# Postprandial gallbladder emptying in patients with type 2 diabetes: potential implications for bile-induced secretion of glucagon-like peptide 1

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## Abstract

*Objective*: Recent preclinical work has suggested that postprandial flow of bile acids into the small intestine potentiates nutrient-induced glucagon-like peptide 1 (GLP1) secretion via bile acid-induced activation of the G protein-coupled receptor TGR5 in intestinal L cells. The notion of bile-induced GLP1 secretion combined with the findings of reduced postprandial gallbladder emptying in patients with type 2 diabetes (T2DM) led us to speculate whether reduced postprandial GLP1 responses in some patients with T2DM arise as a consequence of diabetic gallbladder dysmotility.

*Design and methods*: In a randomised design, 15 patients with long-standing T2DM and 15 healthy age-, gender- and BMI-matched control subjects were studied during 75-g oral glucose tolerance test (OGTT) and three isocaloric (500 kcal) and isovolaemic (350 ml) liquid meals: i) 2.5 g fat, 107 g carbohydrate and 13 g protein; ii) 10 g fat, 93 g carbohydrate and 11 g protein; and iii) 40 g fat, 32 g carbohydrate and 3 g protein. Basal and postprandial plasma concentrations of glucose, insulin, C-peptide, glucagon, GLP1, glucose-dependent insulinotropic polypeptide (GIP), cholecystokinin and gastrin were measured. Furthermore, gallbladder emptying and gastric emptying were examined.

*Results*: Gallbladder emptying increased with increasing meal fat content, but no intergroup differences were demonstrated. GIP and GLP1 responses were comparable among the groups with GIP levels being higher following high-fat meals, whereas GLP1 secretion was similar after both OGTT and meals.

*Conclusions*: In conclusion, patients with T2DM exhibited normal gallbladder emptying to meals with a wide range of fat content. Incretin responses were similar to that in controls, and an association with postprandial gallbladder contraction could not be demonstrated.

## Introduction

It has been shown previously in 1983 that bile acids stimulated the release of gut glucagon-like immunoreactive materials in dogs (1). More recently, it has become clear that bile acids not only act as simple fat solubilisers in the digestive tract, but also represent complex hormonal metabolic integrators (2). The most important receptors activated by bile acids are the farnesoid X receptor – a receptor (expressed in high concentrations in the liver and intestines), which is implicated in glucose control – and the G protein-coupled cell membrane receptor TGR5 (2, 3). Interestingly, in enteroendocrine cells, TGR5 activation leads to the secretion of glucagon-like peptide 1 (GLP1) and

possibly other L cell products (e.g. the other satiety hormone peptide YY), suggesting that bile acids may modulate glucose homoeostasis, appetite and body weight via TGR5 (2). GLP1 and glucose-dependent insulinotropic polypeptide (GIP) are incretin hormones and they act in concert to generate the so-called incretin effect (i.e. the augmentation of insulin secretion after oral administration compared with an isoglycaemic i.v. glucose stimulus (4)). Moreover, GLP1 has a range of additional effects including inhibition of glucagon secretion, appetite and gastric motility, all of which ultimately impact on glucose homoeostasis (5). Secretion of GLP1 is stimulated by the arrival of digested nutrients in the intestinal lumen (5). However, GLP1 is rapidly metabolised by the ubiquitous enzyme dipeptidyl peptidase 4 (DPP4), whereby the biological activity is abolished. In accordance, it is believed that afferent neurons in the intestinal mucosa mediate the major glucose-lowering effect of GLP1 (5). The predominant, but not unchallenged, view on GLP1 secretion in patients with type 2 diabetes (T2DM) is that some, but not all, patients (6) have defective postprandial responses (7, 8, 9, 10), whereas levels during oral glucose tolerance tests (OGTTs) usually are comparable to controls (11, 12, 13, 14, 15, 16). As bile acids may influence nutrient-induced GLP1 secretion from the L cells (via TGR5), postprandial flow of bile from the gallbladder into the intestine may potentiate the post-prandial L cell response. Conversely, gallbladder dysmotility – a common, albeit not consistent (17), finding in patients with T2DM (18, 19, 20, 21) – may result in impaired postprandial flow of bile into the intestine and subsequently reduced bile acid-induced TGR5 activation (22).

We hypothesised that patients with T2DM of relatively long duration (>5 years) would be characterised by decreased postprandial gallbladder contraction (as shown in previous studies) and, consequently, reduced post-prandial intestinal TGR5 activation, resulting in attenuated GLP1 responses compared with healthy age-, gender- and BMI-matched control subjects. According to this hypothesis, an impairment of postprandial GLP1 secretion following high-fat meal intake in patients with T2DM would not be evident during low-fat meals or oral glucose, which ostensibly do not stimulate gallbladder emptying to the same extent.

## Subjects and methods

The protocol was approved by the Scientific-Ethical Committee of the Capital Region of Denmark (registration no. H-1-2011-008) and registered with the Danish Data Protection Agency (registration no. 2011-41-6274) and at ClinicalTrials.gov (clinical trial reg. no. NCT01374594). The study was conducted according to the principles of the Helsinki Declaration II. Written informed consent was obtained from all participants.

### Study population

Following screening for prespecified inclusion and exclusion criteria (see below), 15 patients with a mean diabetes duration of 7.5 years (range: 6–20) and 15 healthy age-, gender- and BMI-matched control subjects were included in the study (Table 1). At the screening visit, all potential subjects ($n = 58$) underwent a physical examination and had standard haematological and clinical biochemistry parameters measured. Exclusion criteria included acute or chronic illnesses (including hypercholesterolaemia), gall-bladder disease, taking ongoing medication (which could not be paused for 12 h), having first-degree relatives with diabetes (control group) and showing repeated abnormalities in haemoglobin, plasma liver enzymes (alanine or aspartate aminotransferase), creatinine concentration or urinary albumin-to-creatinine ratio. The diabetic group consisted of patients with non-insulin-requiring T2DM of relatively long duration (>5 years) diagnosed according to the criteria of the World Health Organization (WHO). All patients underwent a 75 g OGTT (1–2 weeks before the first day of study). None of the patients suffered from any overt diabetic complications. Four patients received only dietary treatment, eight were also treated with metformin and three with sulphonylurea. Patients were instructed to avoid their blood glucose-lowering drugs, if any, for

**Table 1** Characteristics of subjects. Data are mean values with ranges in parentheses.

|  | Controls | T2DM |
|---|---|---|
| Age (years) | 60 (42–71) | 60 (43–70) |
| Sex (M/F) | 9/6 | 9/6 |
| Height (m) | 1.76 (1.58–1.92) | 1.75 (1.60–1.97) |
| Weight (kg) | 86.5 (66.0–106.0) | 85.9 (63.6–121.5) |
| BMI (kg/m²) | 27.9 (24.0–31.7) | 28.0 (24.5–31.9) |
| Waist:hip ratio | 0.97 (0.85–1.11) | 0.98 (0.86–1.14) |
| Systolic blood pressure (mmHg) | 127 (110–142) | 133 (109–168) |
| Diastolic blood pressure (mmHg) | 81 (62–88) | 82 (66–97) |
| Fasting plasma glucose (mmol/l) | 5.3 (4.8–6.0) | 10.2 (6.3–15.5) |
| HbA1c (%) | 5.2 (4.9–5.7) | 7.5 (6.0–9.9) |
| HbA1c (mmol/mol) | 33 (30–39) | 58 (42–85) |
| Duration of T2DM (years) | – | 7.5 (6–20) |

a period of not less than a week before each experimental day (see below). Other medications included angiotensin II receptor blockers: 2; angiotensin-converting enzyme inhibitors: 7; calcium antagonists: 4; thiazide diuretics: 4; acetylsalicylic acid: 7; protein-pump inhibitors: 1; and statins: 13. Control subjects were without family history of diabetes and had normal glucose tolerance according to a 75 g OGTT performed immediately before inclusion in the study. The control subjects were not taking any form of medication.

### Study design

On all occasions, subjects were studied in a semi-recumbent position, lying on their backs with the upper part of the body 45° upright, after an overnight (10 h) fast including liquids, medication and tobacco use. Subjects were instructed to avoid strenuous physical exercise for 12 h before arriving in the morning. The experiments were carried out for 4 days randomly, separated by at least 48 h, and consisted of a 75 g OGTT (75 g of water-free glucose dissolved in 300 ml water) and three isocaloric (500 kcal) and isovolaemic (350 ml) liquid meals (low fat: 2.5 g fat, 107 g carbohydrate and 13 g protein; medium fat: 10 g fat, 93 g carbohydrate and 11 g protein and high fat: 40 g fat, 32 g carbohydrate and 3 g protein). Meals were made by combining two nutritional drinks (Nutridrink Juice Style and Calogen Strawberry, Nutricia, Allerød, Denmark) and mixing them with 1.5 g paracetamol for evaluation of gastric emptying. The amount of fat in the meals was based on the threshold for effective fat-induced gallbladder contraction ($\sim$10 g) (23). Water was added to standardise the volume to 350 ml. On each day, a cannula was inserted into a cubital vein for collection of arterialised blood samples (cannulated forearm was placed in a heating box (50 °C) throughout the experiment). The OGTT or meals were ingested over the first 10 min of the experiment (after baseline sampling).

### Data collection

Arterialised blood samples were drawn 20, 10 and 0 min before and 15, 30, 45, 60, 90, 120, 180 and 240 min after ingestion of the OGTT or meals. Blood was collected into chilled tubes containing EDTA and a specific DPP4 inhibitor (valine pyrrolidide at a final concentration of 0.01 mmol/l) for plasma analyses of gut hormones. For analyses of insulin and C-peptide, blood was distributed into chilled tubes containing heparin. EDTA and heparin tubes were kept on ice until centrifugation. Blood for analysis of paracetamol (acetaminophen) was distributed into dry tubes for coagulation (20 min at room temperature). All samples were centrifuged for 20 min at 1200 $\mathbf{g}$ and 4 °C. Plasma samples for GLP1, GIP, glucagon, cholecystokinin (CCK) and gastrin analyses and serum samples for paracetamol were stored at −20 °C, and plasma samples for insulin and C-peptide analyses were stored at −80 °C until analysis. For bedside measurements of plasma glucose, blood was distributed into fluoride tubes and centrifuged immediately at 7400 $\mathbf{g}$ for 2 min at room temperature.

Gallbladder measurements were performed using a Pro Focus ultrasound scanner (B&K Medical, Herlev, Denmark) with a 3.5 MHz convex transducer. The gallbladder was measured in three dimensions, one longitudinal ($D1$) and two cross-sectional diameters ($D2$ and $D3$), and the volume was calculated using the ellipse formula (volume $= \pi/6 \times D1 \times D2 \times D3$) (24). Basal gallbladder volumes were based on the average of two sequential measurements followed by measurements every 10 min for the first hour. A single investigator performed gallbladder measurements. Intraobserver variation of fasting images was within 10% (smallest when the gallbladder was large), which is in agreement with Everson et al. (25). Gallbladder ejection fraction (EF%) was defined as the reduction in the gallbladder volume at any time point from baseline divided by the baseline gallbladder volume multiplied by 100%. EF% time curves were characterised descriptively by calculating the maximum response ($E_{max}$) and time to maximum response ($T_{max}$), and further by calculating the area under the curve (AUC) from 0–60 min, using the trapezoidal rule.

### Laboratory methods

Plasma glucose was measured by the glucose oxidase method, using a glucose analyser (Yellow Springs Instrument model 2300 STAT plus analyzer; YSI, Inc., Yellow Springs, OH, USA). Plasma insulin and C-peptide concentrations were measured using a two-sided electro-chemiluminescence immunoassay (Roche/Hitachi Modular analytics; Roche Diagnostic GmbH). Intra- and interassay coefficient of variation (CV) values are <3% (insulin) and <6% (C-peptide) respectively. Plasma concentrations of total GLP1, GIP, amidated gastrin and CCK were measured by RIAs as described previously (26, 27, 28, 29). Intra- and interassay CV values for GIP, GLP1 and glucagon are <6 and <15% respectively. The glucagon assay was directed against the C-terminal of the glucagon molecule and, therefore, measures glucagon of

pancreatic origin (14). For CCK and amidated gastrin, the intra- and interassay CV values are $<6$ and $<10\%$ respectively. Serum paracetamol was measured by the Vitros ACET slide as described previously (30, 31). Intra- and interassay CV value for paracetamol is $<5\%$.

### Statistical analyses

Results are expressed as means $\pm$ s.e.m. unless otherwise stated. The AUC values were calculated using the trapezoidal rule and are presented as incremental AUC (iAUC) values if nothing else is stated. For analysis of variations and differences between the AUC values, repeated measures ANOVA with Tukey's *post hoc* test was used. The assumptions of a Gaussian distribution of observations and homogeneity of variances were assessed visually by drawing histograms and probability plots. If assumptions could not be met, data were transformed logarithmically or reciprocally before statistical analysis was performed. A two-sided $P$ value of $<0.05$ was used to indicate significant differences. Statistical analyses were performed using GraphPad Prism version 6.0b for Windows/Mac (GraphPad Software, San Diego, CA, USA) and R (3.0.1) for Windows/Mac (http://cran.r-project.org).

## Results

### Glucose

Time courses for plasma concentrations of glucose are shown in Fig. 1 and the AUC values are given in Table 2. On all study days, mean fasting and postprandial plasma glucose concentrations were higher in patients compared with the control group. In both groups, postprandial glucose excursions were comparable following the OGTT, low-fat meal and medium-fat meal, but became markedly reduced in response to the high-fat meal.

### Insulin and C-peptide

Time courses for plasma concentrations of insulin and C-peptide are shown in Fig. 1 and AUC values are given in Table 2. Basal concentrations of insulin and C-peptide, respectively, were slightly higher in patients with T2DM compared with controls, and during the first 90 min of the OGTT, low-fat meal and medium-fat meal (Fig. 1), the concentrations were markedly lower in patients when compared with controls ($P=0.001$–0.005). By contrast, no intergroup differences were demonstrated comparing the responses after the high-fat meal ($P=0.16$). In both

groups, high fat resulted in very small increments of insulin and C-peptide concentrations (Fig. 1).

### Glucagon

Basal concentrations of glucagon were higher in patients with T2DM compared with controls ($14.0\pm0.7$ vs $8.7\pm0.3$ pmol/l$\times$min, $P<0.0001$). As illustrated in Fig. 1, the glucagon concentrations were markedly augmented in patients with T2DM compared with controls. From 0–30 min, peak concentrations reached $\sim$20 pmol/l (OGTT) to $\sim$25 pmol/l (meals) in patients compared with a peak of $\sim$12 pmol/l in controls during the high-fat meal. Controls were capable of suppressing glucagon secretion during OGTT ($-134\pm40$ pmol/l$\times$min), low-fat ($-45\pm52$ pmol/l$\times$min) and medium-fat ($-19\pm32$ pmol/l$\times$min) meals (Fig. 1 and Table 2), with greater suppression following OGTT in comparison with low- and medium-fat meals ($P=0.002$–0.013). However, glucagon was augmented throughout following high-fat meals in controls (Fig. 1).

### GLP1 and GIP

Time courses for plasma concentrations of GLP1 and GIP are shown in Fig. 2 and AUC values are given in Table 3. Intergroup differences of peak values and iAUC values of both GLP1 and GIP were comparable (Table 2). On the contrary, within group comparisons in both groups revealed that the high-fat meal resulted in the highest peak and iAUC values ($P=0.0001$–0.01). This was particularly clear for GIP, which increased with increasing fat content in the meals ($P<0.0001$). Notably, following the high-fat meal, patients with T2DM exhibited earlier peak values of GIP ($\sim$30 min) compared with controls ($\sim$90 min) (Fig. 2).

### CCK and gastrin

Time courses for the plasma concentrations of CCK and gastrin are shown in Fig. 2 and AUC values are given in Table 3. Basal levels of CCK were comparable among patients with T2DM and controls on all study days ($0.7\pm0.03$ vs $0.7\pm0.04$ pmol/l$\times$min, all days grouped). In controls, peak CCK concentrations were similar and no clear dose–response relationship towards augmented CCK responses with increasing fat content was indicated (Table 3). Patients with T2DM exhibited augmented meal CCK responses compared with the OGTT response (Table 3). The peak concentrations of CCK following medium- and high-fat meals were higher than those of
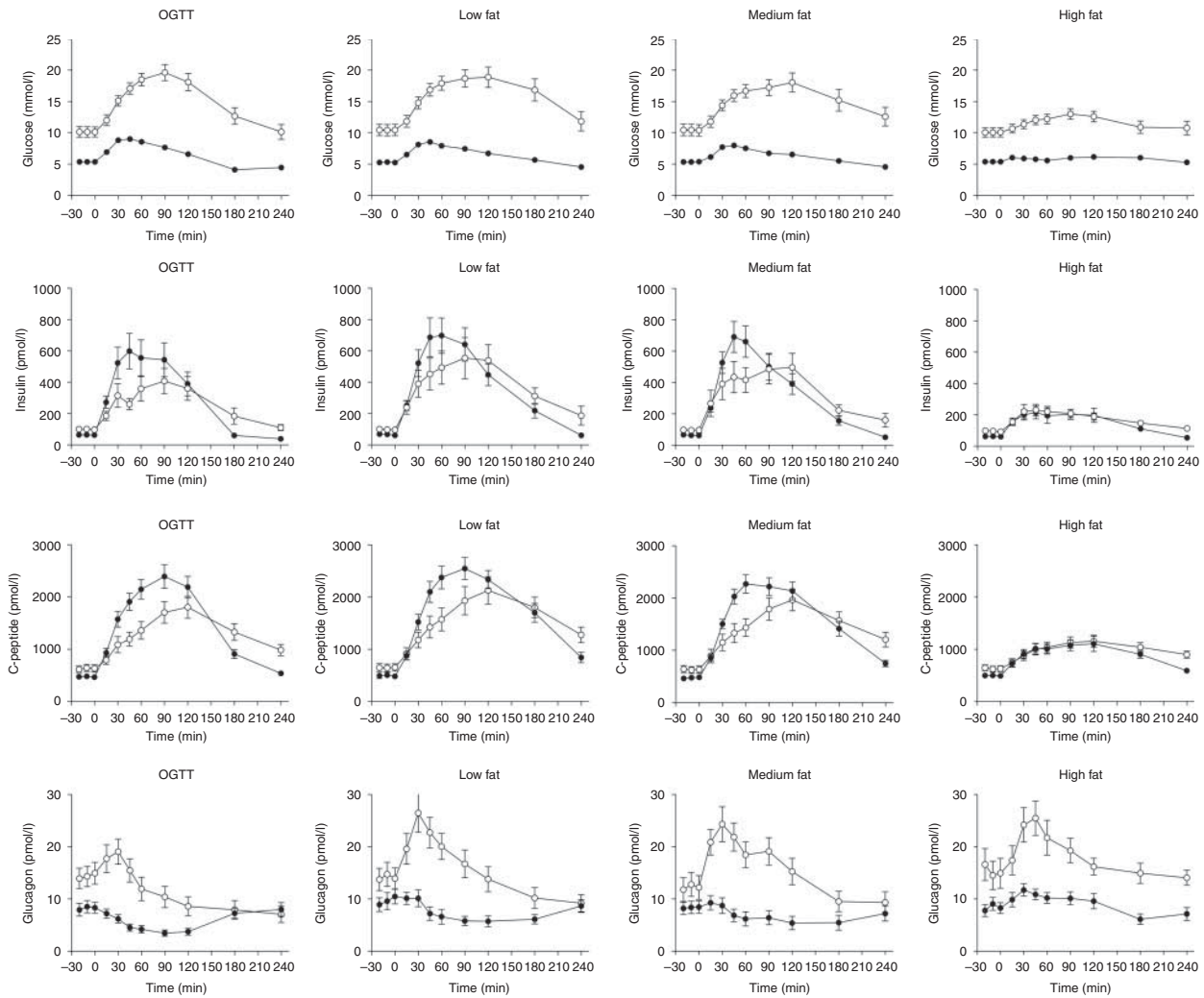
**Figure 1**

Plasma levels of glucose, insulin, C-peptide and glucagon during a 75 g OGTT and three isocaloric (500 kcal) and isovolaemic (350 ml) liquid meals with low fat (2.5 g fat, 107 g carbohydra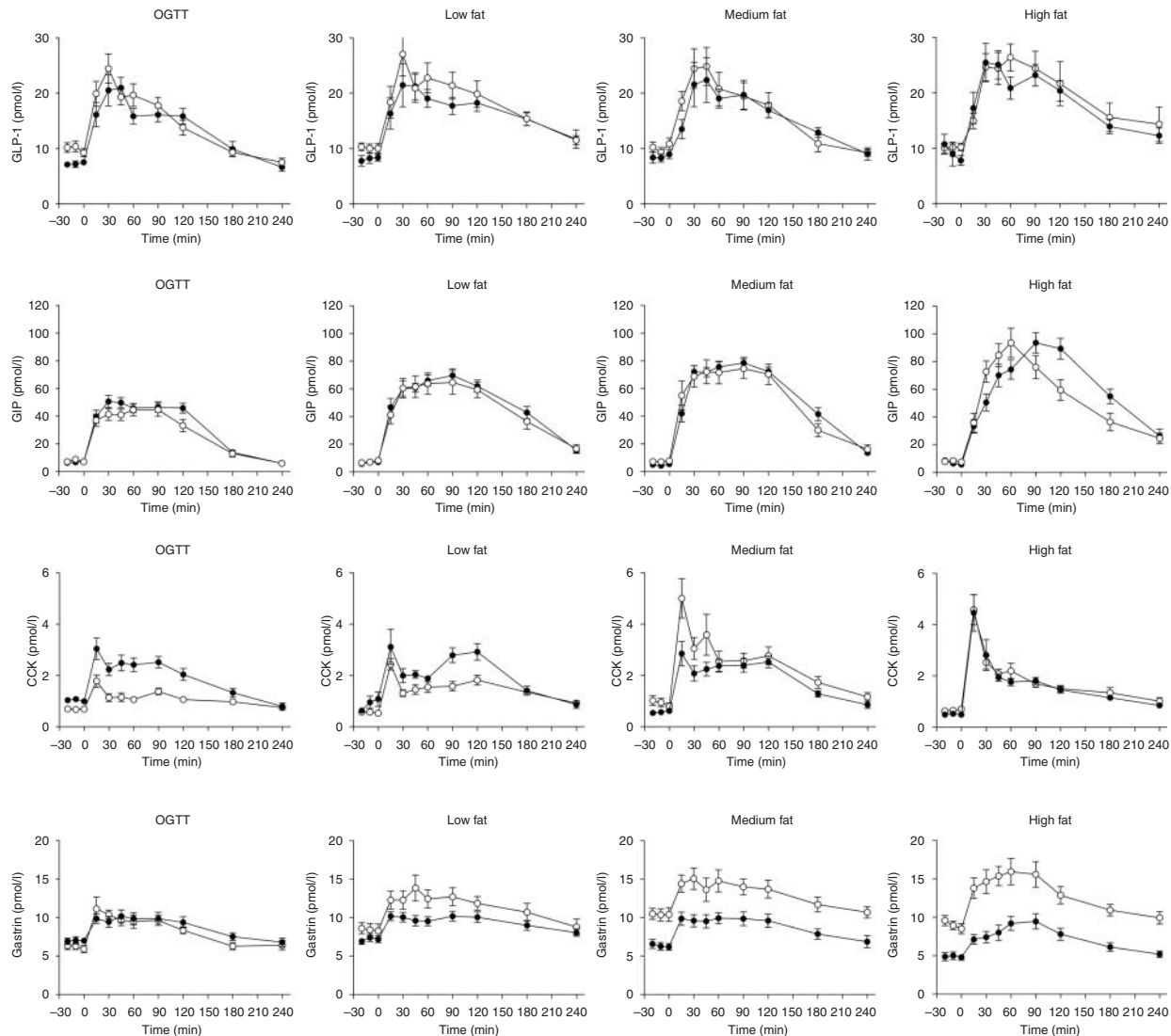te and 13 g protein), medium fat (10 g fat, 93 g carbohydrate and 11 g protein) and high fat (40 g fat, 32 g carbohydrate and 3 g protein) in patients with T2DM ($n=15$, open symbols) and healthy control subjects ($n=15$, closed symbols). Means $\pm$ s.e.m. are shown.

OGTT and low-fat meals ($P<0.05$). OGTT resulted in significantly higher $AUC_{CCK}$ values among controls compared with patients (Table 3). However, $AUC_{CCK}$ values after meals were not significantly different between the two groups. Fasting concentrations of gastrin varied considerably among study days, but tended to be higher in patients compared with controls although statistical significance was only evident comparing medium and high fat ($10.4\pm 0.5$ vs $6.4\pm0.5$ and $9.0\pm0.6$ vs $4.9\pm0.4$ pmol/l$\times$min, $P<0.001$; Table 3). In patients with T2DM, gastrin responses following OGTT were similar to that of controls, but more robust and protracted following meals (Fig. 2).

However, statistical significance was only observed comparing iAUC values during the high-fat meal, although all meal peak values were higher in patients compared with controls (Table 3). All postprandial tAUC values of gastrin were higher in comparison with controls (Fig. 2).

**Gallbladder emptying**

EF% time curves are depicted in Fig. 3 and calculations of gallbladder motility indices are given in Table 4. In both groups, gallbladder contractility increased with increasing meal fat content ($P<0.001$). Nevertheless, a comparable

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**Figure 2**

Plasma levels of GLP1, GIP, CCK and gastrin during a 75 g OGTT and three isocaloric (500 kcal) and isovolaemic (350 ml) liquid meals with low fat (2.5 g fat, 107 g carbohydrate and 13 g protein), medium fat (10 g fat, 93 g carbohydrate and 11 g protein) and high fat (40 g fat, 32 g carbohydrate and 3 g protein) in patients with T2DM ($n=15$, open symbols) and healthy control subjects ($n=15$, closed symbols). Means$\pm$s.e.m. are shown.

magnitude of gallbladder contraction was observed following the medium- and high-fat meals. No statistically significant intergroup differences were demonstrated in any of the calculated gallbladder motility indices (Table 4).

### Gastric emptying

Time courses for the rate of gastric emptying are shown in Fig. 3 and calculations are given in Table 5. No intergroup difference in gastric emptying was demonstrated after low- or medium-fat meals (Table 5). However, as indicated by time-to-peak values, gastric emptying following OGTT or high-fat meals was significantly faster in patients with T2DM compared with controls.

### Discussion

The reported role of bile-induced TGR5 activation in L cell secretion combined with previous findings of reduced

**Table 2** Glucose, insulin, C-peptide and glucagon data. Plasma glucose, insulin, C-peptide and glucagon during (1) 75 g-OGTT and three isocaloric (500 kcal) and isovolaemic (350 ml) liquid meals with low fat, (2) (2.5 g fat, 107 g carbohydrate, 13 g protein), medium fat (3) (10 g fat, 93 g carbohydrate, 11 g protein) and high fat (4) (40 g fat, 32 g carbohydrate, 3 g protein). Data are reported as means±s.e.m.

| | Basal | | | Peak | | | iAUC | | |
|---|---|---|---|---|---|---|---|---|---|
| | T2DM | Controls | P value | T2DM | Controls | P value | T2DM | Controls | P value |
| Glucose[a] | | | | | | | | | |
| (1) OGTT | 10.1±0.8 | 5.3±0.1 | <0.0001 | 19.9±1.2*[3,4] | 9.4±0.3*[3,4] | <0.0001 | 1189±105*[4] | 225±34*[2,4] | <0.0001 |
| (2) Low fat | 10.5±0.9 | 5.3±0.1 | <0.0001 | 19.6±1.5*[4] | 8.9±0.3*[4] | <0.0001 | 1422±147*[3,4] | 302±41*[1,4] | <0.0001 |
| (3) Medium fat | 10.4±0.9 | 5.4±0.1 | <0.0001 | 18.7±1.4*[1,4] | 8.4±0.3*[1,4] | <0.0001 | 1198±125*[2,4] | 215±41*[4] | <0.0001 |
| (4) High fat | 10.1±0.7 | 5.4±0.1 | <0.0001 | 13.6±0.8*[1,2,3] | 7.1±0.3*[1,2,3] | <0.0001 | 370±83*[1,2,3] | 113±16*[1,2,3] | <0.0001 |
| rmANOVA (P) | 0.76 | 0.35 | | <0.0001 | <0.0001 | | <0.0001 | <0.0001 | |
| Insulin[b] | | | | | | | | | |
| (1) OGTT | 100±13 | 64±9 | 0.03 | 445±87*[4] | 716±117*[4] | 0.07 | 17±4*[2,3,4] | 36±7*[4] | 0.02 |
| (2) Low fat | 98±14 | 66±10 | 0.08 | 630±137*[4] | 826±126*[4] | 0.30 | 28±7*[1,4] | 42±6*[4] | 0.04 |
| (3) Medium fat | 96±17 | 64±8 | 0.09 | 559±107*[4] | 761±101*[4] | 0.18 | 25±6*[1,4] | 39±5*[4] | 0.03 |
| (4) High fat | 95±13 | 62±7 | 0.03 | 280±48*[1,2,3] | 350±52*[1,2,3] | 0.33 | 9±2*[1,2,3] | 11±2*[1,2,3] | 0.5 |
| rmANOVA (P) | 0.13 | 0.19 | | 0.002 | <0.0001 | | 0.002 | <0.0001 | |
| C-peptide[b] | | | | | | | | | |
| (1) OGTT | 631±67 | 474±41 | 0.05 | 1886±227*[2,4] | 2501±213*[2,4] | 0.05 | 84±14*[2,4] | 166±15*[4] | 0.0005 |
| (2) Low fat | 649±73 | 495±54 | 0.10 | 2195±275*[1,4] | 2729±202*[1,4] | 0.13 | 105±14*[1,4] | 177±15*[4] | 0.006 |
| (3) Medium fat | 632±68 | 473±37 | 0.04 | 2002±216*[4] | 2473±168*[4] | 0.09 | 95±15*[4] | 163±12*[4] | 0.001 |
| (4) High fat | 632±58 | 497±44 | 0.07 | 1213±116*[1,2,3] | 1376±135*[1,2,3] | 0.36 | 43±6*[1,2,3] | 55±6*[1,2,3] | 0.15 |
| rmANOVA (P) | 0.65 | 0.34 | | <0.0001 | <0.0001 | | <0.0001 | <0.0001 | |
| Glucagon[c] | | | | | | | | | |
| (1) OGTT | 14.4±2.0 | 8.3±1.1 | 0.0002 | 20.0±2.6*[2,3,4] | 11.0±1.1 | 0.003 | 21±60*[2,3,4] | −267±66*[3,4] | <0.0001 |
| (2) Low fat | 14.1±2.1 | 9.7±1.4 | <0.0001 | 27.5±3.6*[1] | 13.3±1.4 | 0.001 | 569±80*[1] | −148±87*[4] | <0.0001 |
| (3) Medium fat | 12.3±2.3*[4] | 8.4±1.1 | <0.0001 | 26.9±3.0*[1] | 12.3±1.7 | 0.0002 | 697±66*[1,4] | −81±59*[1,4] | <0.0001 |
| (4) High fat | 15.4±2.9*[3] | 8.4±1.1 | <0.0001 | 27.0±3.2*[1] | 13.3±1.0 | 0.0004 | 517±76*[1,3] | 177±51*[1,2,3] | <0.0001 |
| rmANOVA (P) | 0.09 | 0.38 | | 0.0016 | 0.16 | | <0.0001 | <0.0004 | |

Significant differences ($P<0.05$) within groups (*post hoc* analysis after one-way repeated measures ANOVA (rmANOVA)) are indicated (*followed by the number of the stimulus compared with).
Differences between groups were compared using two-way rmANOVA and Student's t-test.
[a]Basal and peak values are in mmol/l and iAUC is $iAUC_{0-240\ min}$ (mmol/l×min).
[b]Basal and peak values are in pmol/l and iAUC is $iAUC_{0-90\ min}$ (nmol/l×min).
[c]iAUC is $iAUC_{0-90\ min}$ (pmol/l×min).

**Table 3** Gut hormones. Data are expressed as means ± s.e.m.

| | Basal (pmol/l) | | | Peak (pmol/l) | | | iAUC$_{0-240 \text{ min}}$ (pmol/l × min) | | |
|---|---|---|---|---|---|---|---|---|---|
| | T2DM | Controls | P value | T2DM | Controls | P value | T2DM | Controls | P value |
| **GLP1 total (pmol/l)** | | | | | | | | | |
| (1) OGTT | 10.0±0.8 | 7.3±0.5 | 0.01 | 26.8±2.5 | 25.4±2.5 | 0.70 | 1025±286*[2,4] | 1521±232*[2,4] | 0.19 |
| (2) Low fat | 10.2±0.7 | 8.2±0.7 | 0.06 | 30.9±3.8 | 26.8±3.4 | 0.43 | 1954±340*[1] | 2045±289*[1] | 0.84 |
| (3) Medium fat | 10.2±0.9 | 8.6±0.8 | 0.20 | 28.5±3.6 | 25.1±3.8 | 0.52 | 1448±382 | 1700±361 | 0.64 |
| (4) High fat | 10.1±0.8 | 9.2±1.5 | 0.57 | 34.5±3.6 | 31.8±2.5 | 0.55 | 2271±505*[1] | 2161±433*[1] | 0.87 |
| rmANOVA (P) | 0.96 | 0.28 | | 0.08 | 0.07 | | 0.01 | 0.003 | |
| **GIP total (pmol/l)** | | | | | | | | | |
| (1) OGTT | 8.1±0.7 | 6.6±1.2 | 0.07 | 51.5±4.9*[2,3,4] | 55.0±4.0*[2,3,4] | 0.59 | 5033±674*[2,3,4] | 5921±472*[2,3,4] | 0.29 |
| (2) Low fat | 7.4±0.9 | 6.5±1.2 | 0.65 | 71.9±8.2*[1,3,4] | 74.1±4.6*[1,4] | 0.82 | 9478±1169*[1,3,4] | 10 380±647*[1,3,4] | 0.50 |
| (3) Medium fat | 7.6±0.7 | 4.9±0.6*[4] | 0.01 | 87.7±9.8*[1,2] | 85.1±3.5*[1,4] | 0.71 | 10 480±1110*[1,2] | 11 863±581*[1,2] | 0.28 |
| (4) High fat | 7.9±0.9 | 6.8±0.5*[3] | 0.28 | 99.5±10.0*[1,2] | 106.8±6.8*[1,2,3] | 0.55 | 11 035±1210*[1,2] | 13 297±729*[1,2] | 0.12 |
| rmANOVA (P) | 0.90 | 0.15 | | <0.0001 | <0.0001 | | <0.0001 | <0.0001 | |
| **CCK (pmol/l)** | | | | | | | | | |
| (1) OGTT | 0.7±0.1 | 1.0±0.1*[3,4] | 0.001 | 2.1±0.2*[3,4] | 3.7±0.3 | 0.0004 | 95±17*[2,3,4] | 200±34*[3] | 0.01 |
| (2) Low fat | 0.6±0.1 | 0.9±0.2 | 0.09 | 2.7±0.2*[3,4] | 4.3±0.5 | 0.008 | 221±24*[1,3] | 269±43 | 0.33 |
| (3) Medium fat | 0.9±0.1 | 0.6±0.1*[1] | 0.05 | 5.4±0.7*[1,2] | 3.6±0.4 | 0.04 | 358±53*[1,2] | 315±25*[1] | 0.47 |
| (4) High fat | 0.7±0.1 | 0.5±0.1*[1] | 0.05 | 4.7±0.6*[1,2] | 4.7±0.7 | 0.96 | 262±36*[1] | 276±32 | 0.15 |
| rmANOVA (P) | 0.06 | 0.02 | | <0.0001 | 0.30 | | <0.0001 | 0.07 | |
| **Gastrin (pmol/l)** | | | | | | | | | |
| (1) OGTT | 6.2±0.4*[4] | 7.0±0.4*[3,4] | 0.26 | 12.8±1.3*[3,4] | 11.6±0.9 | 0.46 | 478±99*[4] | 413±82 | 0.67 |
| (2) Low fat | 8.3±0.8*[4] | 7.2±0.4*[3] | 0.21 | 15.6±1.7 | 12.3±0.7 | 0.03 | 726±132 | 535±83 | 0.30 |
| (3) Medium fat | 10.4±0.7 | 6.4±0.5*[2] | 0.001 | 16.8±1.3*[1] | 11.3±0.9 | 0.006 | 617±145 | 587±78 | 0.90 |
| (4) High fat | 9.0±0.6*[1,2] | 4.9±0.4*[1] | 0.0001 | 18.4±1.6*[1] | 10.9±1.0 | 0.0007 | 922±132*[1] | 567±109 | 0.04 |
| rmANOVA (P) | <0.0001 | <0.0001 | | 0.001 | 0.36 | | 0.007 | 0.16 | |

Plasma GLP1, GIP, CCK and gastrin during (1) 75 g OGTT and three (2, 3 and 4) isocaloric (500 kcal) and isovolaemic (350 ml) liquid meals with low fat (2) (2.5 g fat, 107 g carbohydrate and 13 g protein), medium fat (3) (10 g fat, 93 g carbohydrate and 11 g protein) and high fat (4) (40 g fat, 32 g carbohydrate and 3 g protein). Significant differences (P<0.05) within groups (post hoc analysis after one-way repeated measures ANOVA (rmANOVA)) are indicated (*followed by the number of stimuli compared with). Differences between groups were compared using two-way rmANOVA and Student's t-tests.
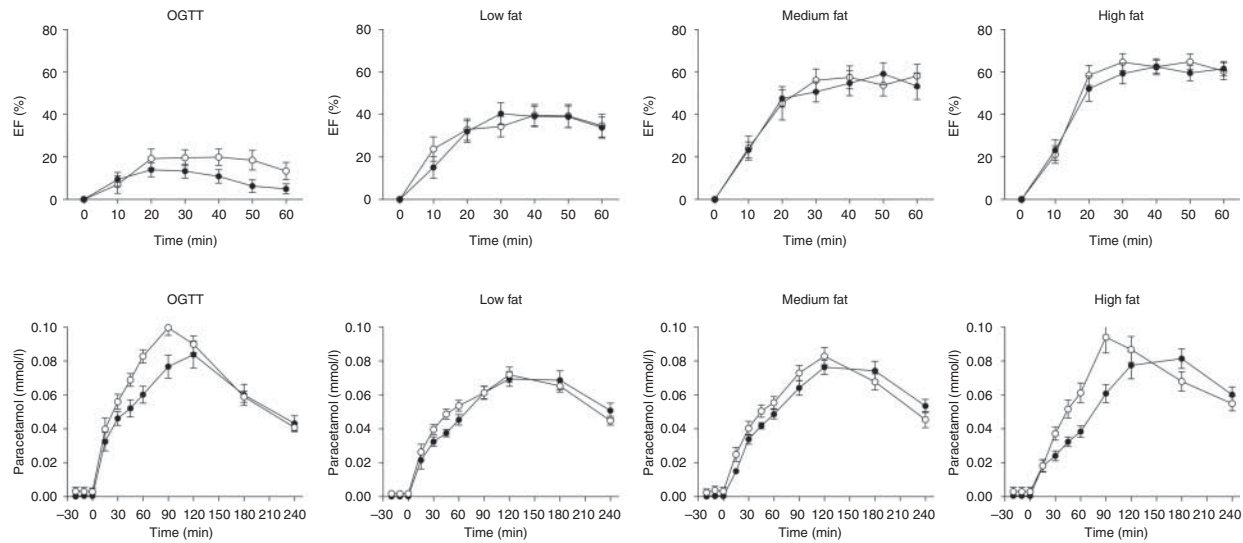
**Figure 3**

Gallbladder ejection fraction (EF%) and gastric emptying (serum paracetamol) during a 75 g OGTT and three isocaloric (500 kcal) and isovolaemic (350 ml) liquid meals with low fat (2.5 g fat, 107 g carbohydrate and 13 g protein), medium fat (10 g fat, 93 g carbohydrate and 11 g protein) and high fat (40 g fat, 32 g carbohydrate and 3 g protein) in patients with T2DM ($n = 15$, open symbols) and healthy control subjects ($n = 15$, closed symbols). Means $\pm$ s.e.m. are shown.

postprandial gallbladder emptying in patients with T2DM [18, 19] prompted us to hypothesise that gallbladder dysmotility associated with T2DM with less flow of bile into the small intestine and, thereby, reduced activation of TGR5 on L cells might explain the lower postprandial GLP1 responses frequently reported in patients with T2DM. We also hypothesised that oral glucose would not affect gallbladder emptying noticeably in either healthy subjects or patients with T2DM and thereby explain the, often intact (or even exaggerated), post-OGTT GLP1 responses in T2DM. Finally, we aimed to address whether the pathophysiology of T2DM

encompasses reduced nutrient-induced GLP1 secretion, which, as noted, is still a subject of debate [6].

Our study illustrates that in both healthy subjects and patients with T2DM, gallbladder emptying increased with increasing meal fat content (Fig. 3). Unexpectedly, however, patients with T2DM did not exhibit any signs of reduced gallbladder motility as reported previously [18, 19, 20, 21]. Hence, it seems unlikely that a difference in gallbladder emptying is of relevance to the variability of GLP1 responses observed in patients with T2DM, acknowledging that this study design and the relatively low numbers of patients in our study do not allow causal inferences in this regard.

**Table 4** Gallbladder motility indices measured as gallbladder ejection fraction (EF%). Data are presented as mean $\pm$ s.e.m.

| | $E_{max}$ | | | $TE_{max}$ (min) | | | $AUC_{0-60\ min}$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | T2DM | Controls | P value | T2DM | Controls | P value | T2DM | Controls | P value |
| Gallbladder emptying (EF%) | | | | | | | | | |
| (1) OGTT | $32 \pm 4$*,[2,3,4] | $23 \pm 3$*,[2,3,4] | 0.08 | $32 \pm 3$ | $27 \pm 3$*,[2,4] | 0.34 | $910 \pm 130$*,[2,3,4] | $563 \pm 111$*,[2,3,4] | $<0.06$ |
| (2) Low fat | $50 \pm 4$*,[1,3,4] | $49 \pm 4$*,[1,3,4] | 0.83 | $41 \pm 3$ | $43 \pm 3$*,[1] | 0.55 | $1873 \pm 234$*,[1,3,4] | $1823 \pm 217$*,[1,3,4] | $<0.87$ |
| (3) Medium fat | $64 \pm 5$*,[1,2] | $64 \pm 4$*,[1,2] | 0.99 | $41 \pm 4$ | $42 \pm 4$ | 0.90 | $2659 \pm 290$*,[1,2] | $2620 \pm 216$*,[1,2] | $<0.92$ |
| (4) High fat | $73 \pm 3$*,[1,2] | $70 \pm 3$*,[1,2] | 0.59 | $40 \pm 4$ | $45 \pm 3$*,[1] | 0.28 | $3016 \pm 173$*,[1,2] | $2874 \pm 196$*,[1,2] | $<0.59$ |
| rmANOVA (P) | $<0.0001$ | 0.0004 | | 0.21 | 0.005 | | $<0.0001$ | $<0.0001$ | |

Gallbladder motility indices (paracetamol absorption) during (1) 75 g OGTT and three (2, 3 and 4) isocaloric (500 kcal) and isovolaemic (350 ml) liquid meals with low fat (2) (2.5 g fat, 107 g carbohydrate and 13 g protein), medium fat (3) (10 g fat, 93 g carbohydrate and 11 g protein) and high fat (4) (40 g fat, 32 g carbohydrate and 3 g protein). Significant differences ($P < 0.05$) within groups (*post hoc* analysis after one-way repeated measures ANOVA (rmANOVA)) are indicated (*followed by the number of stimuli compared with). Differences between groups were compared using two-way rmANOVA and Student's *t*-tests.

**Table 5** Gastric emptying measured as paracetamol absorption during 75 g OGTT and three isocaloric and isovolaemic liquid meals with low fat (2.5 g fat, 107 g carbohydrate and 13 g protein), medium fat (10 g fat, 93 g carbohydrate and 11 g protein) and high fat (40 g fat, 32 g carbohydrate and 3 g protein). Data are presented as mean ± s.e.m.

| | Basal (mmol/l) | | | Peak (mmol/l) | | | iAUC$_{0-240 \, min}$ (mmol/l×min) | | |
|---|---|---|---|---|---|---|---|---|---|
| | T2DM | Controls | P value | T2DM | Controls | P value | T2DM | Controls | P value |
| Paracetamol (mmol/l) | | | | | | | | | |
| (1) OGTT | 0.102±0.005*,[2,3] | 0.085±0.008 | 0.07 | 95±7*,[2,3] | 112±4*,[3,4] | 0.03 | 14.2±1.3 | 15.4*,[2,3] | 0.41 |
| (2) Low fat | 0.074±0.004*,[1,3,4] | 0.075±0.005*,[4] | 0.86 | 140±11*,[1] | 137±12 | 0.87 | 12.9±0.8 | 12.8*,[1,4] | 0.92 |
| (3) Medium fat | 0.087±0.005*,[1,2] | 0.080±0.005*,[4] | 0.38 | 127±10*,[1] | 144±8*,[1] | 0.19 | 13.8±0.8 | 13.6*,[1] | 0.86 |
| (4) High fat | 0.105±0.008*,[1,2] | 0.091±0.006*,[2,3] | 0.16 | 118±13 | 154±11*,[1] | 0.04 | 13.9±0.8 | 14.8*,[2] | 0.47 |
| rmANOVA (P) | 0.0014 | 0.064 | | 0.007 | 0.006 | | 0.35 | 0.002 | |

Significant differences ($P<0.05$) within groups (*post hoc* analysis after one-way repeated measures ANOVA (rmANOVA)) are indicated (*followed by the number of stimuli compared with). Differences between groups were compared using two-way rmANOVA and Student's *t*-tests.

However, in a previous study, we found that cholecystomised patients exhibited preserved postprandial GLP1 responses compared with matched healthy controls, thus confirming that gallbladder contraction is not a prerequisite for postprandial GLP1 release (32). In terms of incretin responses, we show that GLP1 levels increased comparably following OGTT (~300 kcal) and meals of a wide range of nutrient composition (~500 kcal). By contrast, postprandial GIP responses were higher compared with OGTT and were further augmented with the increasing fat content of the meals, confirming the important physiological role of fat in GIP release as demonstrated previously in obese (33) as well as lean subjects (34).

Both reduced, normal and increased GLP1 and GIP responses have previously been observed in patients with T2DM, but studies are difficult to compare (6) and only few studies directly assess incretin responses following both OGTT and meals in T2DM vs matched controls (16, 35). Possibly, our findings of similar incretin responses reflect the disease duration, as deteriorating glycaemic control might impair L cell secretion only in long-standing disease, but our results may also reflect the careful matching of patients and controls in terms of factors that modulate incretin release (6). Metformin use (eight patients) could have masked the defects in L cell secretion (36); however, we applied a washout period of 7 days for antidiabetic medications to ensure that metformin use would not result in carry-over stimulatory effects on GLP1 secretion. Thus, in these studies we did not find reduced postprandial GLP1 secretion during oral or liquid meal tests (in line with a recent meta-analysis of GLP1 responses in patients with T2DM and healthy control subjects (6)). This may be because of the small sample size and the fact that some of our patients had only mild diabetes and were not particularly obese. In a recent study of twin pairs discordant for obesity, liver fat and insulin resistance, a defective GLP1 response emerged concomitantly with increase in liver fat, insulin resistance and, ultimately, obesity (37).

In both groups, insulin levels were only modestly augmented, and strikingly similar, following the high-fat meal, despite high incretin levels, which might reflect incretin effects glucose dependency, although both GIP and GLP1 have been shown to increase insulin secretion at the given glycaemic levels (38). Notably, recent studies have suggested that incretins may also contribute to the islet response to fat and protein, although responses – including plasma concentrations of glucose and lipids – correlate poorly with insulin levels (34, 39). These findings might suggest that the primary role of GLP1 and GIP as mediators of insulin release after pure fat and protein ingestion is negligible. By contrast, under normal physiological circumstances, GIP and GLP1 seem to influence the insulinotropic capability of whey proteins and various amino acids (40, 41). Moreover, although lipids may stimulate insulin secretion directly (42), it has been recently shown that lipids evoke a similar incretin effect as is known for glucose (39). Notably, this is also evident for amino acids (43). Thus, the idea of widening the definition of the incretin concept, involving also non-carbohydrate macronutrients, is intriguing, although other insulin-stimulating factors may be involved such as direct effects of nutrients on the islet cells, neural factors or other gut hormones affecting insulin release (33, 34).

The fact that, in controls, glucagon secretion increased with increasing fat content in the meal is in keeping with the relatively recent finding that lipids stimulate glucagon secretion (34). This may be owing to GIP-induced glucagon secretion, and low glucose and

insulin levels during the high-fat meal might also have played a role (44). In patients with T2DM, who were characterised by massive glucagon hypersecretion, nutrient composition in meals did not give rise to different glucagon profiles, suggesting that meal calories rather than composition is the prime regulator of postprandial glucagon secretion in patients with T2DM.

Our observation of preserved gallbladder emptying in patients with T2DM is in contrast to the majority of studies of gallbladder function in T2DM (17, 18, 19). However, some studies report preserved gallbladder emptying, but deviate from this study in terms of patient selection, control group, methods of investigation and stimuli used for inducing gallbladder contraction (17, 45). To provide an explanation for this disparity, it is relevant to review our selection of patients with T2DM. These were broad representatives for a dysregulated population of patients with T2DM (HbA1c >7%, disease duration >5 years), but without clinical signs of autonomic neuropathy. Our choice of patients was based on the fact that in several studies reporting impaired gallbladder emptying in diabetic populations, the relationship to autonomic neuropathy has been subtle (17, 18, 45). Other 'diabetic' factors may be of greater importance for diabetic gallbladder dysmotility (17). For example, high plasma glucose levels decrease the sensitivity of the smooth muscles of the gallbladder to circulating CCK (46, 47). Furthermore, hyperglycaemia may suppress cholinergic activity, and thus dampen the neural part of CCK-induced gallbladder emptying (48, 49). Thus, we believe that the patients included in our study were good representatives of the 'typical' phenotype of T2DM having a high risk of gallbladder dysmotility. Nevertheless, we cannot completely exclude the possibility of random sampling having resulted in the inclusion of T2DM patients with normal gallbladder function. Another explanation might be rooted in the rate of gastric emptying in the patients. In this study, we observed both normal (low- and medium-fat meals) and increased (OGTT and high-fat meal) gastric emptying in patients with T2DM compared with healthy controls. As gastric emptying determines nutrient delivery into the small intestine and thereby the release of CCK and GLP1, it is likely that the gallbladder contracts sufficiently despite the presence of some of the inhibitory factors mentioned above. Finally, methodological issues could have played a role, as the impairment of gallbladder emptying in the diabetic patients might have been too small for proper ultrasound assessment. Indeed, even relatively large impairments of gallbladder emptying, assessed by cholescintigraphy, can be overlooked by

ultrasound assessment (17). However, in this study, the method was sensitive enough to detect differences following the different meal stimuli.

Our results on gastric emptying and gallbladder motility may reflect the impairment (responsiveness or secretion) of gut hormones that are important for intestinal motor function (i.e. motilin, CCK and GLP1). Low postprandial CCK concentrations have been reported in patients with T2DM (50). This may result in accelerated gastric emptying (and slowing of gallbladder emptying), and thus worsen postprandial hyperglycaemia. In this study, postprandial CCK concentrations were slightly lower in the diabetic group, but only following oral glucose (Fig. 2). To our knowledge, reduced CCK responses after glucose stimuli – together with the finding of similar gastrin responses – have never previously been reported in patients with T2DM.

In conclusion, our patients with T2DM exhibited normal gallbladder emptying to meals with a wide range of fat content. After both OGTT and meals, no evidence of a secretory defect in incretin secretion in patients with T2DM was observed. In both groups, postprandial incretin responses were unaffected by gallbladder emptying. Therefore, postprandial flow of bile from the gallbladder into the intestine is not likely to play a physiological role in potentiating the L cell response (via TGR5) in humans.

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