A). Given that THCV does not mediated GLP-1 secretion through CB1 and CB2, its combination with OEA cannot be explained by the modulation of GPR55-CB1 or GPR55-CB2 heteromers. To validate the possible the presence of similar functional heteromers of GPR119-CB1 and GPR119-CB2, we used the GPR119 specific O-LPI in combination with either THC or THCV. However, as shown in panel C and D figure 21, there appears to be no more than additive effect on GLP-1 secretion, indicating that GPR119 and either CB1 or CB2, do not functionally interact.

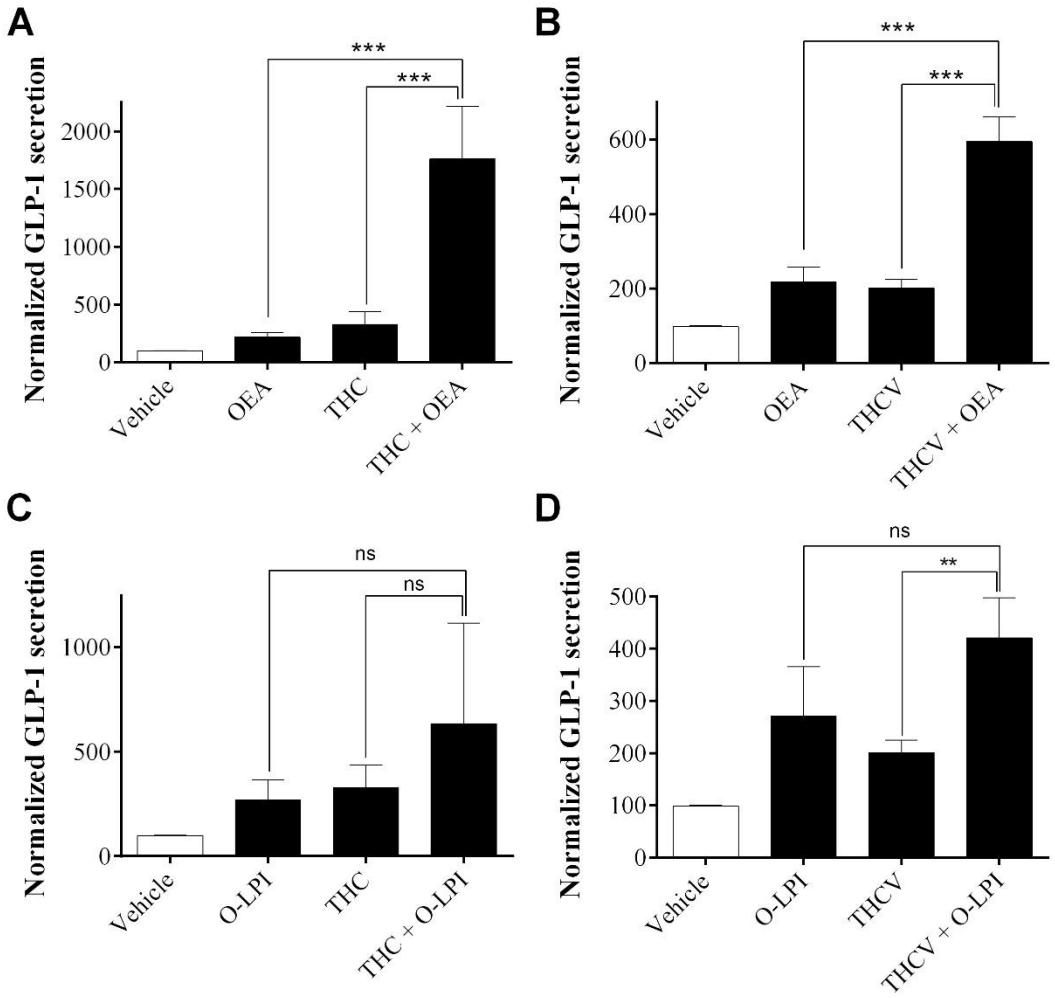



**Figure 21: OEA synergizes with the GLP-1 secreting properties of phytocannabinoids.** STC-1 cells treated with the indicated drugs, phytocannabinoids 5 µM, either alone or in combination with OEA 20 µM, or (O-LPI) Oleoyl-LPI 10 µM. Secreted GLP-1 was quantified from cleared supernatants and is shown as averages normalized on vehicle controls. Error bars represent ± SEM of at least 5 independent experiments. Oleoyl-LPI combinations are preliminary results shown as averages of 2 independent experiments ± SD. Statistical significance was assessed by one-way ANOVA corrected by multiple comparisons with Dunnet's test. Significant increase in secretion from either combination is indicated as follows. ** p-value < 0.01, *** p-value < 0.001

### 4.3.4 Enteroendocrine cells express all cannabinoid receptors

The prototypical cannabinoid receptors, CB1 and CB2, engage in different intracellular transduction pathways, which have been described in detail especially in neurons (Kendall & Yudowski, 2016). Both these receptors are known to engage with intracellular heterotrimeric G proteins of the α subclass Gi and Go, which negatively modulate the activity of Adenylate Cyclase (AC), leading to lower intracellular levels of cAMP and all downstream elements of its cascade. Beyond the Gα mediated activity, the Gβγ subunits are also released and engaged to positively modulate downstream pathways such as Akt and mTORc1. The ERK1/2 activation, as well as cAMP inhibition, cascades shared by CB2-specific activity, indicate that both pathways have evolved in conjunction to regulate the proliferation and differentiation of neurons, migration as well as inhibition proliferation and apoptosis of immune cells (Bouaboula et al., 1996; Díaz-Alonso, Guzmán, & Galve-Roperh, 2012; Kaminski, 1996; Klemke et al., 1997; Y. Kobayashi, Arai, Waku, & Sugiura, 2001; Shoemaker et al., 2005). Nonetheless, CB2 can also activate stimulatory Gα proteins in leukocytes, leading to the release of various cytokines (Saroz, Kho, Glass, Graham, & Grimsey, 2019). As shown in the previous section §4.3.2, the GLP-1 secretagogues THC and THCV, as well as CBD, can all stimulate the activation of ERK1/2, CREB and Akt in STC-1 cells. These are known signalling pathways downstream of both CB1 and CB2 receptors, nonetheless pharmacological antagonism of either CB1 or CB2 abolished only THC-mediated GLP-1 secretion (figure 19) indicating that THCV exerts its pharmacology through other receptors, possibly GPR55 as shown in (Anavi-Goffer et al., 2012). As introduced earlier, different types of enteroendocrine cells have been described to express both CB1 and CB2 at the transcript levels, and agonism of the former receptor with mAEA, acutely inhibits cAMP/PKA/CREB mediated GLP-1 secretion (Moss et al., 2012), contrasting with our observation of phytocannabinoid-driven GLP-1 secretion, that in the case of THC depends on both CB1 and CB2. We sought than to validate the expression of CB1, and CB2 across our 3 cell lines both at the transcript, and protein level (Figure 22).

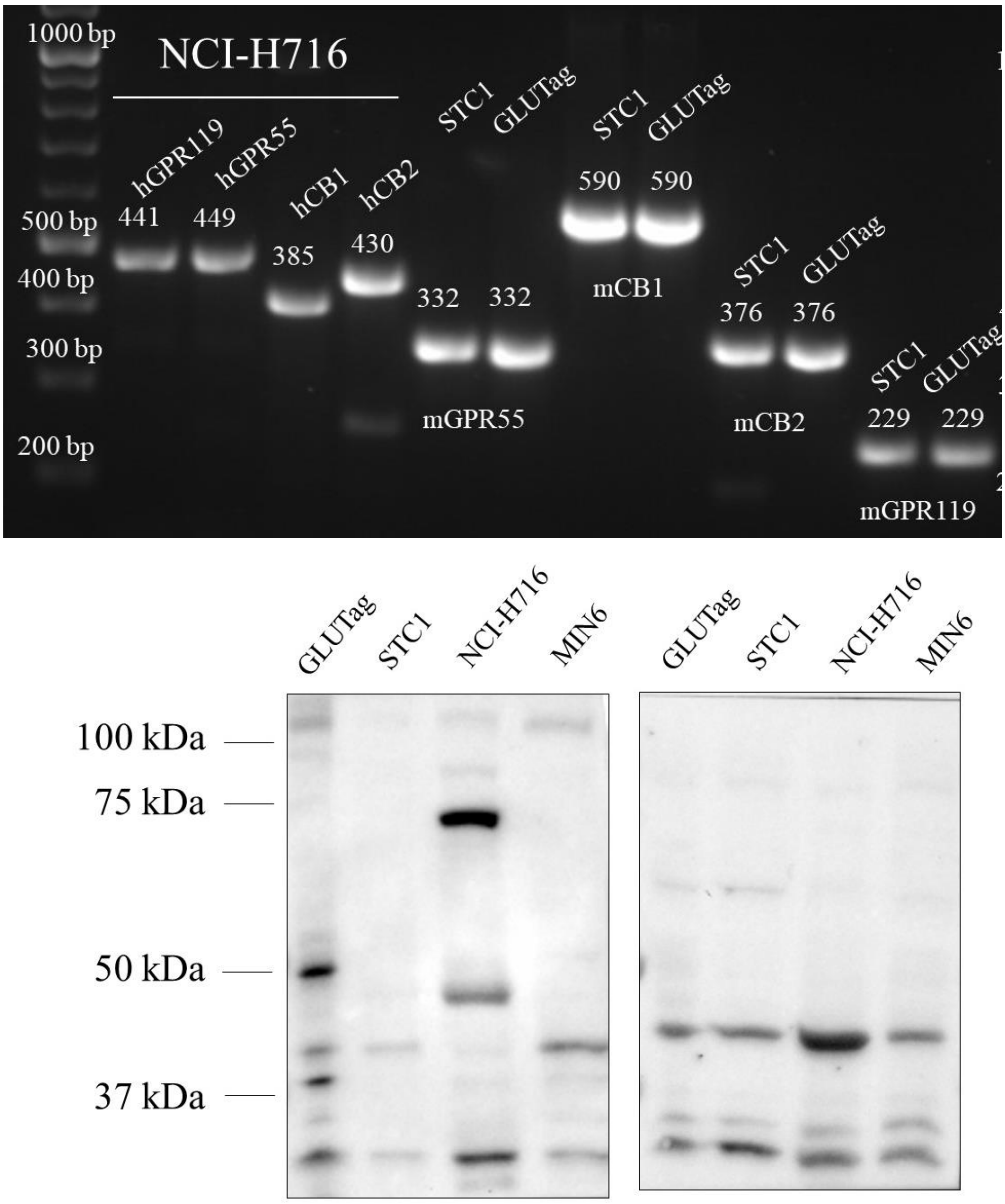



**Figure 22: Enteroendocrine cells express cannabinoid receptors.** (Top panel) Agarose gel electrophoresis of PCR products amplified from cDNA of the indicated cell lines. (Human) hGPR119, hGPR55, hCB1, hCB2 and (murine) mGPR119, mGPR55, mCB1, mCB2. Molecular marker sizes are indicated on the left side, and expected size of each amplicon is indicated next to each band. (Bottom panel) Lysates of the indicated cell lines, were immunoblotted with polyclonal antibodies raised against CB1 (left panel) and CB2 (right panel). Relative size marks are indicated on the left side.

The immunoblot analysis of CB1 protein, shows multiple bands of different size across the cell lines, to be possibly rationalized by the presence of isoforms, as described in human liver and β-cells (González-Mariscal, Krzysik-Walker, Doyle, et al., 2016), or different post-translational glycosylation patterns. In particular, the mouse derived STC-1 cell line shares its main bands at ca. 25 and 42 kDa with the mixed α-β-cell line MIN-6. GLUTag beyond the bands at 25 and 42 kDa, shows main immunoreactive bands at 40 and 50 kDa. The human derived NCI-H716 shares a main band at 25 kDa, in addition to a signal at 45 and 70 kDa. The antibody that we used, was raised against a peptide corresponding to the C-terminal portion of human CB1. The provider (Cat #ab23703, Abcam) reports that a band at ca. 60 kDa should be detected from human brain tissue, and indicates cross-reactivity with murine, rat, canine and human CB1. A recently published study using the same antibody, reports a 60 kDa band in western blots of rats heart lysates (Remiszewski et al., 2020), while another indicates two bands, one at 53 and another at 60 kDa (Lowin, Apitz, Anders, & Straub, 2015), especially from lysate of nuclear fractions, but they also show a maintained staining for the 53 kDa band in cytosolic lysates, possibly indicating endosomal-specific possibly not glycosilated isoforms of the receptor (Lowin et al., 2015). Another report using genetic CB1 knockout, validates the sensitivity of the antibody we used via electron immunogold detection in skeletal muscle mitochondria in rats (Arrabal et al., 2015). The same authors show a western blot with multiple bands below and above the main band reported at a molecular size of 52 kDa. Another study further confirms the specific and sensitive immunodetection of CB1 with the same antibody, by both heterologous overexpression, and downregulation of the receptor with siRNA technology *in vitro* (Xian, Tang, Wu, & Huang, 2018). The CB2 immunodetection that we report, appears much clearer and consistent across all L-cells, including in the αβ-cell line MIN-6, with a main band at 42 kDa, as reported in multiple studies in different cellular systems (H. Y. Zhang et al., 2019; R. Zhang et al., 2007), giving us further confidence that corroborates the functional expression of the prototypical cannabinoid receptors.

### 4.4 Conclusions and significance

The physiology and pathophysiology of the endocannabinoid system, and especially its relationship with the enteroendocrine cell system, is not completely understood at the molecular level. A layer of complexity that still needs to be dissected in a more physiological context, is the multiple contrasting evidence that indicates that THC and THCV can act both as agonists or antagonists of CB1 and CB2 *in vitro* and *in vivo*, depending on their concentration (Pertwee, 2008), with THCV showing different pharmacology *in vivo*, possibly due to its impact on the physiological metabolism of endocannabinoids (McPartland et al., 2015). In this study, we present novel evidence indicating that THC and THCV induce a dose-dependent release of GLP-1, with THC displaying a more than additive effect at 10 μM with intriguing therapeutical implications in metabolic diseases. We also show that STC-1 cells express functional CB1, and CB2 receptors, in addition to the non-canonical, GPR55 and GPR119. We report that THC-mediated GLP-1 secretion is exerted through CB1 and CB2, while THCV is not affected by the antagonism of either receptor. Surprisingly, CB1 and CB2, do not appear to significantly mediate phytocannabinoid-driven CREB, ERK1/2 and Akt activation. Indeed, CBD activates the same pathways, albeit not eliciting any significant release of GLP-1 at 5 μM. Both THC and THCV have been shown to be able to activate ERK1/2 via GPR55 (Anavi-Goffer et al., 2012; Duncan et al., 2005), indicating that this receptor might be involved. On the other hand, we also show that the endocannabinoid OEA, is possibly modulating GPR55-CB1 and GPR55-CB2 heteromers, by increasing the sensitivity to THC and THCV-mediated GLP-1 secretion. The promising proof of concept *in vitro* evidence that this study provides, lays the groundwork for future investigations. To build upon these findings, the functional expression of CB1 and CB2 along the enteroendocrine cell system must be yet demonstrated, furthermore the physical and functional interaction of these two receptors with other known proteins such as GPR55 and possibly GPR119 must also be explored. The available studies reporting the use of THC and THCV in humans, indicate opposite pharmacologies to the ones reported *in vitro*. To address these contrasting reports in the context of metabolic diseases, drug delivery of phytocannabinoids to distal GLP-1 secreting cells is required, furthermore drug dosage, especially in combination with OEA holds vast therapeutical potential, and should therefore be tailored to maximize pharmacological GLP-1 secretion.

# Chapter 5

# Chapter 5: Discussion, limitations and future directions

## 5.1 Discussion, limitations and future directions

The vast biology of GLP-1 as reviewed in chapter §1.1, cannot be replicated by currently available GLP-1 mimetic drugs on the market for type 2 diabetes and obesity treatment. Their systemic action fails to reproduce the localized, and time restricted release of GLP-1 into the portal system, before reaching the systemic circulation. We do not know have yet a complete understanding of what is the pathological role of GLP-1 in obesity and diabetes. Early reports, indicated that diabetic patients, have an impaired meal-stimulated GLP-1 secretion, nonetheless more recent evidence contrasts with this tenet (Hira et al., 2020), questioning the idea of over-stimulating systemically the GLP-1 axis with GLP-1RAs. On the other hand, novel approaches are showing that the parallel activation of GLP-1, Glucagon and GIP receptor holds a much broader therapeutical potential (Brandt, Götz, Tschöp, & Müller, 2018).

Nonetheless, the tissue specific biology of GLP-1 and other gut hormones, can indeed be replicated only with gut acting secretagogues. As discussed in chapter §1.2 cancers like PDAC remind us that metabolic diseases are much more complex, and don't just reflect a pathological condition of glucose management. Many other hormones can be secreted by the EEC, and as described in chapter §1.3 we have just started to characterize their biology, anyhow preliminary studies indicate that they modulate a vast number of biological processes.

The manuscript in chapter §2 suggests that O-LPI, is the main endogenous GPR119 ligand. This activity modulates intracellular MAPK-ERK1/2, and cAMP/PKA/CREB pathways that ultimately induce GLP-1 secretion. As further characterized in chapter §3, O-LPI administered to healthy mice at 10 mg/kg, does induce a small increase in peripheral GLP-1 levels, nonetheless future studies should characterize better approaches to deliver it quantitatively to more distal ileal L cells. This activity does appear promising especially in comparison to a structurally related GPR119 agonist, 2-Oleoylglycerol (2-OG). The authors use a metabolically stable ether form that does not increase the levels of plasmatic GIP in mice gavaged with the considerable dose of 1,000 mg/kg (Hassing, Engelstoft, et al., 2016), while GLP-1 was not even reported.

The compound ps297b, that we have characterized as an efficient GLP-1 secretagogue *in vitro* and *in vivo* at 20 mg/kg in a mouse model of diabetes, holds also a vast potential for future optimization. Indeed, its tropism for GPR119 will need to be evaluated, and if confirmed, it would represent an ideal candidate to be studied in a chronic setting. Mice models of diet-induced obesity and diabetes, will need to be administered daily, ideally with a more distally targeted dose of ps297b to maximize the release of GLP-1, and quantify the clinical impact this has on obesity, and metabolic health, and contrast it to currently available, systemically acting GLP-1 mimetics. It is important to recognize that GPR119 agonism, is indeed still a valid target especially metabolic diseases considering that its expression appears to be upregulated in these pathologies (Peiris et al., 2018).

A-LPI does not elicit GLP-1 secretion in the two cell lines of colonic origin, GLUTag and NCI-H716, while STC-1 of duodenal origin appeared much more responsive. It must also be noted that this same LPI specie, can indeed activate, and modulate GLP-1 secretion via other receptors such as the ionic channel TRPV2 (Harada et al., 2017). Therefore validation, and dissection of the pharmacology of either LPI species, will need to be replicated in a more physiological relevant cellular system. This is indeed currently under further investigation in *ex vivo* gut tissue preparations of human origin (Prof. Damien Keating, Flinder University, South Australia).

The surprising activity of THC and THCV, especially in combination with the endocannabinoid OEA, as detailed in chapter §4, provides novel evidence that the enteroendocrine cell system is under control of the endocannabinoid system. Indeed two studies indicate that the endocannabinoid OEA enhances synergistically the pharmacology of GLP-1 by physically interacting with it, therefore acting as a positive allosteric modulator (PAM) on the ligand, rather than any receptor (Brown et al., 2018; Cheng, Ho, Huang, Chou, & King, 2015). The allosteric modulation of receptors is indeed another plausible explanation for the phenomenon we observe, possible involving different cannabinoid receptors. PAM-like drugs have a valuable pharmacological profile, because they enforce a more physiological topographic and dynamic signalling cascade (Gado et al., 2019). Indeed, OEA is already on the market as a food supplement that induces satiety, therefore it could easily be implemented with drugs like GLP-1RAs or phytocannabinoids for the management of type 2 diabetes. Nonetheless before drawing further therapeutic conclusions, we need to

acknowledge that the preliminary data that we provide is based on the pharmacology of a single L-cell line which will need to be further validated, ideally in a more physiologically relevant animal or *ex-vivo* model. We have demonstrated that THC elicits a CB1- and CB2-dependent secretion of GLP-1. The activity of THCV on the other hand, is independent from CB1 and CB2, and might possibly be mediated by GPR55. Nonetheless, understanding the clinical implications of the therapeutical agonism of this receptor in metabolic diseases is yet not clear, especially considering that GPR55 expression is upregulated in obesity (Moreno-Navarrete et al., 2012), and its activation in the liver has been reported to cause steatohepatitis (Fondevila et al., 2020). Future studies will need to corroborate this tropism. The receptor mediating THCV-induced GLP-1 secretion in L-cells can be pinpointed by use of cell lines, or animals genetically deficient for the known cannabinoid receptors, namely CB1, CB2, GPR55 or GPR119. The role of the endocannabinoid system needs to be considered; indeed, its homeostatic balance is known to be highly dynamic, nonetheless its physiology cannot be entirely replicated in *in vitro* cellular systems. To obtain more translatable evidence and dissect the impact that drugs targeting cannabinoid receptors might have on the endocannabinoid system itself, *in vivo* models replicating the disease should be used (Little et al., 2018).

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Date: 6/3/2020

**Publication 4)**

**Paternoster S**, Falasca M. Dissecting the Physiology and Pathophysiology of Glucagon-Like Peptide-1. *Front Endocrinol (Lausanne)*. 2018;9:584. Published 2018 Oct 11. doi:10.3389/fendo.2018.00584



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I, *Silvano Paternoster,* researched and interpreted all the data from available scientific literature on the PUBMED database, organized, wrote and revised the whole manuscript. I also conceptualized and drew all the figures assembling the final format of the publication entitled Dissecting the Physiology and Pathophysiology of Glucagon-Like Peptide-1.

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**Publication 5)**

Arifin SA, **Paternoster S**, Carlessi R, et al. Oleoyl-lysophosphatidylinositol enhances glucagon-like peptide-1 secretion from enteroendocrine L-cells through GPR119. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2018;1863(9):1132–1141. doi:10.1016/j.bbalip.2018.06.007

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## List of secondary publications

For the following secondary publication, no parts of these studies have been presented in this thesis

Caporale C., Ranieri A.M., **Paternoster S**., Bader C.A., Falasca M., Plush S.E., Brooks D.A., Stagni S., Massi M. Photophysical and Biological Properties of Iridium Tetrazolato Complexes Functionalised with Fatty Acid Chains. Inorganics 2020, 8, 23.

Domenichini A, Edmands JS, Adamska A, Begicevic RR, **Paternoster S**, Falasca M. Pancreatic cancer tumorspheres are cancer stem-like cells with increased chemoresistance and reduced metabolic potential. *Adv Biol Regul*. 2019;72:63–77. doi:10.1016/j.jbior.2019.02.001

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