EJE PRIZE 2018

A gut feeling about glucagon

Filip K Knop[1,2,3]

[1]Clinical Metabolic Physiology, Steno Diabetes Center Copenhagen, Gentofte Hospital, University of Copenhagen, Hellerup, Denmark,
[2]Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, and
[3]Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Correspondence
should be addressed
to F K Knop
Email
filip.knop@regionh.dk

This article is based on the presentation for the *European Journal of Endocrinology* Prize Lecture 2018 at the European Society for Endocrinology Meeting at Barcelona, Spain.

Abstract

Hyperglucagonaemia (in the fasting as well as in the postprandial state) is considered a core pathophysiological component of diabetes and is found to contribute substantially to the hyperglycaemic state of diabetes. Hyperglucagonaemia is usually viewed upon as a consequence of pancreatic alpha cell insensitity to the glucagon-suppressive effects of glucose and insulin. Since we observed that the well-known hyperglucagonaemic response to oral glucose in patients with type 2 diabetes is exchanged by normal suppression of plasma glucagon levels following isoglycaemic intravenous glucose administration in these patients, we have been focusing on the gut and gut-derived factors as potential mediators of diabetic hyperglucagonaemia. In a series of clinical experiments, we have elucidated the role of gut-derived factors in diabetic hyperglucagonaemia and shown that glucose-dependent insulinotropic polypeptide promotes hyperglucagonaemia and that glucagon, hitherto considered a pancreas-specific hormone, may also be secreted from extrapancreatic tissues – most likely from proglucagon-producing enteroendocrine cells. Furthermore, our observation that fasting hyperglucagonaemia is unrelated to the diabetic state, but strongly correlates with obesity, liver fat content and circulating amino acids, has made us question the common 'pancreacentric' and 'glucocentric' understanding of hyperglucagonaemia and led to the hypothesis that steatosis-induced hepatic glucagon resistance (and reduced amino acid turnover) and compensatory glucagon secretion mediated by increased circulating amino acids constitute a complete endocrine feedback system: the liver–alpha cell axis. This article summarises the physiological regulation of glucagon secretion in humans and considers new findings suggesting that the liver and the gut play key roles in determining fasting and postabsorptive circulating glucagon levels.

Invited Author's profile

Filip K Knop, MD PhD, is a consultant endocrinologist, Professor of Endocrinology and Head of Clinical Metabolic Physiology at Steno Diabetes Center Copenhagen, Gentofte Hospital, University of Copenhagen, Denmark. Prof. Knop has been involved in diabetes research since 1999. He is particularly interested in the integrative role of the gut in human (patho)physiology and has authored 270 scientific peer-reviewed publications. He serves as a referee for several international journals and is a member of numerous professional societies and committees.

Published by Bioscientifica Ltd.

Introduction

For almost a century, the 29 amino acid hormone glucagon, derived from the precursor proglucagon encoded by the glucagon gene (1), has been considered a pancreas-specific hormone secreted from alpha cells in the islets of Langerhans (2, 3). Of importance for the findings outlined in the present paper, proglucagon is also produced in enteroendocrine L cells found in the intestinal epithelium (4). In contrast to pancreatic alpha cells in which proglucagon is processed to 29 amino acid glucagon by prohormone convertase 2 (PC2), processing of proglucagon in enteroendocrine L cells is undertaken by PC1/3 leading to several active peptides including the glucose-lowering and satiety-inducing glucagon-like peptide 1 (GLP-1) and the intestinotrophic and glucagonotropic glucagon-like peptide 2 (GLP-2) (5) (Fig. 1). Thus, the notion of glucagon's pancreas-specific origin is based on pancreas-specific expression of PC2
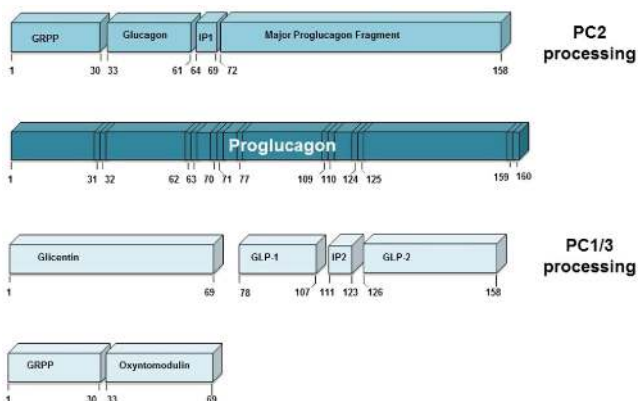

Figure 1

Differential processing of proglucagon by prohormone convertase 2 (PC2) and prohormone convertase 1/3 (PC1/3). The posttranslational processing of proglucagon is based on the presence and activity of the enzymes PC1/3 and PC2, which hitherto have been believed to be tissue-specific with PC2 being restricted to the pancreatic alpha cells and PC1/3 to the enteroendocrine L cells. PC2 processes proglucagon into glucagon and three presumably biologically inactive fragments (glicentin-related pancreatic polypeptide (GRPP), intervening peptide 1 (IP1) and a major proglucagon fragment (MPGF)), whereas PC1/3 processes proglucagon into glicentin, which is further processed into oxyntomodulin and GRPP, intervening peptide 2 (IP2) and to the glucagon-like peptides: GLP-1 and GLP-2. Numbers indicate amino acid positions corresponding to amino acid positions in the 160 amino acid proglucagon.

rather than pancreas-specific expression of proglucagon itself.

Glucagon is recognised as a hormone that primarily maintains glucose homeostasis via its stimulatory effects on hepatic glucose production and, thus, plays a fundamental role in securing supply of easily accessible and energy-rich glucose to bodily tissues, particularly the central nervous system and muscles, when needed (6). This critical role of glucagon obviously requires a strict regulatory coordination of glucagon secretion, and it has been known for decades that circulating glucagon concentrations increase when blood glucose concentrations fall below normal fasting levels (6). In addition to hypoglycaemia, other stimulatory factors are elicited during situations where increased energy supply is needed to sustain physiological functions, e.g. during stress and cold exposure (7) and include activity in the sympathetic nervous system and elevated levels of adrenaline and noradrenaline (8, 9). Glucagon release is also stimulated by non-carbohydrate nutritional stimulators of insulin secretion, e.g. protein meals and administration of most amino acids, which without glucagon release would cause hypoglycaemia (10, 11). Furthermore, exogenous administration of the gut hormones glucose-dependent insulinotropic polypeptide (GIP) (12, 13, 14, 15) and GLP-2 (16, 17, 18), respectively, has been shown to stimulate glucagon secretion in healthy individuals and in patients with diabetes. Hyperglycaemia elicited by intravenous (iv) administration of glucose is known to reduce circulating concentrations of glucagon (6). This inhibitory effect of glucose on alpha cells is mediated directly by glucose itself and by glucose-stimulated secretion of insulin and somatostatin from neighbouring beta and delta cells, respectively (19, 20, 21, 22, 23, 24). Furthermore, the insulinotropic and satiety-promoting gut hormone GLP-1 inhibits alpha cell secretion and reduces circulating glucagon concentrations in a glucose-dependent manner (25, 26, 27). These glucagon-suppressive factors are all signs of energy abundance and minimised need for glucagon-induced endogenous glucose production.

It is the general belief that the complex regulatory system controlling circulating glucagon concentrations outlined above converges at the level of glucagon-producing pancreatic alpha cells (6). Importantly, perturbations in the regulation of plasma glucagon concentrations are well known in patients with diabetes, who typically are characterised by hyperglucagonaemia (6). And for more than four decades, it has been argued that hypersecretion of glucagon from pancreatic alpha cells contributes importantly to diabetic hyperglycaemia

(28). However, the mechanisms underlying diabetic hyperglucagonaemia remain incompletely understood and are usually explained by resistance of pancreatic alpha cells to the suppressive effects of glucose and insulin, respectively, combined with absolute or relative hypoinsulinaemia pathognomonic for diabetes (29).

In 2007, we reported the first human findings pointing to the gut and/or gut-derived factors as major determinants of the hyperglucagonaemia observed after nutrient ingestion in patients with type 2 diabetes (30), and a few years later, we observed that fasting hyperglucagonaemia is more tightly related to obesity than the diabetic state (31) and suggested that obesity-associated steatosis in the liver leads to hepatic glucagon resistance and compensatory glucagon secretion explaining fasting hyperglucagonaemia (32). These findings have spurred a range of clinical studies on the basis of which we have delineated the causality and aetiology of hyperglucagonaemia (11, 34, 36, 37, 38) and provided evidence to suggest (1) that fasting hyperglucagonaemia occurs due to steatosis-induced disruption of the newly discovered liver–alpha cell axis (32, 33, 34, 35, 36, 37, 38, 39, 40) and (2) that postprandial hyperglucagonaemia occurs on the basis of gut-derived glucagon secretion (41) and, possibly, contribution of glucagonotropic gut factors stimulating pancreatic glucagon secretion (18).

In the present paper, hyperglucagonaemia as a pathophysiological phenomenon will be outlined, and recent findings involving the gastrointestinal tract and the liver in the regulation of glucagon secretion and hyperglucagonaemia will be discussed in the context of the traditional 'pancreacentric' and 'glucocentric' conceptions of glucagon biology.

Hyperglucagonaemia in diabetes

In patients with type 2 diabetes, glucagon concentrations are typically elevated in the fasting state and fail to decrease appropriately or even increase during an oral glucose tolerance test (OGTT) or after ingestion of a carbohydrate-rich meal resulting in undesirably high plasma glucagon concentrations in the context of hyperglycaemia (42, 43). Pioneering studies by Gerich *et al.* pointed to an essential role for glucagon in the development of the hyperglycaemia of type 1 diabetes (44) leading Unger and Orci to propose their 'bihormonal hypothesis' stating that the combination of insulin deficiency and hyperglucagonaemia constitutes a central determinant

for diabetic hyperglycaemia (28). Over the decades, it has been established that elevated glucagon concentrations stimulate endogenous glucose production in the liver (45), and, thus, contribute significantly to the fasting and postprandial hyperglycaemia characterising patients with diabetes (46, 47). Accordingly, hyperglucagonaemia has been pursued as a therapeutic target, and glucose-lowering drugs with glucagon-lowering or glucagon receptor-blocking capabilities have been developed (48). Despite these endeavours, the mechanisms underlying diabetic hyperglucagonaemia are not clear. As alluded earlier, the current dogma states that alpha cell resistance to the glucagon-suppressive effects of glucose and insulin, respectively, constitutes a critical factor for the development of hyperglucagonaemia in diabetes (29, 49). Furthermore, compromised beta cell function and ensuing reduced insulin-mediated inhibition of alpha cell secretion is thought to contribute to diabetic hyperglucagonaemia (29, 50). Likewise, compromised beta cell function in diabetes may involve a reduced paracrine influence of other beta cell products, which have been suggested to inhibit pancreatic alpha cell secretion of glucagon (e.g. gamma aminobutyric acid and amylin (51, 52)). Additionally, reduced postprandial plasma concentrations of the beta cell-stimulating and alpha cell-inhibiting gut hormone GLP-1 observed in some cohorts of patients with type 2 diabetes (compared to healthy control subjects) (53) could, hypothetically, contribute to type 2 diabetic postprandial hyperglucagonaemia. Thus, generally, diabetic hyperglucagonaemia is considered a pancreatic problem of increased glucagon secretion due to diminished or defect suppression of alpha cell secretory activity (6).

As alluded earlier, in 2007, we reported the first human data showing that patients with type 2 diabetes indeed exhibit a hyperglucagonaemic response to oral glucose administration (OGTT), but, surprisingly, react with a normal suppression of circulating glucagon concentrations after an isoglycaemic intravenous glucose infusion (IIGI) administered to copy the plasma glucose curve from the oral glucose challenge (Fig. 2) (30). This strongly suggested that the genesis of type 2 diabetic hyperglucagonaemia was not glucose and/or insulin 'blindness' of the alpha cells or compromised insulin secretion as previously thought and that the gastrointestinal tract or factors originating from glucose stimulation of the gastrointestinal tract may play an important role in the postprandial hyperglucagonaemia characterising patients with type 2 diabetes.
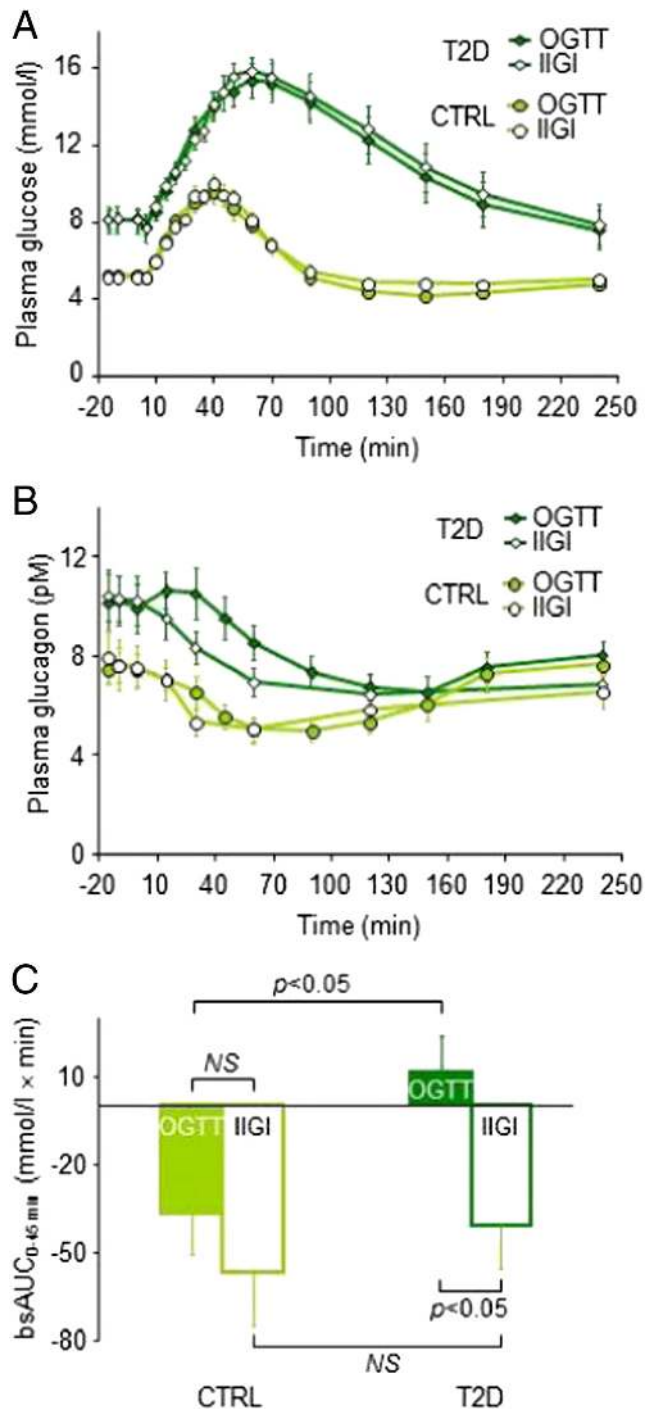
Figure 2

Oral glucose tolerance test (OGTT) and isoglycaemic intravenous glucose infusion (IIGI) performed in patients with type 2 diabetes (T2D) and matched healthy controls (CTRL) (A) revealed a differential glucagon response in the diabetic patients with a hyperglucagonaemic response to oral glucose administration, but, surprisingly, a seemingly normal suppression of circulating glucagon concentrations after IIGI

The OGTT/IIGI phenomenon

Our observation in 2007 that hyperglucagonaemia in type 2 diabetes is aggravated by OGTT, but suppressed similarly as in healthy subjects during IIGI was reproduced by us (54) and others (55, 56) and made us reconsider the genesis of type 2 diabetic postprandial hyperglucagonaemia. Due to the isoglycaemic conditions in these studies, it seemed unlikely that impairment of glucose sensing of the alpha cell plays a major role in the exaggerated glucagon responses after oral glucose ingestion in patients with type 2 diabetes. Neither did insulin resistance of the alpha cell seem to explain this phenomenon as insulin responses during OGTT are markedly higher than those during IIGI due to the strong insulinotropic effects of the incretin hormones GIP and GLP-1 (30) (despite a reduced incretin effect in type 2 diabetes (30, 54, 57)). Thus, if anything, the larger insulin response to oral glucose would be expected to result in greater suppression of glucagon responses compared to IIGI; but the opposite occurred. Taken together, our findings in 2007 strongly suggested that the gastrointestinal tract plays an important role in postprandial glucagon secretion (see sections on 'Potential gut factors contributing to postabsorptive hyperglucagonaemia' and 'Gut-derived glucagon').

In order to establish whether the paradoxical differences in glucagon responses to OGTT and IIGI, respectively, occur as a type 2 diabetes-specific phenomenon, we investigated glucagon responses to OGTT and IIGI in other forms of diabetes and found that patients with secondary diabetes to chronic pancreatitis (54) and patients with hepatocyte nuclear factor 1a diabetes (maturity-onset diabetes of the young type 3) (58) also exhibited the paradoxical glucagon responses to OGTT and IIGI, respectively, suggesting the phenomenon to occur as a consequence of the diabetic state rather than being a specific pathogenetic trait of type 2 diabetes. In order to eliminate potential differences in intra-islet insulin signalling as an explanation of the paradoxical glucagon responses, Hare *et al.* performed OGTT and IIGI in C-peptide-negative patients with type 1 diabetes and showed similar patterns of glucagon responses to the two glucose stimuli as observed in type 2 diabetes (59). We also investigated patients with chronic pancreatitis and

administered to copy the plasma glucose curve from the oral glucose challenge (B and C). NS, non-significant; bsAUC, baseline-subtracted area under curve. Adapted from Knop *et al.* (30).

different degrees of secondary endocrine insufficiency and showed that the difference in glucagon response to oral and iv glucose, respectively, increases from normal glucose tolerance over impaired glucose tolerance to overt diabetic glucose tolerance in these patients – driven by increased glucagon responses to oral glucose (glucagon was suppressed normally by iv glucose in all groups, i.e. independently of glucose tolerance) (60). These findings suggested that post-OGTT hyperglucagonaemia constituted a common phenomenon among patients with different types of diabetes and that IIGI in all cases suppressed circulating glucagon levels.

We then went back to study patients with type 2 diabetes and showed that the paradoxical glucagon responses to OGTT and IIGI, respectively, intensified when larger oral glucose loads were employed suggesting that greater stimulation of the gastrointestinal tract elicits or constitutes a more potent glucagonotropic signal (61). Interestingly, these studies also showed that the difference in glucagon secretion between OGTT and IIGI is present in non-diabetic control subjects when larger glucose loads are ingested (61, 62). Thus, gastrointestinally mediated glucagon secretion seemed to be a consistent phenomenon in diabetic patients that can also be evoked in healthy subjects when larger glucose loads are used. However, the underlying mechanisms and derived effects of gastrointestinally mediated glucagon secretion were still obscure.

Implications of gastrointestinal-stimulated glucagon secretion

As alluded earlier, the incretin effect describes the potentiation of glucose-induced insulin secretion elicited by stimulation of insulinotropic gut hormones, i.e. GIP and GLP-1 (54, 57). The incretin effect is measured by comparing insulin responses to OGTT and IIGI and in healthy subjects. The incretin effect is responsible for 50–70% of the insulin secreted in response to oral glucose, whereas the incretin effect is severely reduced in type 2 diabetes (30, 54, 55, 57, 63) and other forms of diabetes (54, 58, 64). Reduced incretin effect seems to be an early consequence of disturbed glucose metabolism as it has been observed in subjects with prediabetes (65, 66) and in other conditions with high risk of developing type 2 diabetes (e.g. obese insulin-resistant subjects with normal glucose tolerance, patients with non-alcoholic fatty liver disease and psoriasis patients) (31, 38, 67, 68). It is well acknowledged that reduced incretin effect limits

postprandial insulin secretion, but its effect on glucose disposal has been difficult to disentangle – especially in the context of insulin resistance often accompanying a reduced incretin effect. Nevertheless, an incretin effect of any size will, in any event, contribute to improve glucose disposal during OGTT vs IIGI.

In order to better understand how oral and iv glucose differentially affect glucose disposal, we introduced the term 'gastrointestinally mediated glucose disposal' (GIGD) in 2010 (59). GIGD describes the impact of gastrointestinal factors on glucose disposal following OGTT compared with IIGI by relating the difference between the amount of glucose ingested (through the gastrointestinal tract) and the amount of glucose needed to be infused intravenously to mimic the plasma glucose curve from the OGTT curve to the glucose ingested orally. GIGD explains what percentage of an individual's glucose disposal during an OGTT is caused by the oral route of administration. The incretin effect most likely constitutes an important contributor to GIGD, but other factors affecting plasma glucose concentrations differently during OGTT and IIGI, including differences in glucagon secretion, activation of afferent nerves in the intestinal mucosa, first-pass hepatic glucose uptake, differences in portal and venous blood glucose concentrations and/or at the present unknown factors are also taken into account when calculating GIGD. In healthy subjects, GIGD typically amounts to ~60% (31, 56, 65, 67, 68, 69, 70), whereas patients with diabetes exhibit a severely reduced GIGD in the range of ~10–30% (31, 56, 57, 58, 64). As mentioned earlier, Hare *et al*. performed OGTT and IIGI in C-peptide-negative patients with type 1 diabetes and were, thus, able to describe GIGD in the context of abrogated incretin effect (59). Interestingly, mean GIGD came out negative (amounting to −6%) suggesting that the hyperglucagonaemic response elicited by the oral glucose administration deteriorated glucose disposal during OGTT vs IIGI (which suppressed circulating glucagon concentrations) most likely by stimulating hepatic glucose production. This suggested that gastrointestinally mediated glucagon secretion may contribute to postabsorptive hyperglycaemia pathognomonic for diabetes.

In a Korean study, lean and well-controlled Asian patients with type 2 diabetes were shown to have a normal incretin effect, but exhibited a hyperglucagonaemic response to 75 g OGTT, whereas IIGI supressed circulating glucagon concentrations normally (healthy controls exhibited similar glucagon suppression to both glucose stimuli). Interestingly, GIGD was found to be significantly compromised in the type 2 diabetic patients and to

correlate significantly with OGTT glucagon responses (56). This strongly suggests that gastrointestinal-stimulated glucagon secretion contributes to the impaired glucose handling in type 2 diabetes.

In order to further evaluate whether differences in glucagon secretion during OGTT (hypersecretion) and IIGI (suppression) influence postabsorptive glucose excursions, Lund *et al*. investigated glucose disappearance and endogenous glucose production during OGTT and IIGI in patients with type 2 diabetes and matched non-diabetic controls using glucose tracer methodology (71). As expected from our previous studies, inappropriately high glucagon responses were observed during OGTT, whereas IIGI suppressed glucagon levels in a normal fashion, and, importantly, we showed that endogenous glucose production was higher during OGTT than during IIGI even with higher levels of insulin (due to the incretin effect) and, presumably, higher portal glucose concentrations (known to diminish endogenous glucose production) during OGTT. Despite these indications that the differential glucagon responses to OGTT and IIGI translate into meaningful effects on circulating glucose levels, preliminary data from studies using the glucagon receptor antagonist LY2409021 suggest that the hyperglucagonaemic responses to OGTT and mixed meal test, respectively, have little or no effect on postabsorptive glucose excursions (72, 73). However, potential lack of specificity of LY2409021 and possible compensatory mechanisms elicited by glucagon receptor antagonism complicate the interpretation of these results. Taken together, hyperglucagonaemia arsing as a consequence of nutrient stimulation of the gastrointestinal tract seems to be a consistent phenomenon, but the effects and pathophysiologic implications of postabsorptive hyperglucagonaemia are not completely clear and warrant further investigation.

Potential gut factors contributing to postabsorptive hyperglucagonaemia

As glucose (due to the isoglycaemic conditions) and insulin (due to the incretin effect) did not seem to explain the paradoxical glucagon responses to OGTT and IIGI in patients with diabetes, we directed our attention to glucagon-modulating gut-derived factors. An obvious candidate was the glucagonostatic incretin hormone GLP-1, and we speculated that reduced secretion or attenuated glucagonostatic effect of GLP-1 could contribute to explain the OGTT-induced hyperglucagonaemia in diabetic patients vs healthy controls. Despite the general notion of 'hypo-GLP-1-aemia' in type 2 diabetes (74), we observed no difference in GLP-1 responses to OGTT between hyperglucagonaemic patients with diabetes and healthy subjects suppressing glucagon normally during OGTT (30, 54, 58, 59, 63, 71). We also performed a systematic review of studies comparing GLP-1 responses between patients with type 2 diabetes and healthy control subjects and meta-analysis of the data disclosed no differences in GLP-1 responses (53). Thus, reduced GLP-1 response to OGTT did not seem to explain the exaggerated glucagon responses to OGTT in diabetic patients. We then evaluated the glucagonostatic potency of GLP-1 in type 2 diabetic patients and found it to be similar as in matched healthy control subjects (27). With GLP-1 eliminated from the equation, we turned to the two glucagonotropic gut hormones GLP-2 and GIP. As GLP-2 is secreted together with GLP-1 in equimolar amounts (5) and no clear signals from our own or other studies indicated increased OGTT-induced responses of GIP (75), we found it unlikely that increased secretion of GLP-2 and/or GIP constituted an underlying mechanism of the hyperglucagonaemic OGTT responses. Interestingly, Chia *et al*. had shown that exogenous GIP administration increased postprandial glucagon responses in type 2 diabetes and, consequently, worsened postprandial hyperglycaemia (76). Therefore, we designed a study involving an OGTT and five IIGIs (with concomitant saline, GIP, GLP-1, GLP-2 and GIP+GLP-1+GLP2 infusions, respectively) and were able to show that GIP infusion significantly increased glucagon responses during IIGI, whereas GLP-2 had little effect on plasma glucagon levels (as expected, GLP-1 decreased circulating glucagon concentrations during IIGI) (18). On the basis of these results, we suggested that GIP may play a predominant role in the inappropriate hyperglucagonaemic response to orally ingested glucose in type 2 diabetes. However, we could not escape the thought that OGTT-induced hyperglucagonaemia could be a result of glucagon secretion directly from the gut.

Gut-derived glucagon

Extrapancreatic glucagon secretion was suggested 70 years ago by Sutherland & de Duve who showed that a 'glycogenolytic substance' could be extracted from the gastric mucosa of rabbits and dogs (77). Since then, several studies in totally pancreatectomised animals and humans (referenced in detail by Lund *et al*. (41)) have been undertaken with inconsistent and conflicting results

(most likely due to species differences and difficulties in measuring the 29 amino acid proglucagon product, glucagon, due to important shortcomings and low accuracy of many current and previous glucagon assays (78)) making decisive statements about the absence or presence of extrapancreatic glucagon impossible. Importantly, new analytical endeavours have improved sensitivity as well as specificity of glucagon assays dramatically (79), and, furthermore, technical advances within mass spectrometry-based proteomics allow detection of low-abundant peptides, such as glucagon, in human plasma (80).

Lund *et al*. performed 75 g OGTT and IIGI in 10 totally pancreatectomised patients and 10 matched healthy control subjects (79). Applying a recently developed sandwich ELISA utilising a combination of C and N-terminal antiglucagon antibodies, and thus, in theory eliminating, cross-reactivity with elongated or truncated forms of glucagon (79), and mass spectrometry-based proteomics, we were able to show a dramatic increase in circulating glucagon concentrations in response to OGTT, whereas IIGI decreased circulating glucagon to almost undetectable levels in totally pancreatectomised patients (Fig. 3). The healthy subjects suppressed plasma glucagon levels in response to both glucose stimuli, but to a greater extent during IIGI as expected from previous studies applying 75 g OGTT and IIGI in healthy subjects (61, 62). As other pancreas-related products (e.g. C-peptide and pancreatic polypeptide) were absent in our totally pancreatectomised patients, these findings strongly emphasised the existence of extrapancreatic glucagon in man – most likely originating from the gut. With this new piece of evidence of gut-derived glucagon secretion, a number of exciting questions arose including 'What is the localisation(s) of extrapancreatic glucagon secretion and how is it brought about?', 'How is extrapancreatic glucagon secretion regulated?' and 'What may be the physiological and/or pathophysiological implications of extrapancreatic glucagon secretion?'.

Studies in depancreatised dogs from the 1970s had suggested that the mucosa in the antrum of the ventricle and the ileum could constitute places of origin of extrapancreatic glucagon (81, 82). Later, alpha cell-resembling glucagon-positive cells were identified in the foetal human stomach (83), while this finding was not reconfirmed in mucosal biopsies from the stomach of adult humans (84). As alluded earlier, the pancreas-specific notion of glucagon secretion is related to the idea that processing of proglucagon to glucagon (and the presumably biological inactive fragments intervening
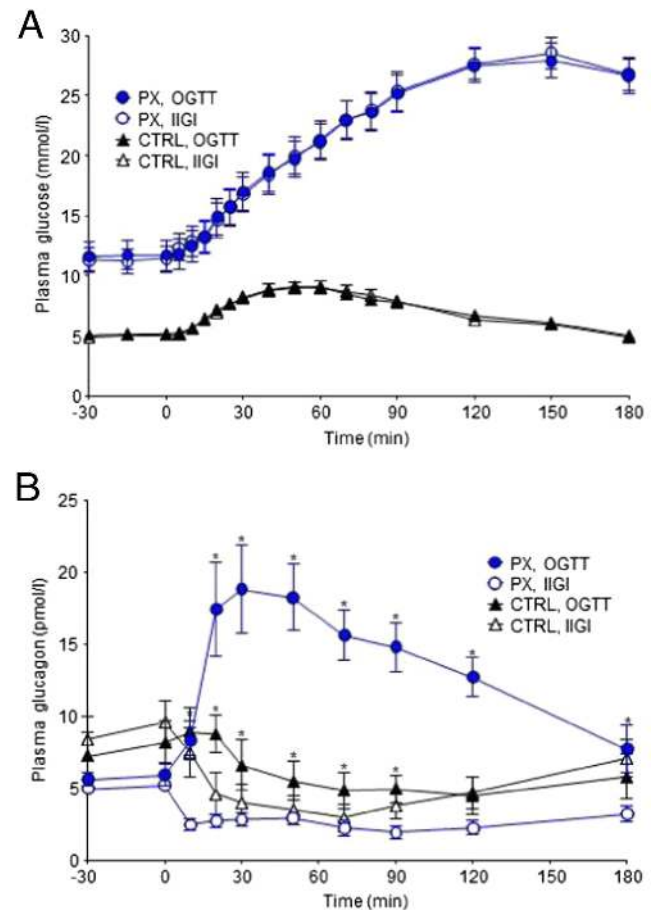

Figure 3

Plasma responses of glucose (A) and glucagon (B) during 75 g oral glucose tolerance test (OGTT) and isoglycaemic intravenous glucose infusion (IIGI) in totally pancreatectomised patients (PX) and non-diabetic control subjects (CTRL). Asterisks (*) indicate significant differences ($P < 0.05$). Adapted from Lund *et al*. (41).

peptide 1 and major proglucagon fragment) by PC2 is restricted to the pancreas (85). In 2009, it was hypothesised that PC2 expression in enteroendocrine L cells, which are thought to process proglucagon exclusively by PC1/3 to GLP-1, GLP-2, glicentin, oxyntomodulin and intervening peptide 2 (5), would result in co-secretion of 'traditional' L cell products and 29 amino acid 'pancreatic' glucagon following luminal nutrient stimulation and, therefore, explain the paradoxical glucagon responses to OGTT and IIGI, respectively (86). In order to explore this hypothesis, we sampled jejunal biopsies from patients with type 2 diabetes (exhibiting grossly postprandial hyperglucagonaemic responses) and matched healthy controls and were able to show (1) the presence of PC2 mRNA and immunohistochemical PC2-positive enteroendocrine

cells, (2) immunohistochemical co-localisation of PC2 and the proglucagon product GLP-1 and (3) 50% more PC2-positive cells in the jejunal epithelium of type 2 diabetic patients as compared to healthy controls (87). We confirmed these findings in a study investigating jejunal mucosa biopsies (obtained during Roux-en-Y gastric bypass surgery) from obese patients with type 2 diabetes and obese non-diabetic patients, respectively (88), and furthermore, we recently observed significantly greater expression of the gene encoding PC2, *PCSK2*, in the small intestine of individuals with type 2 diabetes compared with healthy individuals (4). Recent investigations by Jorsal and colleagues including immunohistochemistry using a new highly glucagon-specific monoclonal antibody and peptide extraction with application of a mass spectrometry-validated sandwich ELISA on human gastric mucosa biopsies found no or negligible signs of glucagon (Jorsal *et al.*, unpublished observation). In this study, however, no co-staining of glucagon and PC2 was evident in small intestinal mucosa biopsies and, thus, the possibility of proglucagon processing to 29 amino acid glucagon by other processing enzymes (e.g. unspecific processing by PC1/3) should be kept open. Held together, current evidence points to enteroendocrine cells in the small intestine as a possible source of extrapancreatic glucagon and suggests that processing of proglucagon in these cells may not be as specific as previously thought.

How is extrapancreatic glucagon secretion regulated? From Lund *et al.*'s studies in totally pancreatectomised patients, it is clear that oral glucose represents a strong stimulus, whereas iv glucose seems to be able to suppress extrapancreatic glucagon secretion (41). In a recent study, Juel *et al.* showed that the well-known stimulator of pancreatic glucagon secretion, arginine, had no impact on circulating glucagon concentrations (as assessed by the recently developed mass spectrometry-validated sandwich ELISA mentioned earlier) in totally pancreatectomised patients (89) suggesting that the stimulus–secretion coupling in the cells responsible for extrapancreatic glucagon is different from pancreatic alpha cells. Also, Juel *et al.* have shown that a mixed meal test in totally pancreatectomised patients elicits clear cut glucagon responses that can be significantly suppressed (concomitantly with reduced postprandial plasma glucose excursions) by single dosing of the short-acting GLP-1 receptor agonist lixisenatide (90). Whether this effect is a direct effect or a result of lixisenatide-induced deceleration of gastric emptying/upper gastrointestinal motility and ensuing diminished stimulation of enteroendocrine glucagon-secreting cells is currently being investigated.

Additionally, we are currently evaluating the effects of a range of factors known to regulate pancreatic glucagon secretion in totally pancreatectomised patients using state-of-the-art glucagon analyses; these include GIP, GLP-1, GLP-2, oxyntomodulin, insulin, amylin and somatostatin. Furthermore, the effects of hypoglycaemia and drugs known to influence glucagon secretion are being investigated.

Presently, the physiological effect of extrapancreatic glucagon secretion remains uncertain. Hypothetically, extrapancreatic glucagon can be thought to counteract the potent glucose-lowering mechanisms (e.g. incretin-induced insulin surges) and the strong suppression of endogenous glucose production unleashed by oral ingestion of carbohydrates, ensuring a 'smooth landing' of postprandial glucose excursions. Such mechanism may be of particular importance in patients who have undergone gastric bypass surgery rerouting nutrients to distal parts of the small intestine where GLP-1-secreting L cells are abundant and often result in a GLP-1-mediated overshoot of insulin relative to the amount of carbohydrate ingested, increasing the risk of postprandial hypoglycaemia (91, 92). In these patients, surgery-induced metabolic benefits are typically accompanied by postprandial hyperglucagonaemia, which seems counterintuitive in the context of surgery-induced improvement in glycaemic control involving massive secretion of GLP-1 and insulin, both of which are known for their suppressive effects on alpha cell secretion (86). Interestingly, the preliminary data from Jorsal *et al.* alluded to above suggest that extrapancreatic glucagon secretion from the gut could help to explain the paradoxical postprandial hyperglucagonaemia observed after gastric bypass surgery: Proglucagon mRNA increased and glucagon was immunohistochemically identified in mucosa biopsies (using a highly specific glucagon antibody) and extractable glucagon appeared in all biopsies retrieved postoperatively vs preoperatively – correlating with postoperative increments in postprandial plasma glucagon concentrations (Jorsal *et al.*, unpublished observation). In addition to the potential contribution of gut-derived glucagon to protection from postprandial hypoglycaemia, glucagon-secreting cells in the gut may also act as nutrient sensors and convey glucagon-mediated satiety signals alongside other gut hormones such as GLP-1 and peptide YY – a view compatible with the reduced appetite observed after gastric bypass surgery (93). Perhaps more intriguingly, gut-derived glucagon may have far-reaching pathophysiological implications and, potentially, play an important role in the postabsorptive

hyperglycaemia pathognomonic for diabetes. Thus, the hyperglucagonaemic response to oral ingestion of carbohydrates and its contribution to postabsorptive hyperglycaemia (46, 94) may completely depend on secretion of glucagon from the gut as iv administration of glucose suppresses glucagon levels independently of dose and condition examined (30, 54, 55, 56, 58, 61, 62). Further studies are needed to address these issues.

Fasting hyperglucagonaemia and the liver-alpha cell axis

In early studies involving relatively lean patients with type 2 diabetes, fasting plasma glucagon concentrations appeared normal (95), but over the decades – as patients grew in size and numbers – fasting hyperglucagonaemia became a well-established fact among the majority of patients with type 2 diabetes (43, 96). Furthermore, Baron *et al*. provided evidence that elevated fasting plasma glucagon levels contribute to increased basal rate of hepatic glucose production, and thus, demonstrated the pivotal role of fasting hyperglucagonaemia in the pathogenesis of fasting hyperglycaemia in type 2 diabetes (45). Nevertheless, in humans, extreme glucagon excess and lack of glucagon signalling, respectively, do not bring about changes completely compatible with this evidence (33). For instance, in patients with glucagon-producing tumours (glucagonomas) the most conspicuous sign is a skin lesion, the necrolytic migratory erythema and overt diabetic hyperglycaemia is only observed in about 30% of glucagonoma patients (33). And inactivating mutations of the glucagon receptor are neither necessarily associated with disturbances of glucose metabolism (33); rather, these patients exhibit a pancreatic swelling due to alpha cell hyperplasia with gross hypersecretion of glucagon (33). Thus, extreme phenotypes of glucagon excess and lack of glucagon signalling, respectively, do not necessarily involve perturbations in glucose homeostasis. On this basis, our findings in 2012 showing fasting hyperglucagonaemia in completely normoglucose-tolerant obese and insulin-resistant subjects (31), combined with elegant studies by Longuet *et al*. (34) showing that selective knockout of the glucagon receptor in the liver results in hyperglucagonaemia (and alpha cell hyperplasia), led us hypothesise that hyperglucagonaemia occurs as a compensatory response to steatosis-induced hepatic insensitivity to glucagon and that this provides
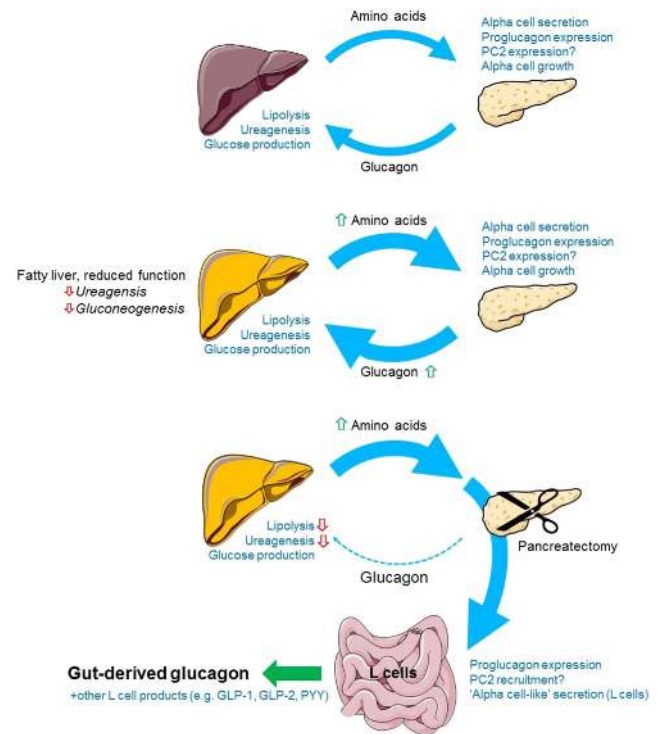

Figure 4

Proposed mechanisms underlying the liver–alpha cell axis and its disruption. Upper panel: Under normal circumstances, amino acids seem to maintain and regulate the secretion of glucagon, which in turn seems to play a vital role in controlling amino acid clearance in the liver by accelerating ureagenesis. Mid panel: When the liver–alpha cell axis is disrupted by fatty infiltration of the liver, ureagenesis and amino acid turnover is reduced (due to steatosis-induced glucagon resistance) resulting in hyperaminoacidaemia, which in turn stimulates alpha cell secretion in order to compensate for the disruption of the balance, leading to fasting hyperglucagonaemia. Lower panel: When the liver–alpha cell axis is disrupted on the basis of total pancreatectomy, stimulation of the liver by pancreatic glucagon is completely abolished, which compromises glucagon-dependent hepatic processes (e.g. ureagenesis and lipolysis). Consequently, amino acid turnover is reduced giving rise to hyperaminoacidaemia, which under normal circumstances would provide compensatory alpha cell growth and glucagon synthesis in and secretion from the pancreas. But without a pancreas, amino acids seem to target other proglucagon-producing cell (i.e. the enteroendocrine L cells) resulting in the formation of glucagon. Also, the lack of pancreatic glucagon after total pancreatectomy may constitute a major determinant of the hepatic steatosis often observed after pancreatectomy.

a feedback mechanism acting at the level of pancreatic alpha cells, leading to fasting hyperglucagonaemia (32) (Fig. 4). Findings from Solloway *et al*. (35) demonstrated that inhibition of the glucagon receptor leads to reduced amino acid turnover and increased plasma levels of amino acids, which in turn induce proliferation of pancreatic alpha cells, and thus, suggested that single or several circulating amino acids could constitute the feedback signal between the liver and the alpha cells (32, 34) (Fig. 4).

The liver is crucial for the metabolism of amino acids through processes including transamination and deamination, to finally produce urea (eliminated with the urine) – processes critically controlled by glucagon (97). In this way, glucagon is responsible for removing from the body the ammonia that results from transamination of amino acids (97). Therefore, amino acids can be thought to constitute more relevant mediators of liver-pancreas cross-talk compared to ever-fluctuating plasma glucose levels (33). A normal function of amino acids, thus, may be to maintain and regulate the secretion of glucagon, which in turn appears to play a vital role in controlling amino acid clearance in the liver by accelerating ureagenesis (Fig. 4). This feedback loop between the liver and the alpha cells of the pancreas has recently been corroborated in rodent studies showing that specific amino acids and amino acid transporters in alpha cells determine glucagon levels (36, 37).

Similar to observations of fasting hyperglucagonaemia in obese normal glucose-tolerant subjects, our recent studies in patients with non-alcoholic fatty liver disease (with and without type 2 diabetes) show that fasting hyperglucagonaemia occurs independently of the diabetic state (38). Rather, fasting hyperglucagonaemia seems to relate to liver fat content and circulating amino acids (40), suggesting that liver-specific disruption of the liver–alpha cell axis represents an important determinant of fasting hyperglucagonaemia (Fig. 4). This notion is supported by preliminary data from our group showing hepatic glucagon resistance at the level of amino acid turnover in obese subjects with biopsy-verified steatosis compared to lean non-steatotic subjects (Suppli *et al*., unpublished observation). Furthermore, recent reports showing glucagon receptor antagonist-induced build-up of liver fat combined with hyperglucagonaemia (98) strongly support the notion that fasting hyperglucagonaemia in the context of obesity and type 2 diabetes arises a result of disruption of a hitherto unrecognised endocrine system involving the liver and the pancreas: the
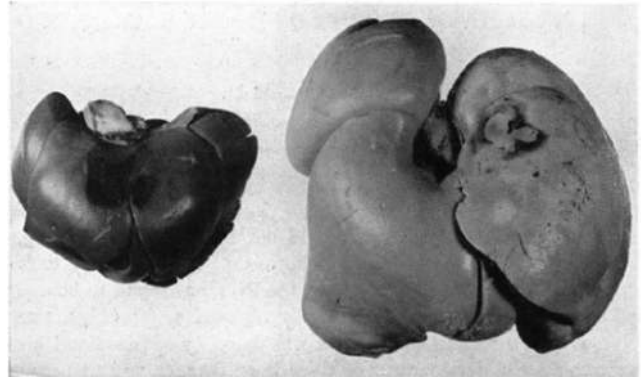
Figure 5

Photograph of normal liver of dog (left) and of the liver of a depancreatised dog (right), approximately the same size, fed a balanced diet and treated adequately with insulin (right). Death occurred 60 days after pancreatectomy. At autopsy, the liver was found to be enlarged and bright yellow in colour. Histology showed marked fatty infiltration of the liver. From Dragsted *et al*. (100).

liver–alpha cell axis (Fig. 4). Another way to disrupt this axis is to remove pancreatic alpha cells. Interestingly, total pancreatectomy is robustly associated with the development of hepatic steatosis (99) and supports the concept that glucagon may play a hitherto unrecognised role in the pathophysiology of non-alcoholic fatty liver disease (as suggested from studies in depancreatised dogs from the 1930s showing increased liver fat content after pancreatectomy (Fig. 5) (100, 101) and later rodent studies (102) in addition to a few and sporadic reports of clinical cases (103, 104)). This total pancreatectomy-associated hepatic steatosis may be a result of glucagon deficiency, which in turn reduces hepatic amino acid turnover and, thus, results in hyperaminoacidaemia perhaps explaining the hyperactivity of extrapancreatic proglucagon-producing cells, i.e. enteroendocrine L cells, in these patients (Fig. 4) (41).

Taken together, fasting hyperglucagonaemia seems to be independent of the diabetic state and rather related to obesity (31, 105), steatosis-associated hepatic glucagon resistance (32, 38) and ensuing reduced amino acid turnover (39, 40). Reduced amino acid turnover ultimately results in hyperaminoacidaemia potentially stimulating glucagon secretion from pancreatic alpha cells (Fig. 4). As suggested from studies in rodents (35, 36, 37), the hyperaminoacidaemic state may not only result in hypersecretion of glucagon, but also increased alpha cell mass, as observed in obese humans (106).

Conclusions

Over the last decade, evidence that the gut plays a hitherto underestimated role in determining postprandial plasma glucagon concentrations has accumulated and human physiology experiments applying OGTT and IIGI experiments have challenged the traditional understanding of postabsorptive hyperglucagonaemia in diabetes (i.e. reduced alpha cell sensitivity to the glucagon-suppressive effects of glucose and insulin combined with diabetic hypoinsulinaemia). This new evidence supports that postabsorptive hyperglucagonaemia occurs as a consequence of gut-derived glucagon secretion and/or glucagonotropic factors elicited by intraluminal stimulation of the gastrointestinal tract. Furthermore, fasting hyperglucagonaemia seems to occur independently of the diabetic state and rather to be related to obesity-associated disruption of the emerging liver–alpha cell axis (hepatic glucagon resistance) involving amino acids as essential mediators of liver–alpha cell cross-talk (triggering compensatory glucagon secretion from alpha cells). Thus, when postabsorptive hyperglucagonaemia is observed, gut-dependent glucagon secretion should be taken into account and when evaluating fasting hyperglucagonaemia, hepatic glucagon resistance and amino acid-mediated compensatory alpha cell secretion need to be considered.

References

1 Bell GI, Sanchez-Pescador R, Laybourn PJ & Najarian RC. Exon duplication and divergence in the human preproglucagon gene. *Nature* 1983 **304** 368–371. (https://doi.org/10.1038/304368a0)

2 Banting FG, Best CH, Collip JB, Campbell WR & Fletcher AA. Pancreatic extracts in the treatment of diabetes mellitus. *Canadian Medical Association Journal* 1922 **12** 141–146.

3 Kimball CP & Murlin JR. Aqueous extracts of pancreas III. Some precipitation reactions of insulin. *Journal of Biological Chemistry* 1923 **58** 337–346.

4 Jorsal T, Rhee NA, Pedersen J, Wahlgren CD, Mortensen B, Jepsen SL, Jelsing J, Dalbøge LS, Vilmann P, Hassan H *et al.* Enteroendocrine K and L cells in healthy and type 2 diabetic individuals. *Diabetologia* 2018 **61** 284–294. (https://doi.org/10.1007/s00125-017-4450-9)

5 Ørskov C, Holst JJ, Knuhtsen S, Baldissera FGA, Poulsen SS & Nielsen OV. Glucagon-like peptides GLP-1 and GLP-2, predicted products of the glucagon gene, are secreted separately from pig small intestine but not pancreas. *Endocrinology* 1986 **119** 1467–1475. (https://doi.org/10.1210/endo-119-4-1467)

6 Sandoval DA & D'Alessio DA. Physiology of proglucagon peptides: role of glucagon and GLP-1 in health and disease. *Physiological Reviews* 2015 **95** 513–548. (https://doi.org/10.1152/physrev.00013.2014)

7 Seitz HJ, Krone W, Wilke H & Tarnowski W. Rapid rise in plasma glucagon induced by acute cold exposure in man and rat. *Pflugers Archiv: European Journal of Physiology* 1981 **389** 115–120. (https://doi.org/10.1007/BF00582100)

8 Holst JJ, Schwartz TW, Knuhtsen S, Jensen SL & Nielsen OV. Autonomic nervous control of the endocrine secretion from the isolated, perfused pig pancreas. *Journal of the Autonomic Nervous System* 1986 **17** 71–84. (https://doi.org/10.1016/0165-1838(86)90045-7)

9 Gerich JE, Karam JH & Forsham PH. Stimulation of glucagon secretion by epinephrine in man. *Journal of Clinical Endocrinology and Metabolism* 1973 **37** 479–481. (https://doi.org/10.1210/jcem-37-3-479)

10 Rocha DM, Faloona GR & Unger RH. Glucagon-stimulating activity of 20 amino acids in dogs. *Journal of Clinical Investigation* 1972 **51** 2346–2351. (https://doi.org/10.1172/JCI107046)

11 Palmer JP, Benson JW, Walter RM & Ensinck JW. Arginine-stimulated acute phase of insulin and glucagon secretion in diabetic subjects. *Journal of Clinical Investigation* 1976 **58** 565–570. (https://doi.org/10.1172/JCI108502)

12 Christensen M, Vedtofte L, Holst JJ, Vilsbøll T & Knop FK. Glucose-dependent insulinotropic polypeptide: a bifunctional glucose-dependent regulator of glucagon and insulin secretion in humans. *Diabetes* 2011 **60** 3103–3109. (https://doi.org/10.2337/db11-0979)

13 Meier JJ, Gallwitz B, Siepmann N, Holst JJ, Deacon CF, Schmidt WE & Nauck MA. Gastric inhibitory polypeptide (GIP) dose-dependently stimulates glucagon secretion in healthy human subjects at euglycaemia. *Diabetologia* 2003 **46** 798–801. (https://doi.org/10.1007/s00125-003-1103-y)

14 Christensen MB, Calanna S, Holst JJ, Vilsbøll T & Knop FK. Glucose-dependent insulinotropic polypeptide: blood glucose stabilizing effects in patients with type 2 diabetes. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** E418–E426. (https://doi.org/10.1210/jc.2013-3644)

15 Christensen M, Calanna S, Sparre-Ulrich AH, Kristensen PL, Rosenkilde MM, Faber J, Purrello F, van Hall G, Holst JJ, Vilsbøll T *et al.* Glucose-dependent insulinotropic polypeptide augments glucagon responses to hypoglycemia in type 1 diabetes. *Diabetes* 2015 **64** 72–78. (https://doi.org/10.2337/db14-0440)

16 Meier JJ, Nauck MA, Pott A, Heinze K, Goetze O, Bulut K, Schmidt WE, Gallwitz B & Holst JJ. Glucagon-like peptide 2 stimulates glucagon secretion, enhances lipid absorption, and inhibits gastric acid secretion in humans. *Gastroenterology* 2006 **130** 44–54. (https://doi.org/10.1053/j.gastro.2005.10.004)

17 Christensen M, Knop FK, Vilsbøll T, Aaboe K, Holst JJ, Madsbad S & Krarup T. Glucagon-like peptide-2, but not glucose-dependent insulinotropic polypeptide, stimulates glucagon release in patients with type 1 diabetes. *Regulatory Peptides* 2010 **163** 96–101. (https://doi.org/10.1016/j.regpep.2010.05.004)

18 Lund A, Vilsbøll T, Bagger JI, Holst JJ & Knop FK. The separate and combined impact of the intestinal hormones, GIP, GLP-1, and GLP-2, on glucagon secretion in type 2 diabetes. *American Journal of Physiology: Endocrinology and Metabolism* 2011 **300** E1038–E1046. (https://doi.org/10.1152/ajpendo.00665.2010)

European Journal of Endocrinology

19 McCulloch DK, Raghu PK, Koerker DJ, Palmer JP & Klaff LJ. Responses of the pancreatic A cell during hypoglycemia and hyperglycemia are dependent on the B cell. *Metabolism: Clinical and Experimental* 1989 **38** 702–707. (https://doi.org/10.1016/0026-0495(89)90111-X)

20 MacDonald PE, De Marinis YZ, Ramracheya R, Salehi A, Ma X, Johnson PRV, Cox R, Eliasson L & Rorsman P. A K ATP channel-dependent pathway within alpha cells regulates glucagon release from both rodent and human islets of Langerhans. *PLoS Biology* 2007 **5** e143. (https://doi.org/10.1371/journal.pbio.0050143)

21 Vieira E, Salehi A & Gylfe E. Glucose inhibits glucagon secretion by a direct effect on mouse pancreatic alpha cells. *Diabetologia* 2007 **50** 370–379. (https://doi.org/10.1007/s00125-006-0511-1)

22 Samols E, Bonner-Weir S & Weir GC. 2 intra-islet insulin – glucagon – somatostatin relationships. *Clinics in Endocrinology and Metabolism* 1986 **15** 33–58. (https://doi.org/10.1016/S0300-595X(86)80041-X)

23 Klaff LJ & Taborsky GJ. Pancreatic somatostatin is a mediator of glucagon inhibition by hyperglycemia. *Diabetes* 1987 **36** 592–596. (https://doi.org/10.2337/diab.36.5.592)

24 Taborsky GJ. Evidence of a paracrine role for pancreatic somatostatin in vivo. *American Journal of Physiology* 1983 **245** E598–E603. (https://doi.org/10.1152/ajpendo.1983.245.6.E598)

25 Kreymann B, Ghatei MA, Williams G & Bloom SR. Glucagon-like peptide-1 7–36: a physiological incretin in man. *Lancet* 1987 **330** 1300–1304. (https://doi.org/10.1016/S0140-6736(87)91194-9)

26 Ørskov C, Holst JJ & Nielsen OV. Effect of truncated glucagon-like peptide-1 (proglucagon-(78–107) amide) on endocrine secretion from pig pancreas, antrum, and nonantral stomach. *Endocrinology* 1988 **123** 2009–2013. (https://doi.org/10.1210/endo-123-4-2009)

27 Hare KJ, Knop FK, Asmar M, Madsbad S, Deacon CF, Holst JJ & Vilsbøll T. Preserved inhibitory potency of GLP-1 on glucagon secretion in type 2 diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism* 2009 **94** 4679–4687. (https://doi.org/10.1210/jc.2009-0921)

28 Unger RH & Orci L. The essential role of glucagon in the pathogenesis of diabetes mellitus. *Lancet* 1975 **1** 14–16. (https://doi.org/10.1016/S0140-6736(75)92375-2)

29 Dunning BE, Foley JE & Ahrén B. Alpha cell function in health and disease: influence of glucagon-like peptide-1. *Diabetologia* 2005 **48** 1700–1713. (https://doi.org/10.1007/s00125-005-1878-0)

30 Knop FK, Vilsbøll T, Madsbad S, Holst JJ & Krarup T. Inappropriate suppression of glucagon during OGTT but not during isoglycaemic i.v. glucose infusion contributes to the reduced incretin effect in type 2 diabetes mellitus. *Diabetologia* 2007 **50** 797–805. (https://doi.org/10.1007/s00125-006-0566-z)

31 Knop FK, Aaboe K, Vilsbøll T, Vølund A, Holst JJ, Krarup T & Madsbad S. Impaired incretin effect and fasting hyperglucagonaemia characterizing type 2 diabetic subjects are early signs of dysmetabolism in obesity. *Diabetes, Obesity and Metabolism* 2012 **14** 500–510. (https://doi.org/10.1111/j.1463-1326.2011.01549.x)

32 Suppli MP, Lund A, Bagger JI, Vilsbøll T & Knop FK. Involvement of steatosis-induced glucagon resistance in hyperglucagonaemia. *Medical Hypotheses* 2016 **86** 100–103. (https://doi.org/10.1016/j.mehy.2015.10.029)

33 Holst JJ, Wewer Albrechtsen NJ, Pedersen J & Knop FK. Glucagon and amino acids are linked in a mutual feedback cycle: the liver-α-cell axis. *Diabetes* 2017 **66** 235–240. (https://doi.org/10.2337/db16-0994)

34 Longuet C, Robledo AM, Dean ED, Dai C, Ali S, McGuinness I, de Chavez V, Vuguin PM, Charron MJ, Powers AC et al. Liver-specific disruption of the murine glucagon receptor produces α-cell hyperplasia. *Diabetes* 2013 **62** 1196–1205. (https://doi.org/10.2337/db11-1605)

35 Solloway MJ, Madjidi A, Gu C, Eastham-Anderson J, Clarke HJ, Kljavin N, Zavala-Solorio J, Kates L, Friedman B, Brauer M et al. Glucagon couples hepatic amino acid catabolism to mTOR-dependent regulation of α-cell mass. *Cell Reports* 2015 **12** 495–510. (https://doi.org/10.1016/j.celrep.2015.06.034)

36 Kim J, Okamoto H, Huang Z, Anguiano G, Chen S, Liu Q, Cavino K, Xin Y, Na E, Hamid R et al. Amino acid transporter Slc38a5 controls glucagon receptor inhibition-induced pancreatic α cell hyperplasia in mice. *Cell Metabolism* 2017 **25** 1348.e8–1361.e8. (https://doi.org/10.1016/j.cmet.2017.05.006)

37 Dean ED, Li M, Prasad N, Wisniewski SN, Von Deylen A, Spaeth J, Maddison L, Botros A, Sedgeman LR, Bozadjieva N et al. Interrupted glucagon signaling reveals hepatic α cell axis and role for L-glutamine in α cell proliferation. *Cell Metabolism* 2017 **25** 1362.e5–1373.e5. (https://doi.org/10.1016/j.cmet.2017.05.011)

38 Junker AE, Gluud L, Holst JJ, Knop FK & Vilsbøll T. Diabetic and nondiabetic patients with nonalcoholic fatty liver disease have an impaired incretin effect and fasting hyperglucagonaemia. *Journal of Internal Medicine* 2016 **279** 485–493. (https://doi.org/10.1111/joim.12462)

39 Wewer Albrechtsen NJ, Færch K, Jensen TM, Witte DR, Pedersen J, Mahendran Y, Jonsson AE, Galsgaard KD, Winther-Sørensen M, Torekov SS et al. Evidence of a liver-alpha cell axis in humans: hepatic insulin resistance attenuates relationship between fasting plasma glucagon and glucagonotropic amino acids. *Diabetologia* 2018 **61** 671–680. (https://doi.org/10.1007/s00125-017-4535-5)

40 Wewer Albrechtsen NJ, Junker AE, Christensen M, Hædersdal S, Wibrand F, Lund AM, Galsgaard KD, Holst JJ, Knop FK & Vilsbøll T. Hyperglucagonemia correlates with plasma levels of non-branched-chain amino acids in patients with liver disease independent of type 2 diabetes. *American Journal of Physiology: Gastrointestinal and Liver Physiology* 2018 **314** G91–G96. (https://doi.org/10.1152/ajpgi.00216.2017)

41 Lund A, Bagger JI, Wewer Albrechtsen NJ, Christensen M, Grøndahl M, Hartmann B, Mathiesen ER, Hansen CP, Storkholm JH, van Hall G et al. Evidence of extrapancreatic glucagon secretion in man. *Diabetes* 2016 **65** 585–597. (https://doi.org/10.2337/db15-1541)

42 Gromada J, Franklin I & Wollheim CB. α-Cells of the endocrine pancreas: 35 years of research but the enigma remains. *Endocrine Reviews* 2007 **28** 84–116. (https://doi.org/10.1210/er.2006-0007)

43 Reaven GM, Chen YDI, Golay A, Swislocki ALM & Jaspan JB. Documentation of hyperglucagonemia throughout the day in nonobese and obese patients with noninsulin-dependent diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism* 1987 **64** 106–110. (https://doi.org/10.1210/jcem-64-1-106)

44 Gerich JE, Lorenzi M, Bier DM, Schneider V, Tsalikian E, Karam JH & Forsham PH. Prevention of human diabetic ketoacidosis by somatostatin. *New England Journal of Medicine* 1975 **292** 985–989. (https://doi.org/10.1056/NEJM197505082921901)

45 Baron AD, Schaeffer L, Shragg P & Kolterman OG. Role of hyperglucagonemia in maintenance of increased rates of hepatic glucose output in type II diabetics. *Diabetes* 1987 **36** 274–283. (https://doi.org/10.2337/diab.36.3.274)

46 Shah P, Basu A, Basu R & Rizza R. Impact of lack of suppression of glucagon on glucose tolerance in humans. *American Journal of Physiology* 1999 **277** E283–E290.

47 Lawrence AM. Glucagon. *Annual Review of Medicine* 1969 **20** 207–222. (https://doi.org/10.1146/annurev.me.20.020169.001231)

48 Grøndahl MF, Keating DJ, Vilsbøll T & Knop FK. Current therapies that modify glucagon secretion: what is the therapeutic effect of such modifications? *Current Diabetes Reports* 2017 **17** 128. (https://doi.org/10.1007/s11892-017-0967-z)

49 Dunning BE & Gerich JE. The role of α-cell dysregulation in fasting and postprandial hyperglycemia in type 2 diabetes and therapeutic implications. *Endocrine Reviews* 2007 **28** 253–283. (https://doi.org/10.1210/er.2006-0026)

50 Hamaguchi T, Fukushima H, Uehara M, Wada S, Shirotani T, Kishikawa H, Ichinose K, Yamaguchi K & Shichiri M. Abnormal

glucagon response to arginine and its normalization in obese hyperinsulinaemic patients with glucose intolerance: importance of insulin action on pancreatic alpha cells. *Diabetologia* 1991 **34** 801–806. (https://doi.org/10.1007/BF00408354)

51 Wan Y, Wang Q & Prud'homme GJ. GABAergic system in the endocrine pancreas: a new target for diabetes treatment. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 2015 **8** 79–87. (https://doi.org/10.2147/DMSO.S50642)

52 Gedulin BR, Rink TJ & Young AA. Dose-response for glucagonostatic effect of amylin in rats. *Metabolism* 1997 **46** 67–70. (https://doi.org/10.1016/S0026-0495(97)90170-0)

53 Calanna S, Christensen M, Holst JJ, Laferrère B, Gluud LL, Vilsbøll T & Knop FK. Secretion of glucagon-like peptide-1 in patients with type 2 diabetes mellitus: systematic review and meta-analyses of clinical studies. *Diabetologia* 2013 **56** 965–972. (https://doi.org/10.1007/s00125-013-2841-0)

54 Knop FK, Vilsbøll T, Højberg PV, Larsen S, Madsbad S, Vølund A, Holst JJ & Krarup T. Reduced incretin effect in type 2 diabetes: cause or consequence of the diabetic state? *Diabetes* 2007 **56** 1951–1959. (https://doi.org/10.2337/db07-0100)

55 Muscelli E, Mari A, Casolaro A, Camastra S, Seghieri G, Gastaldelli A, Holst JJ & Ferrannini E. Separate impact of obesity and glucose tolerance on the incretin effect in normal subjects and type 2 diabetic patients. *Diabetes* 2008 **57** 1340–1348. (https://doi.org/10.2337/db07-1315)

56 Oh TJ, Kim MY, Shin JY, Lee JC, Kim S, Park KS & Cho YM. The incretin effect in Korean subjects with normal glucose tolerance or type 2 diabetes. *Clinical Endocrinology* 2014 **80** 221–227. (https://doi.org/10.1111/cen.12167)

57 Nauck M, Stöckmann F, Ebert R & Creutzfeldt W. Reduced incretin effect in Type 2 (non-insulin-dependent) diabetes. *Diabetologia* 1986 **29** 46–52. (https://doi.org/10.1007/BF02427280)

58 Østoft SH, Bagger JI, Hansen T, Pedersen O, Holst JJ, Knop FK & Vilsbøll T. Incretin effect and glucagon responses to oral and intravenous glucose in patients with maturity-onset diabetes of the young – type 2 and type 3. *Diabetes* 2014 **63** 2838–2844. (https://doi.org/10.2337/db13-1878)

59 Hare KJ, Vilsbøll T, Holst JJ & Knop FK. Inappropriate glucagon response after oral compared with isoglycemic intravenous glucose administration in patients with type 1 diabetes. *American Journal of Physiology: Endocrinology and Metabolism* 2010 **298** E832–E837. (https://doi.org/10.1152/ajpendo.00700.2009)

60 Knop FK, Vilsbøll T, Larsen S, Madsbad S, Holst JJ & Krarup T. Glucagon suppression during OGTT worsens while suppression during IVGTT sustains alongside development of glucose intolerance in patients with chronic pancreatitis. *Regulatory Peptides* 2010 **164** 144–150. (https://doi.org/10.1016/j.regpep.2010.05.011)

61 Bagger JI, Knop FK, Lund A, Holst JJ & Vilsbøll T. Glucagon responses to increasing oral loads of glucose and corresponding isoglycaemic intravenous glucose infusions in patients with type 2 diabetes and healthy individuals. *Diabetologia* 2014 **57** 1720–1725. (https://doi.org/10.1007/s00125-014-3264-2)

62 Meier JJ, Deacon CF, Schmidt WE, Holst JJ & Nauck MA. Suppression of glucagon secretion is lower after oral glucose administration than during intravenous glucose administration in human subjects. *Diabetologia* 2007 **50** 806–813. (https://doi.org/10.1007/s00125-007-0598-z)

63 Bagger JI, Knop FK, Lund A, Vestergaard H, Holst JJ & Vilsbøll T. Impaired regulation of the incretin effect in patients with type 2 diabetes. *Journal of Clinical Endocrinology and Metabolism* 2011 **96** 737–745. (https://doi.org/10.1210/jc.2010-2435)

64 Kosinski M, Knop FK, Vedtofte L, Grycewiczv J, Swierzewska P, Cypryk K & Vilsbøll T. Postpartum reversibility of impaired incretin effect in gestational diabetes mellitus. *Regulatory Peptides* 2013 **186** 104–107. (https://doi.org/10.1016/j.regpep.2013.08.002)

65 Idorn T, Knop FK, Jørgensen M, Holst JJ, Hornum M & Feldt-Rasmussen B. Gastrointestinal factors contribute to glucometabolic disturbances in nondiabetic patients with end-stage renal disease. *Kidney International* 2013 **83** 915–923. (https://doi.org/10.1038/ki.2012.460)

66 Muscelli E, Mari A, Natali A, Astiarraga BD, Camastra S, Frascerra S, Holst JJ & Ferrannini E. Impact of incretin hormones on β-cell function in subjects with normal or impaired glucose tolerance. *American Journal of Physiology: Endocrinology and Metabolism* 2006 **291** E1144–E1150. (https://doi.org/10.1152/ajpendo.00571.2005)

67 Gyldenløve M, Vilsbøll T, Zachariae C, Holst JJ, Knop FK & Skov L. Impaired incretin effect is an early sign of glucose dysmetabolism in nondiabetic patients with psoriasis. *Journal of Internal Medicine* 2015 **278** 660–670. (https://doi.org/10.1111/joim.12388)

68 Hansen KB, Vilsbøll T, Bagger JI, Holst JJ & Knop FK. Reduced glucose tolerance and insulin resistance induced by steroid treatment, relative physical inactivity, and high-calorie diet impairs the incretin effect in healthy subjects. *Journal of Clinical Endocrinology and Metabolism* 2010 **95** 3309–3317. (https://doi.org/10.1210/jc.2010-0119)

69 Plamboeck A, Veedfald S, Deacon CF, Hartmann B, Wettergren A, Svendsen LB, Meisner S, Hovendal C, Knop FK, Vilsbøll T *et al*. Characterisation of oral and i.v. glucose handling in truncally vagotomised subjects with pyloroplasty. *European Journal of Endocrinology* 2013 **169** 187–201. (https://doi.org/10.1530/EJE-13-0264)

70 Rhee NA, Østoft SH, Holst JJ, Deacon CF, Vilsbøll T & Knop FK. The impact of dipeptidyl peptidase 4 inhibition on incretin effect, glucose tolerance, and gastrointestinal-mediated glucose disposal in healthy subjects. *European Journal of Endocrinology* 2014 **171** 353–362. (https://doi.org/10.1530/EJE-14-0314)

71 Lund A, Bagger JI, Christensen M, Grøndahl M, van Hall G, Holst JJ, Vilsbøll T & Knop FK. Higher endogenous glucose production during OGTT vs isoglycemic intravenous glucose infusion. *Journal of Clinical Endocrinology and Metabolism* 2016 **101** 4377–4384. (https://doi.org/10.1210/jc.2016-1948)

72 Hædersdal S, Lund A, Nielsen-Hannerup E, Maagensen H, Holst JJ, Knop FK & Vilsbøll T. The Role of glucagon in gastrointestinal-mediated glucose disposal and incretin effect in patients with type 2 diabetes and normal glucose tolerant individuals. *Diabetes* 2017 **66** A496–A565.

73 Hædersdal S, Lund A, Maagensen H, Nielsen-Hannerup E, Holst JJ, Knop FK & Vilsbøll T. Glucagon receptor antagonism lowers fasting glucose but not postprandial glucose excursions in patients with type 2 diabetes. *Diabetes* 2017 **66** A496–A565.

74 Holst JJ. The physiology of glucagon-like peptide 1. *Physiological Reviews* 2007 **87** 1409–1439. (https://doi.org/10.1152/physrev.00034.2006)

75 Calanna S, Christensen M, Holst JJ, Laferrère B, Gluud LL, Vilsbøll T & Knop FK. Secretion of glucose-dependent insulinotropic polypeptide in patients with type 2 diabetes. *Diabetes Care* 2013 **36** 3346–3352. (https://doi.org/10.2337/dc13-0465)

76 Chia CW, Carlson OD, Kim W, Shin YK, Charles CP, Kim HS, Melvin DL & Egan JM. Exogenous glucose-dependent insulinotropic polypeptide worsens post prandial hyperglycemia in type 2 diabetes. *Diabetes* 2009 **58** 1342–1349. (https://doi.org/10.2337/db08-0958)

77 Sutherland EW & de Duve C. Origin and distribution of the hyperglycemic-glycogenolytic factor of the pancreas. *Journal of Biological Chemistry* 1948 **175** 663–674.

78 Bak MJ, Albrechtsen NW, Pedersen J, Hartmann B, Christensen M, Vilsbøll T, Knop FK, Deacon CF, Dragsted LO & Holst JJ. Specificity and sensitivity of commercially available assays for glucagon and oxyntomodulin measurement in humans. *European Journal of Endocrinology* 2014 **170** 529–538. (https://doi.org/10.1530/EJE-13-0941)

79 Wewer Albrechtsen NJ, Hartmann B, Veedfald S, Windeløv JA, Plamboeck A, Bojsen-Møller KN, Idorn T, Feldt-Rasmussen B, Knop FK, Vilsbøll T *et al*. Hyperglucagonaemia analysed by glucagon sandwich ELISA: nonspecific interference or truly elevated levels? *Diabetologia* 2014 **57** 1919–1926. (https://doi.org/10.1007/s00125-014-3283-z)

80 Scheltema RA, Hauschild JP, Lange O, Hornburg D, Denisov E, Damoc E, Kuehn A, Makarov A & Mann M. The Q exactive HF, a Benchtop mass spectrometer with a pre-filter, high-performance quadrupole and an ultra-high-field orbitrap analyzer. *Molecular and Cellular Proteomics* 2014 **13** 3698–3708. (https://doi.org/10.1074/mcp.M114.043489)

81 Muller WA, Girardier L, Seydoux J, Berger M, Renold AE & Vranic M. Extrapancreatic glucagon and glucagonlike immunoreactivity in depancreatized dogs. *Journal of Clinical Investigation* 1978 **62** 124–132. (https://doi.org/10.1172/JCI109096)

82 Matsuyama T & Foà PP. Plasma glucose, insulin, pancreatic, and enteroglucagon levels in normal and depancreatized dogs. *Proceedings of the Society for Experimental Biology and Medicine: Society for Experimental Biology and Medicine* 1974 **147** 97–102. (https://doi.org/10.3181/00379727-147-38288)

83 Ravazzola M, Unger RH & Orci L. Demonstration of glucagon in the stomach of human fetuses. *Diabetes* 1981 **30** 879–882. (https://doi.org/10.2337/diab.30.10.879)

84 Holst JJ, Aggestrup S, Loud FB & Olesen M. Content and gel filtration profiles of glucagon-like and somatostatin-like immunoreactivity in human fundic mucosa. *Journal of Clinical Endocrinology and Metabolism* 1983 **56** 729–732. (https://doi.org/10.1210/jcem-56-4-729)

85 Campbell JE & Drucker DJ. Islet α cells and glucagon – critical regulators of energy homeostasis. *Nature Reviews Endocrinology* 2015 **11** 329–338. (https://doi.org/10.1038/nrendo.2015.51)

86 Knop FK. Resolution of type 2 diabetes following gastric bypass surgery: involvement of gut-derived glucagon and glucagonotropic signalling? *Diabetologia* 2009 **52** 2270–2276. (https://doi.org/10.1007/s00125-009-1511-8)

87 Knop FK, Hare KJ, Pedersen J, Hendel JW, Poulsen SS, Holst JJ & Vilsbøll T. Prohormone convertase 2 positive enteroendocrine cells are more abundant in patients with type 2 diabetes – a potential source of gut-derived glucagon. *Diabetes* 2011 **60** A442–A521.

88 Rhee NA, Wahlgren CD, Pedersen J, Mortensen B, Langholz E, Wandall EP, Friis SU, Vilmann P, Paulsen SJ, Kristiansen VB *et al*. Effect of Roux-en-Y gastric bypass on the distribution and hormone expression of small-intestinal enteroendocrine cells in obese patients with type 2 diabetes. *Diabetologia* 2015 **58** 2254–2258. (https://doi.org/10.1007/s00125-015-3696-3)

89 Juel CTBJ, Lund A, Hansen CP, Storkholm JH, Wewer Albrechtsen NJ, Holst JJ, Vilsbøll T & Knop FK. Intravenous arginine has no effect on the secretion of gut-derived glucagon in totally pancreatectomized subjects. *Diabetes* 2017 **66** LB1–LB94.

90 Juel CTB, Lund A, Hansen CP, Storkholm JH, Wewer Albrechtsen NJ, Holst JJ, Vilsbøll T & Knop FK. The GLP-1 receptor agonist lixisenatide reduces postprandial glucose excursions in totally pancreatectomized patients. *Diabetes* 2017 **66** A20. (https://doi.org/10.2337/dbi16-0049)

91 Salehi M, Gastaldelli A & D'Alessio DA. Blockade of glucagon-like peptide 1 receptor corrects post-prandial hypoglycemia after gastric bypass. *Gastroenterology* 2014 **146** 669.e2–680.e2. (https://doi.org/10.1053/j.gastro.2013.11.044)

92 Rabiee A, Magruder JT, Salas-Carrillo R, Carlson O, Egan JM, Askin FB, Elahi D & Andersen DK. Hyperinsulinemic hypoglycemia after Roux-en-Y gastric bypass: Unraveling the role of gut hormonal and pancreatic endocrine dysfunction. *Journal of Surgical Research* 2011 **167** 199–205. (https://doi.org/10.1016/j.jss.2010.09.047)

93 Ullrich J, Ernst B, Wilms B, Thurnheer M & Schultes B. Roux-en Y gastric bypass surgery reduces hedonic hunger and improves dietary habits in severely obese subjects. *Obesity Surgery* 2013 **23** 50–55. (https://doi.org/10.1007/s11695-012-0754-5)

94 Shah P, Vella A, Basu A, Basu R, Schwenk WF & Rizza RA. Lack of suppression of glucagon contributes to postprandial hyperglycemia in subjects with type 2 diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism* 2000 **85** 4053–4059. (https://doi.org/10.1210/jcem.85.11.6993)

95 Unger RH, Aguilar-Parada E, Müller WA & Eisentraut AM. Studies of pancreatic alpha cell function in normal and diabetic subjects. *Journal of Clinical Investigation* 1970 **49** 837–848. (https://doi.org/10.1172/JCI106297)

96 DeFronzo RA. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* 2009 **58** 773–795. (https://doi.org/10.2337/db09-9028)

97 Hamberg O & Vilstrup H. Regulation of urea synthesis by glucose and glucagon in normal man. *Clinical Nutrition* 1994 **13** 183–191. (https://doi.org/10.1016/0261-5614(94)90099-X)

98 Guzman CB, Zhang XM, Liu R, Regev A, Shankar S, Garhyan P, Pillai SG, Kazda C, Chalasani N & Hardy TA. Treatment with LY2409021, a glucagon receptor antagonist, increases liver fat in patients with type 2 diabetes. *Diabetes, Obesity and Metabolism* 2017 **19** 1521–1528. (https://doi.org/10.1111/dom.12958)

99 Lund A, Bagger JI, Albrechtsen NJW, Christensen M, Grøndahl M, Hansen CP, Storkholm JH, Holst JJ, Vilsbøll T & Knop FK. Increased liver fat content in totally pancreatectomized patients. *Diabetes* 2017 **66** A4674–A688.

100 Dragstedt LR, Clark DE & Vermeulen C. The significance of lipocaic in surgery. *Annals of Surgery* 1939 **110** 907–915. (https://doi.org/10.1097/00000658-193911000-00010)

101 Chaikoff IL, Connor CL & Biskind GR. Fatty infiltration and cirrhosis of the liver in depancreatized dogs maintained with insulin. *American Journal of Pathology* 1938 **14** 101–110.7.

102 Chernick SS & Scow RO. Early effects of total pancreatectomy on fat metabolism in the rat. *American Journal of Physiology* 1959 **196** 125–131.

103 Dresler CM, Fortner JG, McDermott K & Bajorunas DR. Metabolic consequences of (regional) total pancreatectomy. *Annals of Surgery* 1991 **214** 131–140. (https://doi.org/10.1097/00000658-199108000-00007)

104 Takemura N, Saiura A, Koga R, Yamamoto J & Yamaguchi T. Risk factors for and management of postpancreatectomy hepatic steatosis. *Scandinavian Journal of Surgery* 2017 **106** 224–229. (https://doi.org/10.1177/1457496916669630)

105 Ferrannini E, Muscelli E, Natali A, Gabriel R, Mitrakou A, Flyvbjerg A, Golay A, Hojlund K & Investigators TR between IS and CDR (RISC) P. Association of fasting glucagon and proinsulin concentrations with insulin resistance. *Diabetologia* 2007 **50** 2342–2347. (https://doi.org/10.1007/s00125-007-0806-x)

106 Ellenbroek JH, Töns HAM, Hanegraaf MAJ, Rabelink TJ, Engelse MA, Carlotti F & de Koning EJP. Pancreatic α-cell mass in obesity. *Diabetes, Obesity and Metabolism* 2017 **19** 1810–1813. (https://doi.org/10.1111/dom.12997)