Effects of sequential treatment with lixisenatide, insulin glargine, or their combination on meal-related glycaemic excursions, insulin and glucagon secretion, and gastric emptying in patients with type 2 diabetes

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The availability of glucagon-like peptide-1 (GLP-1) receptor agonists (GLP-1 RAs) has broadened the therapeutic choices for patients with type 2 diabetes.\(^1\)\(^-\)\(^3\) Owing to their different pharmacokinetic profiles, the pharmacodynamic actions of the different GLP-1 RAs are rather distinct. Thus, long-acting GLP-1 RAs act primarily by stimulating insulin and reducing glucagon secretion, whereas short-acting compounds act predominantly on postprandial glycaemic excursions by delaying gastric emptying.\(^4\)\(^-\)\(^5\)

Lixisenatide is a short-acting GLP-1 RA that is based on the exendin-4 backbone.\(^6\)\(^-\)\(^7\) Although its half-life of \(\sim 3.5\) hours\(^8\) would suggest that 2–3 times daily administration is required to elicit a daily glucose-lowering effect, significant reductions have been observed after breakfast, lunch and dinner even following a once-daily injection.\(^9\)\(^-\)\(^11\) This raises the question of how lixisenatide can lower glycaemia even beyond the actual time of exposure to the drug. One potential explanation could be a (partially) persisting effect on gastric emptying at subsequent meals. Alternatively, it seems possible that lixisenatide exerts an insulin-sparing effect after breakfast, which may enhance beta-cell function at the subsequent meals through the mechanism of beta-cell rest.\(^12\)\(^-\)\(^13\)

In clinical practice, lixisenatide is often used in combination with basal insulin.\(^14\)\(^-\)\(^16\) The mechanistic rationale for such a therapeutic regimen is the observation that basal insulin predominantly lowers fasting glucose concentrations,\(^17\) whereas lixisenatide may act in a complementary fashion by lowering postprandial glycaemia (5,9,10). However, the detailed mechanisms of action by which glucose concentrations are lowered in the case of a combined action of both basal insulin and lixisenatide are still poorly understood. It is also yet unclear whether initiating basal insulin prior to lixisenatide\(^14\)\(^-\)\(^16\) is favourable over an initial treatment with lixisenatide, followed by the addition of basal insulin, analogous to insulin detemir added on to liraglutide treatment.\(^18\)

Therefore, in the current study we investigated (i) by which mechanism the combination of insulin glargine and lixisenatide lowers glucose concentrations after breakfast and after a subsequent late lunch, and (ii) if the sequence of initiating treatment with either lixisenatide or insulin glargine has an impact on the overall glucose-lowering effect.

2 | RESEARCH DESIGN AND METHODS

2.1 | Trial protocol

The study protocol was approved by the ethics committee of the medical faculty of the Ruhr University Bochum (registration no. 4779–13 FF) and of the North Rhine Medical Association (registration no. 2013293) as well as the German federal regulatory authorities (Bundesinstitut für Arzneimittel und Medizinprodukte [BfArM]); the trial was registered at www.ClinicalTrials.com (NCT01910194) before commencing. Written informed consent was obtained from all participants. A previous publication of the present research project described intravenous glucose tolerance tests performed with the same treatment sequence.\(^19\)

2.2 | Trial participants

Twenty-eight male and female subjects aged 18–75 years with type 2 diabetes ≥1 year not adequately controlled under therapy with metformin or metformin plus any sulfonylurea, dipeptidyl peptidase-4 inhibitor or sodium-glucose co-transporter-2 inhibitor (dapagliflozin in all cases), with fasting plasma glucose (FPG) >7.8 mmol/L, HbA1c of 7.0%–9.5% and a body mass index of 20.0–40.0 kg/m\(^2\), were allowed to participate in the trial. Twenty-two subjects (11 in each treatment arm) completed all experiments in the current trial and provided gastric-emptying data. Detailed subject characteristics of the total cohort have been described.\(^19\) Data for the 22 completers are presented in Table 1.

2.3 | Trial design

This was a bicentric, multiple dose, randomized, open-label study with two treatment regimens (lixisenatide treatment first and insulin glargine treatment first) and two treatment periods (4 weeks of monotherapy with either lixisenatide or insulin glargine and another 4 weeks of combination therapy with both medications). Experimental studies (two identical mixed meal tests in the morning [breakfast] and 8 hours later [late lunch]) were performed at baseline, after 4 weeks of monotherapy with either agent alone, and after 8 weeks, ie, after 4 weeks of combined treatment with lixisenatide and insulin glargine. Each randomized treatment period was preceded by a 27-day screening/washout period for any oral antidiabetic drug except metformin.

2.4 | Glucose-lowering treatment

The lixisenatide (Lyxumia) dose per injection was 10 μg (30 minutes before breakfast) initially, and was increased after 2 weeks to 20 μg, if tolerability allowed this. Insulin glargine (Lantus) administered at bedtime was titrated to achieve target self-monitored FPG concentrations in the range of 4.4 to 5.6 mmol/L. Details have been described elsewhere.\(^19\)

2.5 | Experimental procedures

After an overnight fast, an indwelling intravenous catheter was inserted into each subject’s forearm for blood sampling. An identical mixed meal (ie, a muffin containing ~500 kcal, 54% carbohydrates, 10% protein and 35% fat) supplemented by 91 mg\(^1\)\(^3\)C-octanoic acid (for the determination of gastric emptying) was used both at breakfast (actual starting time between 08:30 AM and 10:30 AM, nominal starting time: \(t = 0\) minutes) and late lunchtime (actual starting time between 04:00 PM and 06:30 PM, nominal starting time: \(t = 480\) minutes). Blood was drawn and breath samples were obtained over 240 minutes following each meal in order to assess the impact of the experimental treatments on the meal-related excursions in plasma glucose, insulin, C-peptide, glucagon, serum non-esterified fatty acid and triacylglycerol concentrations, as well as on the velocity of gastric emptying of solid components.
Safety assessments, including vital signs, electrocardiogram, measurement of body weight, questioning about adverse events, hypoglycaemic episodes and concomitant medication, were performed at each ambulatory visit.

2.6 Laboratory determinations

Plasma glucose concentrations were determined from venous blood samples using the glucose analyzer SUPER GL compact, Hitado. Insulin was analyzed by means of an enzyme linked immunosorbent assay (Mercodia AB, Uppsala, Sweden), which had no cross-reactivity with insulin glargine or its metabolites. C-peptide was analyzed by means of an electrochemiluminescence assay (Roche Diagnostics, Mannheim, Germany). Glucagon was analyzed by means of a radioimmunoassay (Euro Diagnostica, Malmö, Sweden). The concentration of free fatty acids in serum was assessed with reagents from WAKO Chemicals (Neuss, Germany) using a Roche COBAS 6000 automatic analyzer. Triglycerides were quantified in serum using reagents from Roche Diagnostics using a Roche COBAS 6000 automatic analyzer.

2.7 Insulin secretion

Insulin secretion rates were calculated by deconvolution analysis based on a two-compartment model of C-peptide elimination using C-peptide concentration-time profiles related to the mixed

| TABLE 1 Patient characteristics as determined at the screening examination |
|---------------------------------------------------------------|
| Characteristic         | Unit | Lixisenatide first | Insulin glargine first | Significance (P-value)¹ |
|------------------------|------|-------------------|------------------------|------------------------|
| Gender                 |      | 1/10 (9.1)        | 1/10 (9.1)             | 1.00                   |
| Age                    | years| 61 ± 6            | 57 ± 11                | 0.27                   |
| Height                 | cm   | 175 ± 6           | 179 ± 5                | 0.11                   |
| Weight                 | kg   | 93.5 ± 13.2       | 101.0 ± 12.5           | 0.19                   |
| Waist                  | cm   | 109 ± 10          | 112 ± 11               | 0.41                   |
| Body mass index        | kg/m²| 30.4 ± 3.5        | 31.5 ± 3.8             | 0.50                   |
| HbA1c                  | %    | 7.9 ± 0.6         | 7.6 ± 0.4              | 0.19                   |
| Diabetes duration      | years| 7.6 ± 4.2         | 8.1 ± 9.0              | 0.85                   |
| Glucose-lowering medication |    |                   |                        | 0.75                   |
| Metformin              | yes/no (% yes) | 11/0 (100.0) | 11/0 (100.0)          | 0.00                   |
| DPP-4 inhibitor        | yes/no (% yes) | 3/8 (27.3)   | 3/8 (27.3)             | 0.66                   |
| Sulfonylurea           | yes/no (% yes) | 1/10 (9.1)    | 2/9 (18.2)             | 0.00                   |
| SGLT-2 inhibitor       | yes/no (% yes) | 0/11 (0.0)    | 1/10 (9.1)             | 0.00                   |
| Hypertension           | yes/no (% yes) | 6/5 (54.5)    | 8/3 (72.7)             | 0.66                   |
| Antihypertensive agents| yes/no (% yes) | 6/5 (54.5)    | 8/3 (72.7)             | 0.66                   |
| Statins                | yes/no (% yes) | 3/8 (27.3)    | 2/9 (18.2)             | 1.00                   |
| Systolic blood pressure| mmHg | 143 ± 8           | 146 ± 8                | 0.46                   |
| Diastolic blood pressure| mmHg | 89 ± 5            | 89 ± 5                 | 0.84                   |
| Pulse                  | beats/minute | 68 ± 7          | 67 ± 7                 | 0.90                   |
| Laboratory data        |      |                   |                        |                        |
| AST                    | U/I  | 27 ± 15           | 27 ± 9                 | 0.88                   |
| ALT                    | U/I  | 37 ± 20           | 37 ± 14                | 0.94                   |
| eGFR (MDRD-equation)   | ml/min⁻¹.1.73m⁻² | 86.7 ± 17.6 | 90.8 ± 13.1           | 0.54                   |
| Triglycerides          | mmol/L| 2.58 ± 1.35       | 2.26 ± 2.26            | 0.69                   |
| HDL-cholesterolb       | mmol/L| 0.80 ± 0.22       | 0.96 ± 0.27            | 0.14                   |
| LDL-cholesterolb       | mmol/L| 2.51 ± 0.82       | 2.68 ± 0.57            | 0.58                   |

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DPP-4, dipeptidyl peptidase-4; eGFR, estimated glomerular filtration rate; MDRD, modification of diet in renal disease; SGLT-2, sodium-glucose co-transporter-2.

Statistical analysis: continuous variables: ANOVA; categorical variables: contingency table analysis (Fisher’s exact test, χ²-test, as appropriate).

¹comparison between lixisenatide first and insulin glargine first; mean ± SD.

²lipoprotein variables were determined at the time of the baseline examination.
FIGURE 1  Plasma glucose (A, B), insulin (C, D), C-peptide (E, F) concentrations and estimated insulin secretion rates (G, H) before and after breakfast and late lunch at baseline (open symbols), after 4 weeks of treating with lixisenatide alone (black triangles) or insulin glargine alone (black diamonds), or after an additional 4 weeks of combined treatment with lixisenatide and insulin glargine. The patient cohort initially treated with lixisenatide (n = 11) is shown in the left-hand panels (A, C, E, G), the other cohort initially treated with insulin glargine (n = 11) is shown in the right-hand panels (B, D, F, H). Long arrows indicate the time point of subcutaneous lixisenatide injection, short arrows indicate the administration of the mixed meals. Results for repeated-measures analysis of variance (separately calculated for breakfast and late lunch) are reported as P-values comparing the three experiments (A), analyzing changes over time (B) or the interaction of experiment and time (AB). Asterisks indicate significant differences (P < 0.05) versus the baseline examination, daggers indicate significant differences (P < 0.05) to the first 4 weeks of monotherapy with either lixisenatide or insulin glargine. For corresponding areas under the curve we refer to Table 2 (area above zero concentrations) or Table S1 (area above mean baseline concentrations)
meals, employing the ISEC computer program,\textsuperscript{20} which uses population-derived estimates for the elimination coefficients.\textsuperscript{21}

### 2.8 | Gastric emptying (13C-octanoate breath tests)

The time course of gastric retention was derived from the time profile of the excess amount of \(^{13}\text{CO}_2\) in exhaled breath specimens, as originally published by Ghoos et al.\textsuperscript{22,23} In addition, the Wagner-Nelson approach, which better considers the delay in the appearance of \(^{13}\text{C}-\text{CO}_2\) in breath samples after \(^{13}\text{C}\)-octanoic acid has left the stomach, by taking into account elimination other than exhalation of \(^{13}\text{C}-\text{CO}_2\), was employed, assuming an elimination coefficient of 0.55. This procedure has generated gastric-emptying profiles almost identical to those generated by scintigraphic assessment of gastric emptying.\textsuperscript{24} Gastric emptying half-time (T\(_{1/2}\)) and retention (%) of the initial gastric content (of 100%) were calculated for all time points of breath sampling.

### 2.9 | Integrated responses, areas under the curve

Integrated meal-related responses above mean baseline values (incremental areas under the curve [AUC]), below mean baseline (decremental AUC) and above 0 (total AUC) were derived from individual meal-related time profiles and calculated according to the linear trapezoidal rule using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA).

### 2.10 | Statistical analysis

Subject characteristics are reported as mean ± SD and results as mean ± SEM. Statistical calculations were carried out as general linear model analysis using Statistica (software system for data analysis) version 13 (Dell). Experimental conditions (assessments at baseline, after monotherapy with lixisenatide or insulin glargine, and after combination therapy with both lixisenatide and insulin glargine) were determined at t = −30 min, prelunch values were determined at t = 450 min; mean ± SEM; statistical analysis: Fisher’s exact test.

#### TABLE 2  Prebreakfast and prelunch measurements

| Variable         | Condition                        | Prebreakfast | Prelunch | Significance (P-value) |
|------------------|----------------------------------|--------------|----------|------------------------|
| Glucose [mmol/L] | Baseline                         | 10.0 ± 0.6   | 7.1 ± 0.4| 0.0009                 |
| Insulin [pmol/L] | Baseline                         | 67 ± 9       | 60 ± 6   | 0.48                   |
| C-peptide [nmol/L]| Baseline                         | 0.37 ± 0.02  | 0.39 ± 0.03 | 0.64                     |
| Glucagon [pmol/L] | Baseline                         | 57 ± 2       | 54 ± 2   | 0.21                   |
| Glucose [mmol/L] | Lixisenatide only                | 8.7 ± 0.5    | 6.3 ± 0.5| 0.0021                 |
| Insulin [pmol/L] | Lixisenatide only                | 74 ± 12      | 71 ± 10  | 0.90                   |
| C-peptide [nmol/L]| Lixisenatide only                | 0.40 ± 0.04  | 0.47 ± 0.06 | 0.37                     |
| Glucagon [pmol/L] | Lixisenatide only                | 56 ± 3       | 49 ± 2   | 0.18                   |
| Glucose [mmol/L] | Lixisenatide plus insulin glargine | 6.2 ± 0.4    | 5.3 ± 0.4| 0.098                  |
| Insulin [pmol/L] | Lixisenatide plus insulin glargine | 52 ± 13      | 40 ± 7   | 0.45                   |
| C-peptide [nmol/L]| Lixisenatide plus insulin glargine | 0.25 ± 0.04  | 0.31 ± 0.04 | 0.34                     |
| Glucagon [pmol/L] | Lixisenatide plus insulin glargine | 52 ± 2       | 49 ± 2   | 0.18                   |

| Variable         | Condition                        | Prebreakfast | Prelunch | Significance (P-value) |
|------------------|----------------------------------|--------------|----------|------------------------|
| Glucose [mmol/L] | Baseline                         | 10.7 ± 0.5   | 7.9 ± 0.4| 0.0003                 |
| Insulin [pmol/L] | Baseline                         | 70 ± 15      | 59 ± 15  | 0.60                   |
| C-peptide [nmol/L]| Baseline                         | 1.08 ± 0.13  | 1.03 ± 0.13 | 0.78                     |
| Glucagon [pmol/L] | Baseline                         | 59 ± 3       | 53 ± 2   | 0.15                   |
| Glucose [mmol/L] | Insulin glargine only            | 6.8 ± 0.4    | 5.4 ± 0.2| 0.011                  |
| Insulin [pmol/L] | Insulin glargine only            | 36 ± 10      | 30 ± 8   | 0.68                   |
| C-peptide [nmol/L]| Insulin glargine only            | 0.65 ± 0.11  | 0.60 ± 0.11 | 0.75                     |
| Glucagon [pmol/L] | Insulin glargine only            | 57 ± 3       | 54 ± 3   | 0.43                   |
| Glucose [mmol/L] | Lixisenatide plus insulin glargine | 5.8 ± 0.2    | 5.4 ± 0.4| 0.46                   |
| Insulin [pmol/L] | Lixisenatide plus insulin glargine | 37 ± 13      | 54 ± 26  | 0.55                   |
| C-peptide [nmol/L]| Lixisenatide plus insulin glargine | 0.58 ± 0.14  | 0.82 ± 0.23 | 0.37                     |
| Glucagon [pmol/L] | Lixisenatide plus insulin glargine | 53 ± 2       | 47 ± 2   | 0.092                  |
were used as independent fixed variables, and patients were imputed as random variables. Concentrations or gastric content at all time points were dependent variables (repeated-measures approach). Each meal was assessed separately. Integrated responses (incremental, decremental and total AUCs) were analyzed in a similar manner. They were also compared between the breakfast and late lunch meals.

Only if a significant influence of the experimental condition was documented by a P-value of <0.05, or by a significant interaction of treatment and time (P < 0.05), then were values at individual time points compared.

### TABLE 3 Variables characterizing glycaemic excursions and insulin secretion following meals at baseline, after treatment with either lixisenatide or insulin glargine alone, or with a combination of lixisenatide and insulin glargine

| Variable | Unit | Conditions/treatment | Breakfast | Late lunch | Significance (P-value) |
|----------|------|----------------------|-----------|------------|------------------------|
| 1. AUC<sup>a</sup> lixisenatide first | | | | | |
| Glucose | mmol l<sup>-1</sup> min | Baseline | 3500 ± 195 | 3050 ± 155 | 0.09 |
| | | Lixisenatide | 2264 ± 116<sup>*</sup> | 2504 ± 106<sup>*</sup> | 0.14 |
| | | Lixisenatide + insulin glargine | 1770 ± 121<sup>†</sup> | 2384 ± 80<sup>†</sup> | 0.0004 |
| | Significance (P-value)<sup>c</sup> | <0.0001 | 0.0007 | |
| Insulin | nmol l<sup>-1</sup> min | Baseline | 48.3 ± 5.3 | 45.5 ± 6.8 | 0.75 |
| | | Lixisenatide | 38.8 ± 6.4 | 50.7 ± 6.9 | 0.22 |
| | | Lixisenatide + insulin glargine | 25.8 ± 5.4<sup>*</sup> | 40.2 ± 6.5 | 0.10 |
| | Significance (P-value)<sup>c</sup> | 0.033 | 0.55 | |
| C-Peptide | nmol l<sup>-1</sup> min | Baseline | 171.5 ± 10.9 | 176.8 ± 15.8 | 0.78 |
| | | Lixisenatide | 160.0 ± 13.1 | 195.5 ± 15.8 | 0.10 |
| | | Lixisenatide + insulin glargine | 106.0 ± 13.4<sup>†</sup> | 161.9 ± 19.4 | 0.028 |
| | Significance (P-value)<sup>c</sup> | 0.0018 | 0.39 | |
| Insulin secretion rates | pmol/kg | Baseline | 1535 ± 142 | 1584 ± 189 | 0.84 |
| | | Lixisenatide | 1436 ± 139 | 1719 ± 165 | 0.21 |
| | | Lixisenatide + insulin glargine | 962 ± 137<sup>†</sup> | 1476 ± 223 | 0.064 |
| | Significance (P-value)<sup>c</sup> | 0.015 | 0.68 | |
| 2. AUC<sup>a</sup> insulin glargine first | | | | | |
| Glucose | mmol l<sup>-1</sup> min | Baseline | 3680 ± 153 | 3209 ± 136 | 0.033 |
| | | Insulin glargine | 2610 ± 112<sup>*</sup> | 2448 ± 115<sup>*</sup> | 0.32 |
| | | Insulin glargine + lixisenatide | 1573 ± 37<sup>†</sup> | 2281 ± 119<sup>†</sup> | <0.0001 |
| | Significance (P-value)<sup>b</sup> | <0.0001 | <0.0001 | |
| Insulin | nmol l<sup>-1</sup> min | Baseline | 41.3 ± 6.4 | 33.0 ± 5.1 | 0.33 |
| | | Insulin glargine | 33.2 ± 5.8 | 26.2 ± 4.3 | 0.34 |
| | | Insulin glargine + lixisenatide | 16.0 ± 2.7<sup>†</sup> | 35.1 ± 7.0 | 0.020 |
| | Significance (P-value)<sup>b</sup> | 0.0059 | 0.51 | |
| C-Peptide | nmol l<sup>-1</sup> min | Baseline | 153.9 ± 14.6 | 138.5 ± 11.3 | 0.41 |
| | | Insulin glargine | 123.9 ± 13.3 | 114.6 ± 10.6 | 0.59 |
| | | Insulin glargine + lixisenatide | 81.1 ± 9.1<sup>†</sup> | 148.1 ± 15.1 | 0.0011 |
| | Significance (P-value)<sup>b</sup> | 0.0002 | 0.17 | |
| Insulin secretion rates | pmol/kg | Baseline | 1296 ± 129 | 1167 ± 99 | 0.43 |
| | | Insulin glargine | 1053 ± 113 | 979 ± 81 | 0.60 |
| | | Insulin glargine + lixisenatide | 721 ± 78<sup>†</sup> | 1325 ± 93<sup>†</sup> | <0.0001 |
| | Significance (P-value)<sup>b</sup> | 0.0031 | 0.039 | |

Mean ± SEM, n = 11.
<sup>a</sup>comparison between breakfast and late lunch.
<sup>b</sup>area under the curve above zero.
<sup>c</sup>comparison between the three experimental conditions (at baseline, after treatment with lixisenatide or insulin glargine alone, or a combination of both).
<sup>*</sup>significant difference (P < 0.05) from baseline.; <sup>†</sup>significant difference (P < 0.05) from lixisenatide or insulin glargine only treatment.
points subsequently analyzed by Duncan’s post hoc test. A two-sided \( P \) -value of <0.05 was taken to indicate significant differences. Correlations were carried out by linear regression analysis.

3 | RESULTS

3.1 | Patient characteristics and flow

Patients randomized to the two treatment sequences (either lixisenatide or insulin glargine treatment first) had similar baseline subject characteristics (Table 1). Of 14 patients randomized to each treatment sequence, three patients in each sequence never completed the three experimental periods or the gastric emptying test could not be analyzed because of technical problems (the increment in \( ^{13} \text{C}-\text{CO}_2 \) enrichment following the administration of \( ^{13} \text{C}-\text{octanoic acid} \) was not sufficient to allow curve fitting). We report on the remaining 11 patients in each treatment sequence who participated in all experiments and had an analyzable gastric-emptying test.

3.2 | Plasma glucose

Treatment with lixisenatide 30 minutes before breakfast, whether used alone or in combination with insulin glargine, substantially reduced
postmeal glycaemic excursions after breakfast (0–240 minutes), and slightly reduced fasting or premeal plasma glucose levels, even in addition to the effects of insulin glargine (Figure 1A,B; Tables 2 and 3; Table S1). After a late lunch, lixisenatide still resulted in improved postmeal excursions (total AUC reduced by 18%; Table 3). However, the incremental AUC decrease was only 7% (not significant; Table 3) when adding lixisenatide to insulin glargine treatment (Figure 1B).

Treatment with insulin glargine mainly reduced fasting and pre-breakfast glucose levels, without major effects on integrated incremental plasma glucose concentrations (Table S1). There was, however, a significant reduction in postbreakfast glucose concentrations, which resulted in a significant reduction of the total AUCs (Figure 1B, Table 2). The glucose-lowering effects of insulin glargine persisted after a late lunch (Figure 1B), resulting in a glycaemic profile shifted towards lower concentrations by approximately the same difference characteristic of prelunch glucose concentrations compared with prebreakfast plasma glucose levels.

### 3.3 Insulin secretion

Insulin secretion was assessed by measuring insulin and C-peptide concentrations and deriving insulin secretion rates from deconvolution.

After breakfast, lixisenatide treatment, alone or in combination with insulin glargine treatment, reduced insulin secretion, in line with decelerated gastric emptying (Figure 1C-H), and reduced increments in plasma glucose concentrations following the meal (Figure 1A,B). The reduction in insulin secretion was most apparent with combination treatment (lixisenatide and insulin glargine; Figure 1C-H), along with a further reduction in postmeal glycaemia versus lixisenatide treatment alone (Figure 1A,B).

After a late lunch, the stimulation of insulin secretion was more similar across the different treatments (Figure 1C-H). However, in the cohort first treated with insulin glargine, adding lixisenatide treatment enhanced insulin secretion after a late lunch. This was a trend for insulin concentrations (Figure 1D), and a significant increase of both C-peptide concentrations (Figure 1F) and insulin secretion rates (Figure 1H). Of note, in patients initially treated with lixisenatide, insulin, C-peptide and insulin secretion rates tended to be higher than at baseline (ie, in the absence of lixisenatide treatment; Figure 1C,E,G).

### 3.4 Glucagon

Plasma glucagon increased after breakfast and, less prominently, after a late lunch at baseline, ie, in patients not treated with either lixisenatide or insulin glargine. After breakfast, these increments in plasma glucagon concentrations were reduced in all experiments with lixisenatide, either alone (by 84.7%) or in combination with insulin glargine (by 85.0% in those treated with lixisenatide first and by 90.3% in those treated with insulin glargine first; Figure 2A,B). After breakfast, treatment with lixisenatide not only reduced increments in plasma glucagon concentrations, but even suppressed glucagon to concentrations below fasting levels, both alone or in combination with insulin glargine (Figure 2A,B).

Insulin glargine therapy slightly reduced breakfast-related glucagon responses, whether alone (Figure 2B) or as an addition to lixisenatide treatment (Figure 2A). These effects, however, did not achieve statistical significance.

Meal-related increments in plasma glucagon concentrations were less prominent after a late lunch compared with breakfast (Figure 2A, B; not significant). There were no treatment-related differences in postmeal glucagon increments after a late lunch (Figure 2A,B), although the combination of lixisenatide and insulin glargine resulted in the lowest glucagon responses in both the cohorts starting with either lixisenatide treatment or insulin glargine treatment first (Figure 2A,B).

![Figure 3](image-url)

**FIGURE 3** Time course of gastric emptying (as a percentage of the initial gastric content) after breakfast and late lunch at baseline (open symbols), after 4 weeks of treating with lixisenatide alone (black triangles) or insulin glargine alone (black diamonds), or after an additional 4 weeks of combined treatment with lixisenatide and insulin glargine. The patient cohort initially treated with lixisenatide (n = 11) is shown in the upper panel (A), the other cohort initially treated with insulin glargine (n = 11) is shown in the lower panel (B). Long arrows indicate the time point of subcutaneous lixisenatide injection, short arrows indicate the administration of the mixed meals. Results for repeated-measures analysis of variance (separately calculated for breakfast and late lunch) are reported as P-values comparing the three experiments (A), analyzing changes over time (B) or the interaction of experiment and time (AB). Asterisks indicate significant differences (P < 0.05) versus the baseline examination, daggers indicate significant differences (P < 0.05) to the first 4 weeks of monotherapy with either lixisenatide or insulin glargine. For corresponding analyses of gastric half-emptying time, and gastric retention after 60 and 120 minutes, refer to Table 3.
3.5 | Non-esterified fatty acids

Fasting and premeal free fatty acid levels were reduced by insulin glargine treatment, but were not affected by lixisenatide treatment (Figure 2C,D). They were further reduced after both meals, with no significant difference between the different treatments.

3.6 | Triacylglycerols

During the baseline experiments, triacylglycerol levels increased significantly after both breakfast and a late lunch (Figure 2E,F). After breakfast, this rise was attenuated by treating with lixisenatide, either alone or in combination with insulin glargine (Figure 2E). Despite similar plasma triacylglycerol concentrations being reached with the combination of lixisenatide and insulin glargine treatment and at baseline (ie, without injectable glucose-lowering therapy), this difference did not achieve significance in the cohort initially treated with insulin glargine (Figure 2F).

The differences in triacylglycerol concentrations observed after breakfast tended to be smaller after a late lunch (Figure 2E,F).

3.7 | Gastric emptying

After breakfast, the gastric-emptying time course was retarded in all experiments in which patients had been exposed to lixisenatide injected 30 minutes before the meal (Figure 3, Table 4). A similar result was also derived from calculated gastric half-emptying times, which were prolonged by lixisenatide treatment from \(~90\) minutes at baseline to \(~175\) minutes. Treatment with insulin glargine did not affect gastric emptying significantly (Figure 3, Table 4).

After a late lunch, gastric emptying occurred at a similar velocity as after breakfast at baseline (Figure 3). When lixisenatide was

### TABLE 4 Variables characterizing gastric emptying following meals at baseline, after treatment with either lixisenatide or insulin glargine alone, or with a combination of lixisenatide and insulin glargine

| Variable | Unit | Conditions/treatment | Breakfast | Late lunch | Significance (P-value) |
|----------|------|----------------------|-----------|------------|-----------------------|
| **1. Lixisenatide first** | | | | | |
| Gastric half emptying time (T_{1/2}) | min | Baseline | 87.8 ± 11.7 | 85.0 ± 12.5 | 0.92 |
| | | Lixisenatide | 168.0 ± 20.3* | 63.7 ± 10.8 | 0.0002 |
| | | Lixisenatide + insulin glargine | 187.8 ± 17.1* | 63.9 ± 6.8 | <0.0001 |
| | | Significance (P-value)b | 0.0004 | 0.26 | |
| Retention of gastric content at 60 min (% of initial value) | | Baseline | 68.6 ± 4.2 | 72.8 ± 4.3 | 0.50 |
| | | Lixisenatide | 80.6 ± 2.8* | 60.2 ± 5.6 | 0.0041 |
| | | Lixisenatide + insulin glargine | 85.7 ± 3.1* | 68.6 ± 6.0 | 0.020 |
| | | Significance (P-value)b | 0.0041 | 0.25 | |
| Retention of gastric content at 120 min (% of initial value) | | Baseline | 31.4 ± 5.7 | 22.0 ± 7.7 | 0.34 |
| | | Lixisenatide | 57.0 ± 6.4* | −28.1 ± 6.9 | <0.0001 |
| | | Lixisenatide + insulin glargine | 67.0 ± 6.0* | 4.7 ± 7.0 | <0.0001 |
| | | Significance (P-value)b | 0.0007 | 0.11 | |
| **2. Insulin glargine first** | | | | | |
| Gastric half emptying time (T_{1/2}) | min | Baseline | 78.2 ± 8.3 | 104.9 ± 17.7 | 0.20 |
| | | Insulin glargine | 80.0 ± 15.1 | 89.6 ± 16.7 | 0.67 |
| | | Insulin glargine + lixisenatide | 155.8 ± 20.0*† | 59.3 ± 6.3* | 0.0002 |
| | | Significance (P-value)b | 0.0012 | 0.099 | |
| Retention of gastric content at 60 min (% of initial value) | | Baseline | 67.5 ± 4.0 | 79.5 ± 4.5 | 0.060 |
| | | Insulin glargine | 66.3 ± 3.1 | 73.9 ± 4.9 | 0.20 |
| | | Insulin glargine + lixisenatide | 78.7 ± 3.7*† | 61.9 ± 6.8 | 0.043 |
| | | Significance (P-value)b | 0.041 | 0.086 | |
| Retention of gastric content at 120 min (% of initial value) | | Baseline | 30.7 ± 4.1 | 28.7 ± 8.5 | 0.83 |
| | | Insulin glargine | 26.5 ± 5.3 | 23.5 ± 7.7 | 0.75 |
| | | Insulin glargine + lixisenatide | 58.2 ± 6.8*† | −5.5 ± 7.2*† | <0.0001 |
| | | Significance (P-value)b | 0.0006 | 0.0090 | |

Mean ± SEM, n = 11.

*Comparison between breakfast and late lunch.

†Comparison between the three experimental conditions (at baseline, after treatment with lixisenatide or insulin glargine alone, or a combination of both).

*Significant difference (P < 0.05) from baseline; †Significant difference (P < 0.05) from lixisenatide or insulin glargine only treatment.
administered before breakfast, gastric emptying 8 hours later was slightly accelerated compared with baseline (ie, in the absence of lixisenatide treatment). This was the case in two experiments with lixisenatide in the cohort first treated with lixisenatide and then with the combination of lixisenatide and insulin glargine (Figure 3A), and in the one experiment with lixisenatide (added to ongoing treatment with insulin glargine) in those first treated with insulin glargine. Insulin glargine treatment alone had no significant effect on the velocity of gastric emptying after a late lunch (Figure 3B).

3.8 Comparison of the two randomized cohorts

The two populations studied both started with metformin alone as an oral glucose-lowering drug and finally were treated with a combination of lixisenatide and insulin glargine. Both at baseline (ie, in the absence of injectable treatment) and after 8 weeks, ie, with combination treatment (lixisenatide and insulin glargine), all of the variables measured were in a comparable range (Figures 1–3, Tables S1 and S2; Table 2). Thus the sequence of initiating treatment with either lixisenatide or insulin glargine did not impact upon the glycaemic efficacy of the combination therapy.

4 DISCUSSION

The current study was designed to examine the mechanisms of postprandial glucose reduction with lixisenatide and insulin glargine after two subsequent meals. We report that: (i) the delay in gastric emptying is the leading mechanism for glucose reduction at the first meal following lixisenatide injection; (ii) after a second meal, gastric emptying is slightly accelerated with lixisenatide, and insulin secretion is enhanced; (iii) the glucose-lowering effects of insulin glargine and lixisenatide are partly additive; and (iv) the sequence of initiating lixisenatide and insulin glargine does not affect the glucose-lowering efficacy of both drugs combined.

A number of previous studies have examined the mechanisms of postprandial glucose reductions with lixisenatide or exenatide when the drugs were administered immediately (within 30 minutes) prior to meal ingestion.5,25,26 Consistent with these studies, we have observed that lixisenatide alone has a profound effect on postmeal glycaemic excursions (by 81% based on integrated incremental glucose responses), and this was similar with added insulin glargine treatment (74% in patients treated with lixisenatide first, and 83% in those treated with insulin glargine first). Lixisenatide treatment immediately led to decelerated gastric emptying, reduced postmeal insulin secretory responses, and caused a suppression of plasma glucagon below baseline values, indicating that α-cells are truly suppressed with lixisenatide, and that not just a meal-induced rise in glucagon (which occurs at baseline, ie, in the absence of lixisenatide or insulin glargine treatment) was attenuated because of retarded gastric emptying.

The glucose-lowering mechanisms after the meals not immediately following the injection of the drug have not yet been examined. In the current study, postprandial glucose concentrations were also reduced after the second meal compared with baseline levels. However, even although gastric emptying was significantly delayed after the first meal, gastric emptying was even slightly accelerated after the second meal. There was also just a small increase in insulin secretion after the second meal, and the reduction in glucagon was less pronounced compared with the first meal. Thus, these mechanisms may not fully account for the glucose-lowering effects of lixisenatide after the second meal. However, upon closer inspection, the reduction in postprandial glycaemia after the second meal appeared to be also partly driven by a concomitant reduction in premeal glucose levels. Thus, rather than representing a true reduction in glycaemic excursions, the plasma glucose concentrations were parallel-shifted downwards in a quantitatively similar manner to the reduction in premeal plasma glucose concentrations. In line with this observation, a significant correlation of the difference in premeal plasma glucose concentrations and the difference in integrated plasma glucose responses comparing the first (breakfast) and the second (late lunch) meal was found. A multivariate regression analysis indicated that this reduction in premeal glucose concentrations between the second and first meal was related to gastric half-emptying time, postbreakfast glucose increments and, in particular, the stimulation of insulin secretory responses.

The acceleration in gastric emptying (more than) 8.5 hours after lixisenatide injection is an interesting phenomenon. One hypothetical explanation can be derived from the known phenomenon of tachyphylaxis (desensitization) for GLP-127 or its receptor agonists leading to decreased or even abolished effects on gastric emptying upon prolonged exposure.3–5,25 If prolonged stimulation to GLP-1 RAs leads to reduced effects, the sharp drop in lixisenatide concentrations following the early morning injection, which putatively occurred immediately prior to the late lunch in our protocol,26 may lead to increased sensitization to the effects of endogenously released GLP-1 (which was not measured in the current study) in response to the second meal. Endogenous GLP-1 has previously been shown to effectively decelerate gastric emptying29,30 because antagonization of the GLP-1 receptor blockade leads to an acceleration in gastric emptying. In addition, it appears possible that the acceleration of gastric emptying after the second test meal was partly attributable to the lower levels of prelunch glycaemia. The current study confirms that lixisenatide produces significant, albeit modest reductions in fasting glucose, but has profound effects in reducing postmeal glycaemic excursions immediately following its administration, mainly mediated by a retardation in gastric emptying5,11 as well as a suppression in glucagon secretion.31–33 It also confirms that insulin glargine more substantially reduces fasting glucose,17 but exerts only a modest effect on postmeal rises in glycaemia and does not affect gastric emptying at all. When administered together, the result is an excellent control of fasting glucose (in the near-normal range of concentrations) and a substantial reduction in postmeal glucose concentrations. Some of these glycaemic benefits are preserved after a second meal served 8 hours after breakfast, although most probably there is no longer exposure to effective drug concentrations of lixisenatide during this
period,\(^{20}\) and also despite the fact that a deceleration of gastric emptying and a significant suppression of glucagon are no longer observed. The current study, therefore, provides a mechanistic explanation for the combined use of lixisenatide (or GLP-1 RAs, in general) and insulin glargine (or long-acting insulin preparations, in general). Clinical trials have shown excellent glycaemic control when these agents or classes are used in free\(^{14-16,34}\) or fixed-dose\(^{35-38}\) combinations. These trials have been performed looking at patients who had either used insulin glargine first and then added a GLP-1 RA\(^ {15,34}\) or vice versa (i.e., li拉glutidie first, then insulin detemirl\(^ {18}\). Our study shows that the sequence of initiating lixisenatide and insulin glargine is almost without influence on the therapeutic results with this combination.

A hypothetical explanation for the stimulation of insulin secretion by lixisenatide after the second meal might be an induction of beta-cell rest. Thus, because the lixisenatide-induced deceleration of gastric emptying has led to a marked reduction in the typical postprandial insulin release after breakfast, it is conceivable that the beta-cell insulin secretory capacity was partly restored, thereby providing greater insulin responses after the subsequent meal. A similar phenomenon has previously been observed after transient inhibition of insulin secretion using, eg, somatostatin or potassium channel openers\(^ {12}\) as well as during exogenous insulin therapy.\(^ {13}\) Considering the fact that the amount of insulin secreted after breakfast typically represents the largest increment in insulin throughout the day,\(^ {39}\) such mechanisms would also argue in favour of administering lixisenatide prior to breakfast rather than other meals later during the day. In support of this, a previous study has shown that administration of lixisenatide before breakfast also led to reductions in postprandial glycaemia after lunch and dinner, whereas no effects on postbreakfast glycaemia were observed when the drug was injected before lunch or dinner on the previous day.\(^ {7,10}\)

The current study has some limitations. The number of patients with type 2 diabetes studied was small, and was further reduced by some patients (three out of 14 in each treatment sequence) who either did not complete the series of experiments or did not provide gastric emptying data that was suitable for quantitative analyses. However, irrespective of the sequence of introducing the treatment with lixisenatide and insulin glargine, data from 22 patients were available on the combination of both treatments, including gastric-emptying data. In addition, this study did not include a placebo arm without any treatment escalation. Even though this limitation was partly addressed by the different sequence of treatment escalation with either lixisenatide or insulin glargine, such a potential confounding effect cannot fully be excluded. Furthermore, the meal composition was not representative of the typical composition of breakfast and lunch meals. However, it was a solid meal with a calorific content and nutrient mixture fairly comparable with a standard Western diet. It might have also been helpful to have GLP-1 measurements in order to judge the potential changes in endogenous GLP-1 secretion. Unfortunately, such measurements were not available in the current study. Finally, the timing of the late lunch was not representative of typical lunchtimes in Western Europe, but our emphasis was upon completion of gastric emptying after breakfast and a complete return of plasma glucose concentrations and insulin secretory responses to premeal values. Such normalization of postprandial glucose levels has previously been shown to take up to 7 hours.\(^ {30}\) The strengths of the current study are the number of plasma and breath samples defining the time courses of postmeal glycaemic and insulin as well as glucagon secretory responses, and the analysis of gastric emptying using a method that provides results in terms of gastric emptying of solid meal components that is very similar to the gold standard, ie, scintigraphy.

In conclusion, our detailed study of sequentially initiating a GLP-1 RA, lixisenatide, and a basal insulin, insulin glargine, before using both medications together, provides a mechanistic explanation for the glycaemic benefits gained from this combination. The glucose-lowering effect of lixisenatide is not restricted to the postbreakfast period (before which the short-acting GLP-1 RA was injected), but also extends to a late lunch. Thus, the glycaemic benefits of lixisenatide appear to last significantly longer than the 6–8 hours after injection, where an exposure to effective lixisenatide concentrations can be expected. The different mechanisms of action of lixisenatide and insulin glargine affecting both postprandial and fasting glycaemia provide a rationale for the combined use of these agents in the treatment of patients with type 2 diabetes.

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CONFLICT OF INTEREST

J.J.M. has received lecture honoraria and consulting fees from AstraZeneca, Berlin-Chemie, Boehringer Ingelheim, Eli Lilly, Merck Sharp & Dohme (MSD), Novo Nordisk, Novartis and Sanofi; has received reimbursement of congress participation fees and travel expenses from MSD, Novo Nordisk and Sanofi; and has initiated projects supported by Boehringer-Ingelheim, MSD, Novo Nordisk and Sanofi. C.K. has received travel grants from Eli Lilly and Company; is a co-owner of Profil, which received research funds from ADOCIA, Biocon, Boehringer Ingelheim Pharmaceuticals Inc., Dance Biopharm Holdings Inc., Eli Lilly and Company, Gan & Lee Pharmaceuticals, MedImmune, Mylan, Nordic Bioscience, Nestlé, Novo Nordisk A/S, Poxel SA, Sanofi-Aventis, Woekhardt, Xeris Pharmaceuticals Inc. and Zealand Pharma A/S. M.A.N. has been a member of advisory boards or has consulted with AstraZeneca, Boehringer Ingelheim, Eli Lilly & Co., Fractyl, GlaxoSmithKline, Hoffman La Roche, Menarini/Berlin Chemie, Merck, Sharp & Dohme, NovoNordisk and Versatis; he has received grant support from Eli Lilly & Co., Menarini/Berlin-Chemie, Merck, Sharp & Dohme and Novartis Pharma; he has also served on the speakers’ bureau of AstraZeneca, Boehringer Ingelheim, Eli Lilly & Co., GlaxoSmithKline, Menarini/Berlin Chemie, Merck, Sharp & Dohme, NovoNordisk and Sun Pharma. B.A.M., N.S., M.K.S., S.E. and F.S. have no conflicts of interest to report.
AUTHOR CONTRIBUTIONS

J.J.M. designed the study, contributed to the experimental procedures and analyses and wrote the manuscript. B.A.M. contributed to the experimental procedures and analyses and discussed the manuscript. N.S. contributed to the experimental procedures and analyses and discussed the manuscript. S.E. and M.K.S. contributed to the experimental procedures and analyses and discussed the manuscript. F.S. contributed to the analyses and wrote the manuscript. C.K. designed the study and discussed the manuscript. M.A.N. contributed to the experimental procedures and analyses and wrote the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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