

# Intestine-selective reduction of *Gcg* expression reveals the importance of the distal gut for GLP-1 secretion



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## ABSTRACT

**Objective:** Glucagon-like peptide-1 is a nutrient-sensitive hormone secreted from enteroendocrine L cells within the small and large bowel. Although GLP-1 levels rise rapidly in response to food ingestion, the greatest density of L cells is localized to the distal small bowel and colon. Here, we assessed the importance of the distal gut in the acute L cell response to diverse secretagogues.

**Methods:** Circulating levels of glucose and plasma GLP-1 were measured in response to the administration of L cell secretagogues in wild-type mice and in mice with (1) genetic reduction of *Gcg* expression throughout the small bowel and large bowel (*Gcg*<sup>Gut<sup>-/-</sup>) and (2) selective reduction of *Gcg* expression in the distal gut (*Gcg*<sup>DistalGut<sup>-/-</sup>).</sup></sup>

**Results:** The acute GLP-1 response to olive oil or arginine administration was markedly diminished in *Gcg*<sup>Gut<sup>-/-</sup> but preserved in *Gcg*<sup>DistalGut<sup>-/-</sup> mice. In contrast, the increase in plasma GLP-1 levels following the administration of the GPR119 agonist AR231453, or the melanocortin-4 receptor (MC4R) agonist LY2112688, was markedly diminished in the *Gcg*<sup>DistalGut<sup>-/-</sup> mice. The GLP-1 response to LPS was also markedly attenuated in the *Gcg*<sup>Gut<sup>-/-</sup> mice and remained submaximal in the *Gcg*<sup>DistalGut<sup>-/-</sup> mice. Doses of metformin sufficient to lower glucose and increase GLP-1 levels in the *Gcg*<sup>Gut<sup>+/+</sup> mice retained their glucoregulatory activity, yet they failed to increase GLP-1 levels in the *Gcg*<sup>Gut<sup>-/-</sup> mice. Surprisingly, the actions of metformin to increase plasma GLP-1 levels were substantially attenuated in the *Gcg*<sup>DistalGut<sup>-/-</sup> mice.</sup></sup></sup></sup></sup></sup></sup></sup>

**Conclusion:** These findings further establish the importance of the proximal gut for the acute response to nutrient-related GLP-1 secretagogues. In contrast, we identify essential contributions of the distal gut to (i) the rapid induction of circulating GLP-1 levels in response to pharmacological selective agonism of G-protein-coupled receptors, (ii) the increased GLP-1 levels following the activation of Toll-Like Receptors with LPS, and (iii) the acute GLP-1 response to metformin. Collectively, these results reveal that distal gut *Gcg* + endocrine cells are rapid responders to structurally and functionally diverse GLP-1 secretagogues.

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## 1. INTRODUCTION

The gastrointestinal enteroendocrine system contains dozens of phenotypically distinct plurihormonal cell types linking signals from ingested nutrients and bacterial metabolites to the control of peptide hormone secretion [1,2]. Among the best characterized enteroendocrine cells, the L cell, notable for the synthesis and secretion of multiple proglucagon-derived peptides (PGDPs), has received considerable scrutiny. Indeed, posttranslational processing of proglucagon in the small intestine and large intestine yields glicentin, oxyntomodulin, GLP-1 and GLP-2 [3,4], PGDPs with roles in the regulation of gut motility, mucosal integrity, nutrient absorption, the control of appetite, and nutrient assimilation [5]. Moreover, the clinical development of

GLP-1R agonists for the treatment of diabetes and obesity and a GLP-2R agonist for the therapy of intestinal failure [6] has heightened interest in understanding the translational biology of gut PGDP secretion and action.

The analysis of the location and distribution of L cells suggests an increasing gradient of the L cell number and PGDP content from the proximal gut to the distal gut, with the highest levels of *Gcg* mRNA transcripts and PGDPs being detected within the terminal ileum and colon [7–9]. Paradoxically, however, plasma levels of gut PGDPs, exemplified by GLP-1, increase within minutes of food ingestion, timing inconsistent with the notion that ingested complex macronutrients would be enzymatically digested and transported to the distal gut. Accordingly, several competing theories have evolved to reconcile

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these observations. First, considerable evidence, largely from pre-clinical studies, supports the existence of a proximal-distal gut axis, whereby neural or hormonal signals are rapidly conveyed to distal gut L cells enabling GLP-1 secretion [10]. A second hypothesis invokes the functional importance of proximal gut L cells in the jejunum as sufficient to generate a rapid initial rise in GLP-1 secretion accounting for increased circulating GLP-1 levels within minutes of food intake [9,11,12].

More recently, the putative importance of pancreatic islet GLP-1 has received renewed attention. Although the levels of processed bioactive GLP-1 are very low in the normal mouse and human pancreas [13], islets examined *ex vivo* secrete GLP-1 [14]. Moreover, the development of diabetes and/or pancreatic injury has been associated with increased expression of prohormone convertase-1 (Pcsk1) in islet  $\alpha$ -cells, accompanied by the enhanced biosynthesis and liberation of bioactive islet GLP-1 [15]. Strikingly, mice with selective reactivation of *Gcg* expression in the pancreas reveal an important glucoregulatory role for islet glucagon and/or GLP-1 production [16], rekindling interest in the physiological and pathological circumstances under which the pancreatic islets may represent an important source of glucoregulatory PGDPs, including GLP-1.

To better understand the relative role of the proximal gut and the distal gut in the generation of circulating GLP-1, we recently generated lines of mice with substantial elimination of *Gcg* expression in both the small intestine and large intestine (*Gcg*<sup>Gut<sup>-/-</sup>) or more selective loss of distal gut *Gcg* expression in the terminal ileum and colon (*Gcg*<sup>DistalGut<sup>-/-</sup>) [13]. The analysis of these mice reinforced the importance of the gut as the predominant source of circulating GLP-1. Unexpectedly, circulating levels of GLP-1 were also lower in the fasting state, and glucose tolerance was impaired in *Gcg*<sup>DistalGut<sup>-/-</sup> mice [13], prompting questions about the relative contributions of the proximal and distal gut to the control of GLP-1 levels in the inter-prandial state and following nutrient ingestion. Here, we examined the contribution of distal gut *Gcg* expression to the acute response to ingested nutrients such as the amino acid arginine and olive oil, as well as pharmacological administration of an oral GPR119 agonist and parenteral administration of a melanocortin 4 receptor (MC4R) agonist, lipopolysaccharide (LPS), and metformin. Our findings reveal the unexpected importance of the distal gut *Gcg* system for the rapid initial response to functionally diverse L cell secretagogues.</sup></sup></sup>

## 2. MATERIALS AND METHODS

### 2.1. Animals

All studies were conducted in accordance with protocols approved by the Sinai Health System and The Centre for Phenogenomics (TCP, Toronto, ON, Canada). *In vivo* studies were performed predominantly in adult male mice beginning at 12 weeks old. As we did not observe sex-specific differences in secretagogue responses, in some cases, littermate-matched female mice were also used as appropriately noted in Figure Legends. The mice were housed in groups of up to five in microisolator cages in a pathogen-free facility on a 12/12 light–dark cycle. All animals had *ad libitum* access to irradiated rodent chow (18% kcal from fat, Harlan Teklad, Mississauga, ON, Canada) and sterile water unless otherwise noted.

*Gcg*<sup>Gut<sup>-/-</sup>, *Gcg*<sup>DistalGut<sup>-/-</sup>, and their littermate control *+/+* mice were generated and genotyped as described previously [13,16]. Following the conclusion of experiments, *Gcg* knockdown was assessed in segments of the gut and pancreas to verify the expression and to remove animals with unintended germline deletion, as described previously [13].</sup></sup>

### 2.2. Acute *in vivo* studies

To assess rapid plasma GLP-1 responses to specified secretory agents (Table 1), mice were subjected to acute experiments to detect peak plasma GLP-1 levels independent of normal food intake. Mice were fasted overnight (~16 h) in wire-bottom cages to minimize the ingestion of feces and bedding, with normal access to water. After the fasting period, mice were given a single bolus of the secretagogue by either oral gavage or intraperitoneal injection. Blood glucose was measured using a glucometer (Aviva glucometer, Accu-Chek, Roche, Toronto, ON, Canada), and blood was collected in lithium-heparin-coated capillary Microvette tubes (Sarstedt, Inc.) at the specified times, including at time 0, immediately before secretagogue treatment. The blood was quickly mixed with 10% TED (vol/vol) (5,000 KIU/mL aprotinin) (Sigma A6279, CAS #9087-70-1), 1.2 mg/mL EDTA, and 0.1 nmol/L diprotin A, (Sigma D3822, CAS #90614-45-5). Plasma was then isolated by centrifugation and then stored at  $-80^{\circ}\text{C}$  until subsequent hormone analysis.

### 2.3. Glucose tolerance tests

Prior to testing, mice were fasted overnight (~16 h). Using a triple-crossover study design, all mice were randomized to receive an oral gavage of water (vehicle), 50 mg/kg metformin, or 150 mg/kg metformin. Treatments were switched for subsequent tests, occurring 2 weeks apart for sufficient recovery/washout, so that all animals received all treatments. Sixty minutes after treatment, a glucose bolus was provided. A dose of 2 g/kg D-glucose in water (Sigma, catalog# G8270) was used for oral glucose tolerance tests (oGTTs), and 1.5 g/kg D-glucose in PBS was used for intraperitoneal glucose tolerance tests (ipGTTs). Blood glucose was monitored at specified time points, and ~60  $\mu\text{L}$  of the whole blood was collected via tail vein at time 0 (relative to glucose administration), 15 min, and 60 min and mixed with 10% TED. Plasma was separated and stored at  $-80^{\circ}\text{C}$  for subsequent hormone analysis.

### 2.4. Hormone assays

Plasma samples frozen at  $-80^{\circ}\text{C}$  were thawed on ice on the day of hormone analyses. Insulin was measured using the Mouse Ultrasensitive Insulin ELISA (Alpco, 80-INSMSU-E01, 5  $\mu\text{L}$  volume). Total GLP-1 was measured using V-PLEX GLP-1 Total Kit (Mesoscale Discovery, K1503PD, 25  $\mu\text{L}$  volume). Unknowns were extrapolated from standard curves run in duplicate, according to provided protocols.

### 2.5. Data analysis

All graphs were produced and data analyzed using GraphPad Prism 7.0e. All graphical values are presented as mean  $\pm$  SD. Statistical significance was calculated using either a two-tailed *t*-test or ANOVA with paired Tukey's multiple comparison test, where appropriate. A *P* value  $< 0.05$  was considered statistically significant.

## 3. RESULTS

To elucidate the importance of the distal gut in acute GLP-1 secretion, we studied *Gcg*<sup>Gut<sup>-/-</sup> and *Gcg*<sup>DistalGut<sup>-/-</sup> mice and their respective wild-type littermate controls. *Gcg*<sup>Gut<sup>-/-</sup> mice were generated by crossing *Gcg*<sup>fllox/fllox</sup> mice with *Vil-Cre* mice and exhibit markedly reduced *Gcg* expression in both small bowel and large bowel [13]. *Gcg*<sup>DistalGut<sup>-/-</sup> mice were generated by crossing *Gcg*<sup>fllox/fllox</sup> mice with *Cdx2-Cre* mice and display substantial attenuation of *Gcg* expression in the distal ileum and colon [13]. For all experiments, wild-type, *Gcg*<sup>fllox/fllox</sup>, *Cdx2-Cre*, or *Vil-Cre* littermates were pooled and studied as controls. To assess the consequences of reduced *Gcg* expression on the secretory</sup></sup></sup></sup>

**Table 1** — GLP-1 secretagogues.

Treatment	Description	Route of Admin	Vehicle	Dose	Manufacturer (CAS#)
Olive oil	Macronutrient	Oral	None	200 $\mu$ L	Sigma 01514 (8000-25-0)
L-Arginine	Amino acid	Oral	Water	2 g/kg	Sigma A5006 (74-79-3)
AR231453	GPR119 agonist	Oral	80% Polyethyleneglycol-400, 10% Tween-80, 10% ethanol	10 mg/kg	PEG 400, Sigma P3265 (25322-68-3) Tween-80 Sigma P4780 (9005-65-6) Abcam (CAS# 733750-99-7) Bachem (819048-44-7)
LY2112688	Melanocortin-4 receptor (MC4R) peptide agonist	Intraperitoneal	PBS	3 mg/kg	Bachem (819048-44-7)
Lipopolysaccharide O55:B5, O111:B4	Inflammatory stimulus	Intraperitoneal	PBS	1 mg/kg	Sigma L2880 and L3024
Metformin	Common diabetes drug	Oral	Water	50 and 150 mg/kg	MP Biomedicals, Solon OH, (1115-70-4)

capacity of gut L cells, we focused on GLP-1 due to its metabolic importance and the simultaneous availability of sensitive validated assays for the detection of circulating GLP-1 in mice [17].

### 3.1. Distal Gut *Gcg* expression is dispensable for nutrient-stimulated increments in plasma GLP-1

Prior studies determined that the secretion of GLP-1 following oral glucose challenge originates predominantly from the proximal gut [13]. We then assessed the GLP-1 levels in response to olive oil or arginine, known as stimulators of PGDP secretion [17]. Olive oil administered by oral gavage produced a modest rise in blood glucose excursion, consistent with a modest stress response, which was similar across all genotypes (Figure 1A,B, E, and F). In contrast to robust plasma GLP-1 excursions in *Gcg*<sup>Gut+/+</sup> mice, *Gcg*<sup>Gut-/-</sup> mice failed to increase the levels of plasma total GLP-1 (tGLP-1) after olive oil (Figure 1C,D). Conversely, plasma GLP-1 levels were not different in *Gcg*<sup>DistalGut+/+</sup> versus *Gcg*<sup>DistalGut-/-</sup> mice (Figure 1G,H), revealing that distal gut *Gcg* expression is not required for acute increments in plasma GLP-1 levels after lipid ingestion.

We next challenged mice with oral arginine, an amino acid known to potently stimulate GLP-1 secretion [18]. Blood glucose levels rose modestly following arginine gavage in all genotypes (Figure 1I, J, M, and N). Notably, *Gcg*<sup>Gut-/-</sup> mice exhibited a markedly reduced plasma GLP-1 excursions in response to arginine (Figure 1K and L). In contrast, maximal GLP-1 levels were not different after arginine administration in *Gcg*<sup>DistalGut+/+</sup> versus *Gcg*<sup>DistalGut-/-</sup> mice (Figure 1 O, P). Taken together with prior studies of glucose-stimulated GLP-1 secretion [13], oral nutrient ingestion, as exemplified by glucose, olive oil, and arginine, stimulates GLP-1 predominantly through L cells residing in the proximal murine gut.

### 3.2. Distal Gut *Gcg* expression is required for GPR119 and MC4R agonist-stimulated increases in plasma GLP-1 levels

We next examined the intestinal sites important for the transduction of GLP-1 secretory signals pursuant to the activation of two L-cell-associated G-protein-coupled receptors (GPCRs). GPR119 is activated by multiple derivatives of dietary fatty acids and regulates metabolism in part via the stimulation of incretin secretion [19–21]. Oral gavage of the GPR119 agonist, AR231453, had no meaningful effect on the glycemic excursion in *Gcg*<sup>DistalGut+/+</sup> versus *Gcg*<sup>DistalGut-/-</sup> mice (Figure 2A,B). Surprisingly, the rise in plasma GLP-1 levels after oral AR231453 was markedly attenuated in *Gcg*<sup>DistalGut-/-</sup> mice, implicating the importance of the distal gut for maximal L cell responses to acute GPR119 agonism (Figure 2C,D).

The MC4R is a GPCR expressed primarily in the brain, yet MC4R has also been detected outside the central nervous system, including within mouse and human L cells [22]. Intraperitoneal injection of the

MC4R-selective agonist LY2112688 had little impact on blood glucose levels in *Gcg*<sup>DistalGut+/+</sup> and *Gcg*<sup>DistalGut-/-</sup> mice (Figure 2E,F). Plasma GLP-1 levels were increased following LY2112688 in *Gcg*<sup>DistalGut+/+</sup> mice, yet there was no GLP-1 response in *Gcg*<sup>DistalGut-/-</sup> mice (Figure 2G,H). Hence, maximal GLP-1 excursions to either GPR119 or MC4R agonism require *Gcg* expression within the distal gut.

### 3.3. LPS requires distal Gut *Gcg* expression for maximal increases in plasma GLP-1

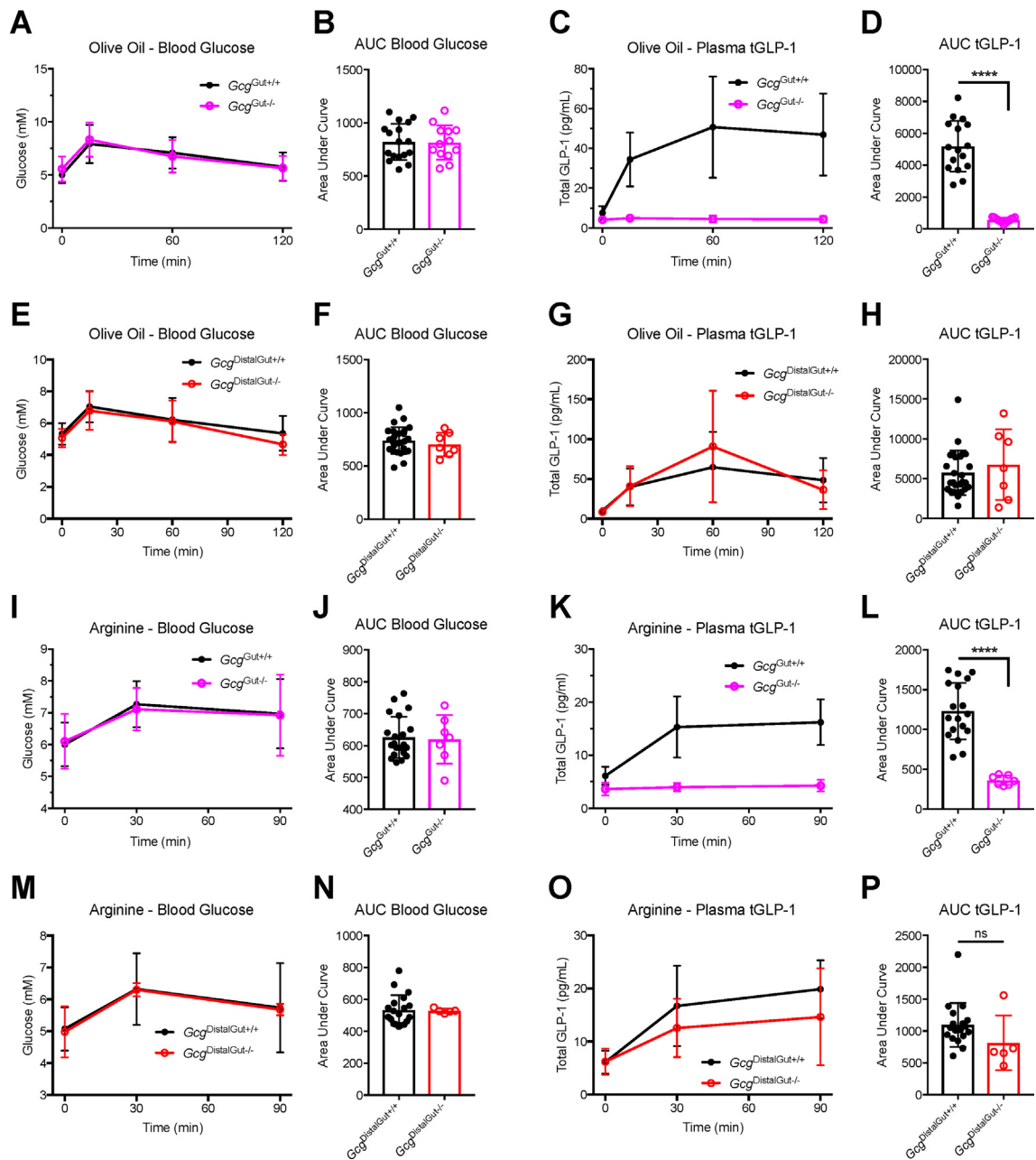
There is considerable evidence that links the administration of bacteria-derived LPS to the augmentation of L cell GLP-1 secretion in mice and humans [23,24]. Intraperitoneal injection of LPS produced a modest reduction in blood glucose levels 3 h after treatment to a similar extent in all genotypes tested (Figure 3A,B, E, and F). The increase in plasma GLP-1 after LPS was clearly dependent on gut *Gcg* expression as it was virtually extinguished in *Gcg*<sup>Gut-/-</sup> mice (Figure 3C,D). Intriguingly, the increase in plasma GLP-1 in LPS-treated mice remained blunted in *Gcg*<sup>DistalGut-/-</sup> mice (Figure 3G,H). These results suggest that LPS-mediated GLP-1 secretion originates from the gut, with a component of the response arising from the distal gut.

### 3.4. Distal Gut *Gcg* expression is essential for acute metformin-induced rises in plasma GLP-1

Among the many actions of metformin that may contribute to its glucoregulatory properties is the enhancement of gut GLP-1 secretion [25–27]. Oral metformin administration at a dose of 150 mg/kg produced a small reduction in blood glucose that was similar across genotypes (Figure 4A,B, E, and F). Notably, metformin failed to increase plasma GLP-1 levels in *Gcg*<sup>Gut-/-</sup> mice (Figure 4C,D). Unexpectedly, metformin also did not increase plasma GLP-1 levels in *Gcg*<sup>DistalGut-/-</sup> mice (Figure 4G,H). Hence, distal gut *Gcg* expression is required for the acute metformin-induced increment in plasma GLP-1.

### 3.5. Gut *Gcg* expression and increases in plasma GLP-1 are not required for metformin-mediated glucoregulation

To determine if a metformin-induced increment in plasma GLP-1 was required for glucoregulation, we treated *Gcg*<sup>Gut+/+</sup> and *Gcg*<sup>Gut-/-</sup> mice with oral metformin at doses of 50 and 150 mg/kg 60 min prior to an oral (oGTT) and intraperitoneal glucose tolerance tests (ipGTT). In the context of an oGTT, a dose-dependent reduction in blood glucose was observed in *Gcg*<sup>Gut+/+</sup> mice (Figure 5A,B). The various metformin doses did not impact insulin levels (Figure 5C); however, plasma GLP-1 levels were increased after metformin administration (Figure 5D). Notably, plasma glucose and insulin responses to metformin were similar in *Gcg*<sup>Gut+/+</sup> and *Gcg*<sup>Gut-/-</sup> mice (Figure 5E–G), despite the lack of increase in plasma GLP-1 levels in *Gcg*<sup>Gut-/-</sup> mice (Figure 5H). Consistent with the oGTT results, metformin lowered glucose during an



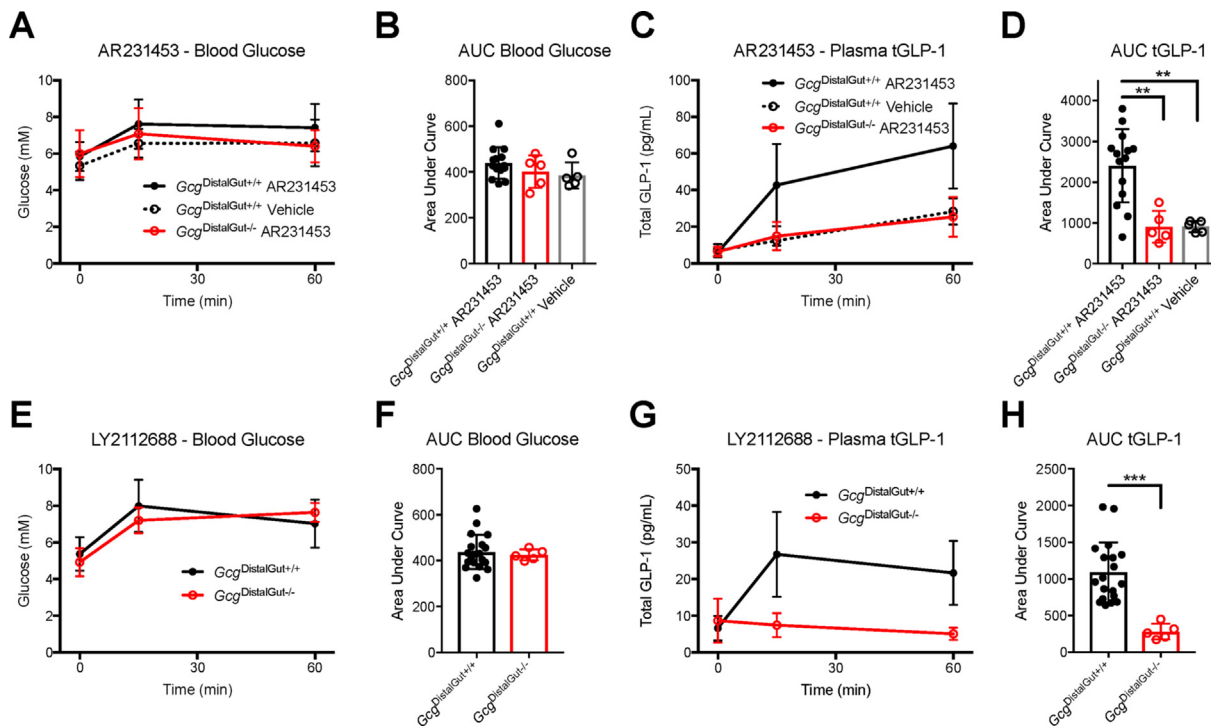
**Figure 1: Distal gut *Gcg* expression is not required for nutrient-stimulated plasma GLP-1 excursion.** *Gcg*<sup>Gut-/-</sup>, *Gcg*<sup>DistalGut-/-</sup>, and littermate control mice were given an oral gavage of olive oil (200  $\mu$ L) or arginine (2 g/kg BW) at time zero, with subsequent monitoring of blood glucose and total GLP-1 (tGLP-1) levels. (A, B) Time course of blood glucose levels during olive oil stimulus and corresponding AUC analysis in *Gcg*<sup>Gut+/+</sup> and *Gcg*<sup>Gut-/-</sup> mice. (C, D) Time course of total tGLP-1 levels during olive oil stimulus and corresponding AUC analysis in *Gcg*<sup>Gut+/+</sup> and *Gcg*<sup>Gut-/-</sup> mice. (E, F) Time course of blood glucose levels during olive oil stimulus and corresponding AUC analysis in *Gcg*<sup>DistalGut+/+</sup> and *Gcg*<sup>DistalGut-/-</sup> mice. (G, H) Time course of total tGLP-1 levels during olive oil stimulus and corresponding AUC analysis in *Gcg*<sup>DistalGut+/+</sup> and *Gcg*<sup>DistalGut-/-</sup> mice. (I, J) Time course of blood glucose levels during arginine stimulus and corresponding AUC analysis in *Gcg*<sup>Gut+/+</sup> and *Gcg*<sup>Gut-/-</sup> mice. (K, L) Time course of total tGLP-1 levels during arginine stimulus and corresponding AUC analysis in *Gcg*<sup>Gut+/+</sup> and *Gcg*<sup>Gut-/-</sup> mice. (M, N) Time course of blood glucose levels during arginine stimulus and corresponding AUC analysis in *Gcg*<sup>DistalGut+/+</sup> and *Gcg*<sup>DistalGut-/-</sup> mice. (O, P) Time course of total tGLP-1 levels during arginine stimulus and corresponding AUC analysis in *Gcg*<sup>DistalGut+/+</sup> and *Gcg*<sup>DistalGut-/-</sup> mice. *Gcg*<sup>Gut+/+</sup>, n = 17–21 (males); *Gcg*<sup>Gut-/-</sup>, n = 7–13 (males); *Gcg*<sup>DistalGut+/+</sup>, n = 19–26 (males + females); *Gcg*<sup>DistalGut-/-</sup>, n = 5–7 (males + females). Statistical significance was determined using the two-tailed *t*-test. \*\*\*\**P* < 0.0001.

intraperitoneal glucose challenge independent of the changes in plasma GLP-1 (Figures 5I–5P). Taken together, these results suggest that the acute glucoregulatory actions of metformin do not require an increase in plasma levels of GLP-1.

#### 4. DISCUSSION

The findings that *Gcg* mRNA transcripts and corresponding levels of PGDPs, including GLP-1, are distributed throughout the small bowel





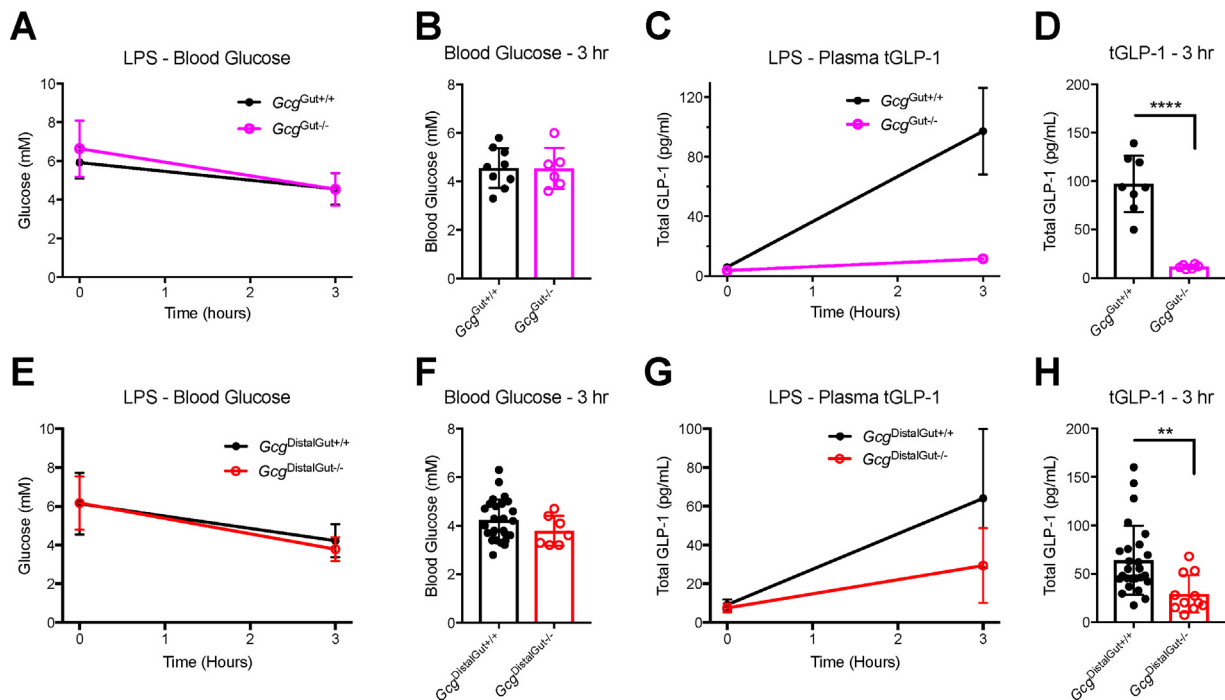
**Figure 2: Distal gut *Gcg* expression is required for acute GPR119- and MC4R-stimulated increases in plasma GLP-1.** *Gcg<sup>DistalGut+/+</sup>* and *Gcg<sup>DistalGut-/-</sup>* mice were treated with either an oral gavage of the GPR119 agonist, AR231453 (10 mg/kg BW), vehicle alone, or an intraperitoneal injection of the MC4R agonist, LY2112688 (3 mg/kg BW), at time zero. Blood glucose and total GLP-1 (tGLP-1) were measured at the indicated time points. (A, B) Time course of blood glucose levels during AR231453 stimulus and corresponding AUC analysis. (C, D) Time course of total tGLP-1 levels during AR231453 stimulus and corresponding AUC analysis. (E, F) Time course of blood glucose levels during LY2112688 stimulus and corresponding AUC analysis. (G, H) Time course of total tGLP-1 levels during LY2112688 stimulus and corresponding AUC analysis. For AR231453 studies: *Gcg<sup>DistalGut+/+</sup>* AR231453, n = 14 (males + females); *Gcg<sup>DistalGut+/+</sup>* Vehicle, n = 5 (males + females); *Gcg<sup>DistalGut-/-</sup>* AR231453, n = 5 (males + females). For LY2112688 studies: *Gcg<sup>DistalGut+/+</sup>*, n = 19 (males + females); *Gcg<sup>DistalGut-/-</sup>*, n = 5 (males + females). Statistical significance was determined using one-way ANOVA (panels B, D) or two-tailed *t*-test (panels F, H). \*\**P* < 0.01; \*\*\**P* < 0.001.

and large bowel have engendered considerable debate toward understanding the relative importance of the proximal and the distal guts in the control of rapid meal-stimulated increases in circulating levels of GLP-1. Considerable evidence suggests that nutrient-stimulated hormonal mediators such as glucose-dependent insulinotropic polypeptide (GIP) liberated from the proximal gut or signals conveyed via neural transmission involving acetylcholine and gastrin-releasing peptide contribute to the amplification of L cell GLP-1 secretion from the distal gut in preclinical studies [10]. Furthermore, luminal perfusion of the proximal and the distal small bowel revealed a considerably greater increment in circulating GLP-1 following the stimulation of distal versus proximal gut L cells [28]. On the other hand, the levels of *Gcg* mRNA transcripts and GLP-1 content in the human proximal small bowel were not substantially different from those found more distally, highlighting potential contributions of the proximal small bowel to GLP-1 biosynthesis and secretion [9]. Moreover, the capacity of the isolated proximal rat gut to secrete GLP-1 in response to GRP or luminal peptone was not meaningfully different relative to the amounts of GLP-1 secreted by distal gut segments in acute short-term studies [12]. Hence, these contrasting findings illustrate the challenges in the interpretation of the relative importance of the proximal and the distal gut for the acute GLP-1 response to various secretagogues.

To better understand the contributions of the distal gut to the acute increase in circulating GLP-1 levels evident following the administration of nutrients and pharmacological agents, we employed mouse lines engineered to exhibit a substantial reduction of the *Gcg*

expression throughout the small bowel and large bowel or predominantly in the terminal ileum and colon [13]. We previously determined that GLP-1 levels rose briskly following oral administration of glucose to *Gcg<sup>DistalGut-/-</sup>* mice, indirectly affirming the functional competence of proximal gut L cells for maximal glucose-regulated GLP-1 secretion. The current studies using the administration of olive oil or arginine further substantiate the redundancy of distal gut L cells for nutrient-stimulated GLP-1 secretion, as plasma levels of GLP-1 were not substantially different after these nutrient challenges in *Gcg<sup>DistalGut+/+</sup>* versus *Gcg<sup>DistalGut-/-</sup>* mice.

In marked contrast, plasma GLP-1 responses to the GPR119 agonist AR231453, or the MC4R agonist LY2112688, were substantially attenuated in *Gcg<sup>DistalGut-/-</sup>* mice. A functional GPR119 recognized by several lipid-related ligands, including cannabinoids, monoacylglycerols such as 2-oleoyl glycerol (2-OG), and oleoylethanolamide, is expressed within murine enteroendocrine L cells in the small bowel and large bowel [29,30]. However, the relative contributions of regional L cell populations to the rise in circulating GLP-1 levels following acute GPR119 activation have not previously been determined. To answer this question, we used the validated pharmacological GPR119 agonist AR231453, an agent we and others have previously shown to be highly selective for the GPR119 receptor [19–21]. Remarkably, although AR231453 was previously shown to stimulate the secretion of GIP, a hormone predominantly secreted from the proximal intestine [19,20], plasma levels of GLP-1 did not rise in response to oral AR231453 in *Gcg<sup>DistalGut-/-</sup>* mice. Intriguingly, the



**Figure 3: LPS requires distal gut *Gcg* expression for maximal increases in plasma GLP-1.** Adult *Gcg*<sup>Gut-/-</sup>, *Gcg*<sup>DistalGut-/-</sup>, and littermate control mice were subjected to acute systemic inflammation with a single intraperitoneal injection of lipopolysaccharide (LPS, 1 mg/kg BW) given at time zero. Blood glucose and total GLP-1 (tGLP-1) were monitored at the start and 3 h later. (A) Time course of blood glucose levels during LPS stimulus, and (B) blood glucose levels at the 3 h time point after LPS treatment in *Gcg*<sup>Gut+/+</sup> and *Gcg*<sup>Gut-/-</sup> mice. (C) Time course of total tGLP-1 levels during LPS stimulus and (D) total tGLP-1 levels at the 3 h time point after LPS treatment in *Gcg*<sup>Gut+/+</sup> and *Gcg*<sup>Gut-/-</sup> mice. (E) Time course of blood glucose levels during LPS stimulus, and (F) blood glucose levels at the 3 h time point after LPS treatment in *Gcg*<sup>DistalGut+/+</sup> and *Gcg*<sup>DistalGut-/-</sup> mice. (G) Time course of total tGLP-1 levels during LPS stimulus and (H) total tGLP-1 levels at the 3 h time point after LPS treatment in *Gcg*<sup>DistalGut+/+</sup> and *Gcg*<sup>DistalGut-/-</sup> mice. *Gcg*<sup>Gut+/+</sup>, n = 9 (males); *Gcg*<sup>Gut-/-</sup>, n = 6 (males); *Gcg*<sup>DistalGut+/+</sup>, n = 26 (males + females); *Gcg*<sup>DistalGut-/-</sup>, n = 7-11 (males + females). Statistical significance was determined using the two-tailed *t*-test. \*\**P* < 0.01; \*\*\*\**P* < 0.0001.

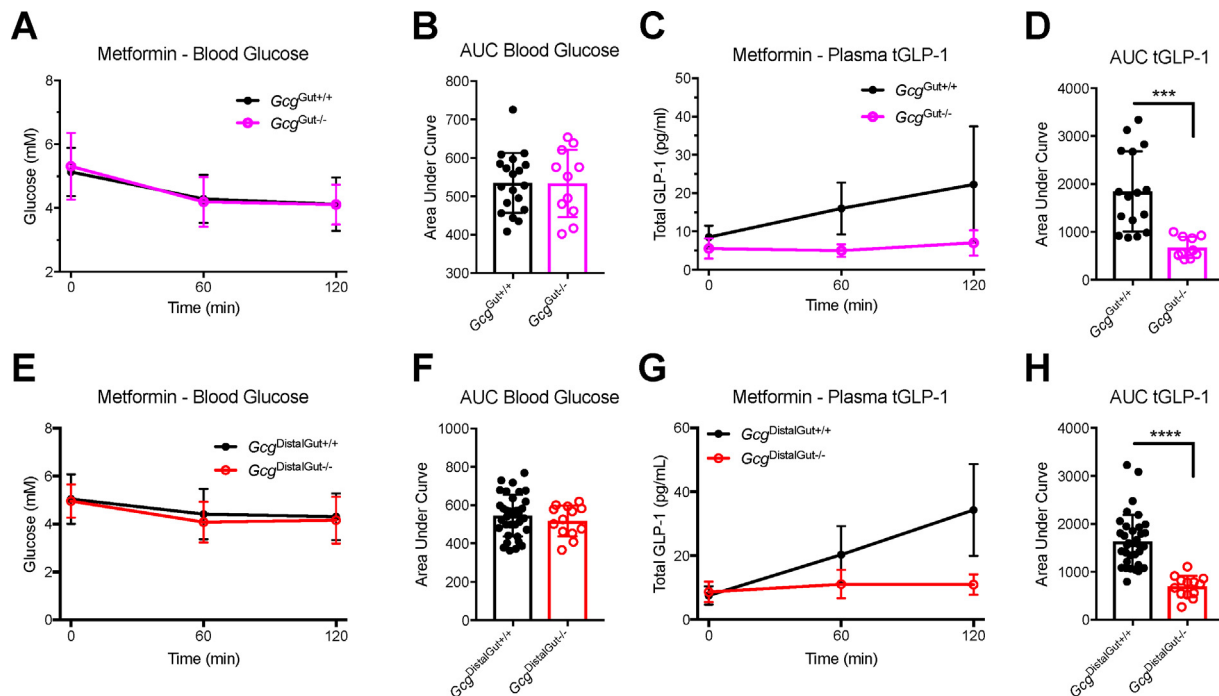
stimulation of GLP-1 secretion by enteral olive oil, while appearing to be mediated through the activation of proximal gut L cells in the current experiments, might theoretically have required contributions to the GLP-1 secretion derived from lipid metabolites acting through GPR119 in the distal gut. Collectively, our findings are consistent with established actions of GPR119 agonists acting predominantly through mouse and human colonic L cells [30,31] and further highlight the importance of the distal gut L cell as an important target for GPR119-dependent GLP-1 secretion.

MC4R is an extensively studied receptor mediating the regulation of food intake and body weight through central and peripheral mechanisms including control of food intake and energy expenditure. Studies in mice and rats demonstrated that GLP-1 reduces food intake through MC4R-independent pathways [32,33], providing a rationale for the successful use of GLP-1R agonists in the treatment of human subjects with obesity arising from genetic mutations in the MC4R pathway [34]. MC4R has also been localized to enteroendocrine L cells, most notably in the colon, functionally linked to the secretion of L cell peptides and increased circulating levels of peptide YY and GLP-1 [22]. Consistent with these findings, the MC4R agonist LY2112688 rapidly increased the circulating levels of GLP-1 in *Gcg*<sup>DistalGut+/+</sup> but not in *Gcg*<sup>DistalGut-/-</sup> mice. Hence, although MC4R is expressed within cell populations of the stomach, small bowel, and large bowel [22], the distal gut is required for the acute GLP-1 response to the MC4R agonism.

Beyond roles in transduction of nutrient-related signals, substantial evidence supports a simultaneous role for L cells as pathogen sensors [35], linking inflammatory signals, including bacterial metabolites and

cell wall products exemplified by LPS, to GLP-1 secretion in animals and humans [23,24,36]. Notably, transient vascular ischemia and mesenteric injury in the proximal small bowel of mice and human subjects produce a rapid rise in plasma GLP-1 levels [23]. Furthermore, LPS increased GLP-1 secretion from small bowel-derived STC-1 L cells [23], raising the possibility that both the small and large bowel L cells are capable of sensing tissue injury and inflammation linked to the enhanced GLP-1 secretion. Our findings reveal that the LPS-induced increment in circulating GLP-1 levels was attenuated, but not abolished, in *Gcg*<sup>DistalGut-/-</sup> mice. Hence, it seems likely that LPS engages L cells in both the proximal and distal guts to enhance GLP-1 secretion.

The pleiotropic actions of metformin have also been linked to the acute GLP-1 secretion in preclinical studies [37] and in humans with type 2 diabetes [25]. Consistent with the importance of the gastrointestinal tract as a site of metformin action, gut-targeted metformin produces a rapid rise in plasma GLP-1 levels detectable within 60 min of metformin administration [38]. Moreover, intraduodenal metformin administration rapidly lowered glycemia and hepatic glucose production in rats through GLP-1R-dependent mechanisms [39], further highlighting the potential importance of the small bowel as a target for metformin-GLP-1 interactions. Nevertheless, our current findings reveal that low doses of enteral metformin are still capable of lowering glucose independent of any detectable changes in plasma GLP-1 levels in *Gcg*<sup>Gut-/-</sup> mice. These observations are consistent with previous results demonstrating the preservation of the glucoregulatory actions of a range of metformin concentrations in *Glp1r*<sup>-/-</sup> mice [27]. Although



**Figure 4: Distal gut *Gcg* expression is essential for acute metformin-induced rises in plasma GLP-1.** Blood glucose and total GLP-1 (tGLP-1) levels in *Gcg*<sup>Gut-/-</sup>, *Gcg*<sup>DistalGut-/-</sup>, and littermate control mice following oral gavage of metformin (150 mg/kg BW). (**A, B**) Time course of blood glucose levels during metformin stimulus and corresponding AUC analysis in *Gcg*<sup>Gut+/+</sup> and *Gcg*<sup>Gut-/-</sup> mice. (**C, D**) Time course of total tGLP-1 levels during metformin stimulus and corresponding AUC analysis in *Gcg*<sup>Gut+/+</sup> and *Gcg*<sup>Gut-/-</sup> mice. (**E, F**) Time course of blood glucose levels during metformin stimulus and corresponding AUC analysis in *Gcg*<sup>DistalGut+/+</sup> and *Gcg*<sup>DistalGut-/-</sup> mice. (**G, H**) Time course of total tGLP-1 levels during metformin stimulus and corresponding AUC analysis in *Gcg*<sup>DistalGut+/+</sup> and *Gcg*<sup>DistalGut-/-</sup> mice. *Gcg*<sup>Gut+/+</sup>, n = 19 (males); *Gcg*<sup>Gut-/-</sup>, n = 11 (males); *Gcg*<sup>DistalGut+/+</sup>, n = 35 (males + females); *Gcg*<sup>DistalGut-/-</sup>, n = 13 (males + females). Statistical significance was determined using the two-tailed *t*-test. \*\*\**P* < 0.001; \*\*\*\**P* < 0.0001.

enteral metformin rapidly augments glucose-stimulated GLP-1 levels following perfusion of both the proximal and distal small bowels in humans [40], the GLP-1-stimulatory actions of metformin were markedly reduced in the absence of concomitant glucose challenge in *Gcg*<sup>DistalGut-/-</sup> mice, implicating distal gut L cells as key intestinal targets of metformin action in mice. It is possible that the method and formulation of metformin administration in our studies preferentially enable metformin targeting to the distal gut. Nevertheless, our findings linking metformin action to the distal gut are also consistent with recent observations demonstrating that oral metformin induces GDF-15 expression predominantly through targeting cells within the distal small bowel and colon [41].

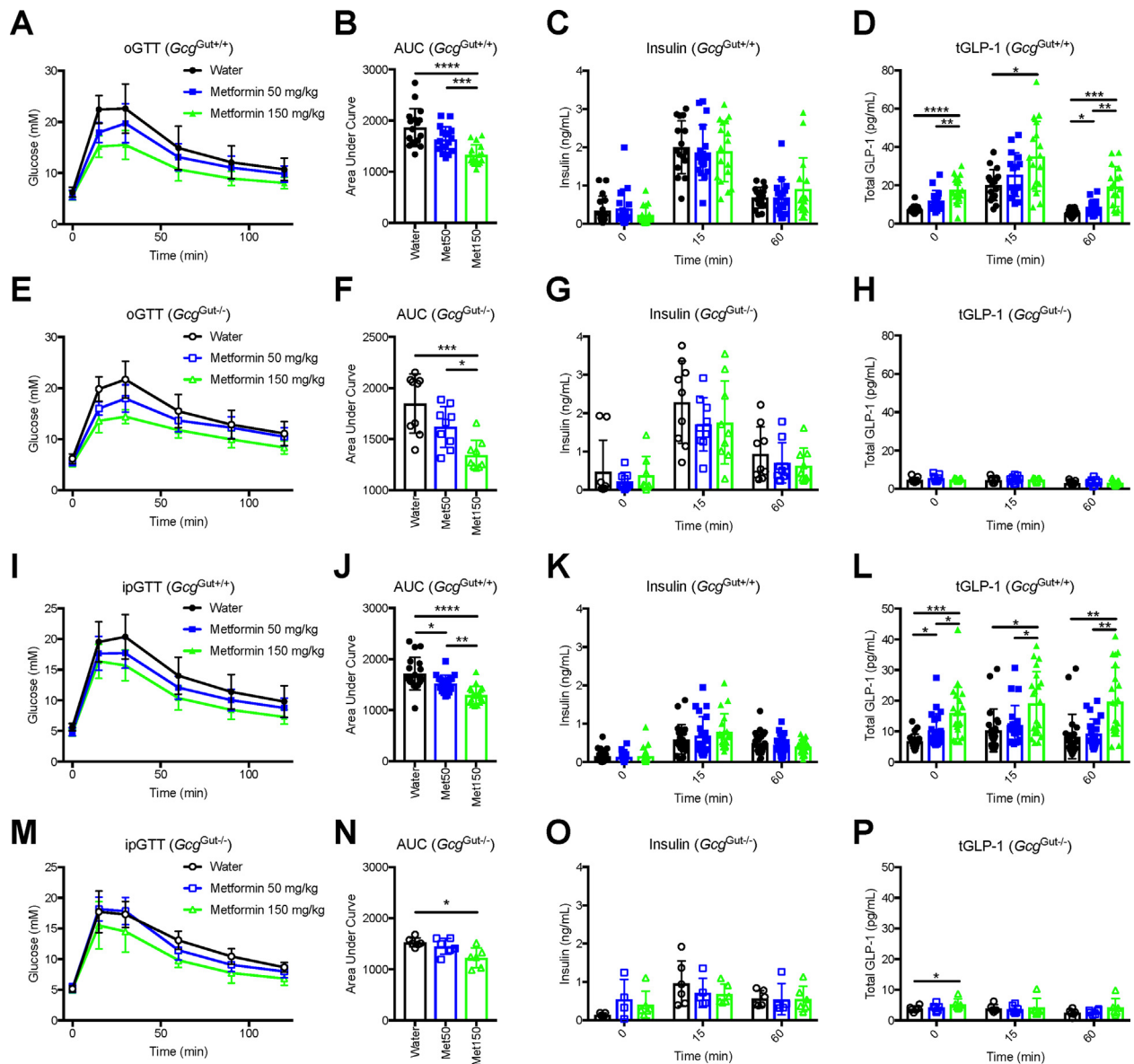
## 5. CONCLUSIONS AND LIMITATIONS

In summary, the current findings highlight the importance of the gastrointestinal tract, and particularly the distal gut, as key sites of the *Gcg* expression linked to the acute stimulation of L cell secretion and increased levels of circulating GLP-1 in mice. Nevertheless, these studies have limitations that restrict conclusions to the experimental models employed herein. It must be noted that rapid pharmacological parenteral administration of secretagogues would reach the distal gut and its relatively greater mass of L cells much faster than oral gavage of nutrient-related L cell secretagogues preferentially targeting the smaller number of L cells in the jejunum. Hence, the interpretation of the data should be tempered by these differences in routes of

administration and rates of access of various secretagogues to L cells distributed within the proximal and distal guts.

Notably, we studied lean, nonobese mice without high-fat feeding, obesity, or diabetes, conditions that might modify the nature and responsiveness of L cells along the gastrointestinal tract [42]. Importantly, GIP is known to increase GLP-1 secretion in mice [10], and some of the secretagogues employed here, such as olive oil and AR231453, are also known to enhance GIP secretion. Moreover, plasma GIP levels are increased in mice following the reduction of gut *Gcg* expression [13]. Hence, it remains possible that, for some secretagogues, the extent of change in plasma GLP-1 levels might also be influenced by simultaneous enhancement of the GIP secretion.

Our analyses of plasma GLP-1 levels were limited to measurements at baseline and only 1 or 2 time points, precluding definitive assessment of any pattern in GLP-1 secretory responses that might become apparent with more frequent blood sampling. Furthermore, experimental obesity and diabetes are frequently associated with pancreatic and islet inflammation and enhanced  $\alpha$ -cell GLP-1 production [35], conditions absent in the lean healthy mice examined in our current studies. Moreover, our studies employed mice with germline elimination of *Gcg* expression within the small and large bowel; hence, it is possible that adaptive compensatory mechanisms arising during growth and development of the gut may have contributed to the pharmacological responses observed herein. Nevertheless, our results, together with recent studies linking selective colonic L cell activation to increased plasma levels of GLP-1 [43], clearly demonstrate that the



**Figure 5: Gut *Gcg* expression and increases in plasma GLP-1 are not required for metformin-mediated gluoregulation.** Oral glucose tolerance tests (oGTT, 2 g/kg BW) and intraperitoneal glucose tolerance tests (ipGTT, 1.5 g/kg BW) were conducted in adult *Gcg*<sup>Gut-/-</sup> and littermate control mice. All mice were given water, metformin 50 mg/kg, or metformin 150 mg/kg by oral gavage 60 min prior to glucose bolus in a triple-crossover study design. (A, B) Glucose levels and AUC analysis from oGTT in *Gcg*<sup>Gut+/+</sup> mice. (C) Insulin and (D) total GLP-1 (tGLP-1) levels during oGTT at 0, 15, and 60 min after glucose in *Gcg*<sup>Gut+/+</sup> mice. (E, F) Glucose levels and AUC analysis from oGTT in *Gcg*<sup>Gut-/-</sup> mice. (G) Insulin and (H) tGLP-1 levels during oGTT at 0, 15, and 60 min after glucose in *Gcg*<sup>Gut-/-</sup> mice. (I, J) Glucose levels and AUC analysis from ipGTT in *Gcg*<sup>Gut+/+</sup> mice. (K) Insulin and (L) Total GLP-1 (tGLP-1) levels during ipGTT at 0, 15, and 60 min after glucose in *Gcg*<sup>Gut+/+</sup> mice. (M, N) Glucose levels and AUC analysis from ipGTT in *Gcg*<sup>Gut-/-</sup> mice. (O) Insulin and (P) tGLP-1 levels during ipGTT at 0, 15, and 60 min after glucose in *Gcg*<sup>Gut-/-</sup> mice. *Gcg*<sup>Gut+/+</sup>, n = 17–22 (males); *Gcg*<sup>Gut-/-</sup>, n = 6–9 (males). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison test. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; \*\*\*\**P* < 0.0001.

distal gut represents an important site for robust L cell secretion, capable of impacting circulating levels of GLP-1. These findings have potential relevance for informing strategies targeting L cells for the treatment of metabolic disorders.

#### AUTHORS' CONTRIBUTIONS

BP, BY, DM, JK, YS, and DD designed the experiments and, together with DS, reviewed and analyzed the data and wrote and/or reviewed

the manuscript. BP, BY, DM, JK, and YS carried out the experiments. DJD secured funding for the studies and is the guarantor of the data.

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## CONFLICTS OF INTEREST

DJD receives consulting honoraria from Intarcia, Merck, Novo Nordisk, Pfizer, and Sanofi within the past 12 months for advisory boards and lectures related to incretin biology. None of the other authors have conflicts of interest. Following the completion of these studies, BP became a full-time employee of Roche Canada Inc.

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