Lixisenatide reduces chylomicron triacylglycerol due to increased clearance

Effect of lixisenatide on postprandial metabolism

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Keywords
Lixisenatide, GLP-1 agonist, postprandial hyperlipidaemia, lipoprotein

I certify that neither I nor my co-authors have a conflict of interest as described above that is relevant to the subject matter or materials included in this work.

ClinicalTrials.gov (NCT 02049034)

Word count abstract: 250
Word count main text: 3811
Number of references: 48
Number of tables: 1
Number of figures: 4

Abstract

Context

GLP-1 agonists control postprandial glucose and lipid excursion in type 2 diabetes; however the mechanism(s) are unclear.

Objective

To determine the mechanism(s) of postprandial lipid and glucose control with lixisenatide (GLP-1 analogue) in type 2 diabetes.

Design

Randomised, double-blind, cross-over study.

Setting

The Centre for Diabetes, Endocrinology, and Research, Royal Surrey County Hospital, Guildford, United Kingdom

Patients

Eight men (57.3±1.9yrs; BMI 30.3±1.0kg/m^2, $HbA1_C$ 66.5±2.6mmol/mol, [8.2±0.3%]).

Interventions

Two metabolic studies, four-weeks after lixisenatide or placebo; with subsequent cross-over and repetition of the studies.

Main outcome measures

Study one: very-low density lipoprotein (VLDL) and chylomicron (CM) triacylglycerol (TAG) kinetics were measured with iv bolus of [$^2H_5$]glycerol in a 12h study, with hourly feeding. Oral [^{13}C]triolein, in a single meal, labelled enterally-derived TAG. Study two: glucose kinetics were measured with [U-^{13}C]glucose in a mixed-meal (plus acetaminophen to measure gastric emptying) and variable iv [6,6-$^2H_2$]glucose infusion.

Results

Study one: CM-TAG (but not VLDL-TAG) pool-size, was lower with lixisenatide (P=0.046). Lixisenatide reduced CM [^{13}C]oleate $AUC_{60\text{-}480min}$ concentration (P=0.048) and increased CM-TAG clearance; with no effect on CM-TAG production rate.

Study two: postprandial glucose and insulin $AUC_{0\text{-}240min}$ were reduced with lixisenatide (P=0.0051, P<0.05). Total glucose production rate (Ra) (P=0.015), Ra_{meal} (P=0.0098) and acetaminophen $AUC_{0\text{-}360min}$ (P=0.006) were lower with lixisenatide than placebo.

Conclusions

Lixisenatide reduced [^{13}C]oleate concentration, derived from a single meal in CM-TAG, as well as glucose Ra_{meal}, through delayed gastric emptying. However chronic CM production, measured with repeated meal feeding, was not reduced by lixisenatide and decreased CM-TAG concentration was due to increased CM-TAG clearance.

Précis

Using stable isotopes; lixisenatide acutely slowed gastric emptying - improving postprandial triacylglycerol (TAG) concentration. Chronically, chylomicron TAG was reduced through increased clearance.

Abbreviations and Definitions

ALT	Alanine aminotransferase
ApoB100	Apolipoprotein B100
ApoB48	Apolipoprotein B48
AUC	Area Under the Curve
BCA	Bi-Cinchoninic acid
BMI	Body Mass Index
CM	Chylomicron
CV	Coefficient of Variation
ELISA	Enzyme-linked immunosorbent assay
FCR	Fractional clearance rate
GLP-1	Glucagon-like peptide 1
GLP-1 RA	GLP-1 receptor agonist
HbA_{1c}	Glycosylated haemoglobin
IMP	Investigational Medicinal product
IV	Intravenous
Kg	Kilogram
LPL	Lipoprotein Lipase
NEFA	Non-esterified fatty acids
PPG	Post-prandial glucose
PR	Production rate
REC	Research Ethics Committee
SAS®	Statistical Analysis System
TAG	Triacylglycerol
TLC	Thin layer chromatography

120	TRL	Triglyceride-rich lipoprotein
121	TTR	Tracer-to-tracee ratio
122	VLDL	Very low density lipoprotein

INTRODUCTION

Postprandial hypertriglyceridaemia is an independent risk factor for cardiovascular disease (1-4). This may occur via the generation of small, dense low-density lipoproteins, oxidative stress, inflammation, and/or endothelial dysfunction. Postprandial hypertriglyceridaemia is due to an excess of triglyceride-rich lipoproteins (TRL) and may result from: the overproduction of very low-density lipoproteins (VLDL) synthesised by the liver, which contain the higher molecular weight form of apolipoprotein (apo)B, apoB100; chylomicrons (CM) synthesised in the enterocytes, in response to dietary fat, which contain the lower molecular weight form of apoB, apoB48; impaired TRL clearance; or a combination of these processes.

GLP-1 receptor agonists (RA) lower postprandial hypertriglyceridaemia (5-7), which may contribute to their cardiovascular benefit, but the mechanism has not been elucidated. Insulin resistance is associated with a postprandial increase in CM apoB48 and triacylglycerol (TAG) (8) and some studies suggest that this is due to impaired clearance (9,10). The small intestine can utilise endogenous substrates to synthesize TRL particles; for instance, an acute elevation of NEFAs in humans increased basal intestinal apoB48 production (11) and CM-TAG production rate was shown to be increased in metabolic syndrome (12). Both animal (13) and human (14) studies suggest that GLP-1 RAs can reduce postprandial TRL-apoB48 independently of their effects on plasma insulin and gastric emptying. In a study of individuals without diabetes, exenatide suppressed the production of apoB48 but with no effect on catabolism (15). However measurement of apoB48 metabolism provides a measure of particle kinetics rather than the kinetics of the TAG substrate within the particle. No studies have previously investigated the effect of GLP-1 RAs on postprandial CM and VLDL TAG

kinetics. Understanding the regulation of enterocyte lipid handling may provide novel strategies to reduce cardiovascular disease risk.

GLP-1 RA also effectively lower postprandial hyperglycaemia (16-18). This may be achieved through delayed gastric emptying (19,20), reduction of hepatic glucose output, and increased peripheral glucose uptake (21). Identifying the relative contribution of delayed gastric emptying to glucose regulation will inform therapeutic decision making in insulinopaenic states and those with pre-existing delayed emptying.

We hypothesized that lixisenatide, a GLP-1 RA, reduces postprandial TAG through a decrease in CM-TAG production, as a result of a direct effect on enterocyte CM assembly, and also from a decrease in VLDL-TAG secretion, due to an improvement in insulin sensitivity. At the study inception, we hypothesized that GLP-1 RA lowered postprandial glycaemia primarily by decreasing endogenous glucose output, although more recent data suggest that the predominant effect of lixisenatide is via gastric emptying (19).

We used a validated constant-feeding methodology and three antibody immunoaffinity method (22), which enables the complete separation of hepatic TRL from intestinal TRL, to measure hepatic VLDL and intestinal CM TAG kinetics, with an intravenous bolus of [$^2H_5$] glycerol. In addition we utilised the validated dual-tracer dilution technique to determine glucose appearance following a meal (23).

MATERIALS AND METHODS

Participants

This study was registered with ClinicalTrials.gov (NCT 02049034) and approved by the UK Medicines and Healthcare Products Regulatory Agency (EudraCT 2013-002826-22), South Central-Hampshire B National Research Ethics Services Committee and University of Surrey Ethics Committee. Prior to initiation of study procedures, written informed consent was obtained from all participants. Inclusion criteria: Caucasian men with type 2 diabetes, age 40-65, receiving metformin monotherapy (HbA1c ≥58 and ≤ 80mmol/mol, 7.5% to 9.5%). Exclusion criteria: insulin use, oral hypoglycaemic agent (other than metformin), alcohol (>12 g of alcohol per day), current smokers, liver or renal disease, previous pancreatitis or gastric surgery.

Experimental Design

This was a double-blind crossover study comparing 4 weeks investigational medicinal product (IMP: lixisenatide or placebo), with a 4-week washout period in-between (**Supplementary Figure 1**). Randomisation was with SAS version 9.1, PROC PLAN software (Statistical Analysis System Institute, NC, USA) prepared by an investigator with no clinical involvement in the trial. The allocation sequence was concealed from the researchers involved in enrolling and assessing participants; in numbered, opaque, sealed envelopes. Lixisenatide was supplied as disposable pre-filled pen-injectors for subcutaneous injection: 10 µg lixisenatide green pens (for 14 days) then 20 µg lixisenatide purple pens (for additional 14 days). Placebo for lixisenatide was supplied as green and purple disposable pen-injectors containing 3 ml of sterile aqueous solution. The injections were self-administered daily, 30 min before breakfast, except on metabolic study days. Medication packs were brought to all study visits to verify adherence.

Metabolic studies occurred on two days, using two distinct studies (one and two), each following completion of 4-weeks treatment. At all visits, subjects attended the Centre for Diabetes, Endocrinology and Research (CEDAR), Royal Surrey County Hospital, Guildford (RSCH) after an over overnight fast, having consumed a standardised low-fat, low-fibre meal of approximately 700 kcal at 8 pm the previous evening.

Study One - postprandial lipid kinetics

Subcutaneous IMP was administered at -270min, 30 minutes prior to the first of twelve, hourly, liquid meals (23.8% carbohydrate, 12.8% protein, 63.5% fat. 178 kcal/80ml) at time -240 min. A cannula was inserted into an antecubital vein for blood sampling. Baseline blood samples were taken to measure fasting glucose, insulin and lipid profile. The feeding study was designed to increase fasting plasma TAG levels twofold and to maintain this throughout the study (12). The first 4 h (−240 to 0 min) allowed a TAG steady-state to be achieved, prior to administration of stable isotope tracers. Meal composition (in 80 ml) was 6.5g sugar, 7g whey powder (Natures Best, UK), 7ml extra virgin olive oil (Tesco brand), 7ml sunflower oil (Tesco brand, UK) and 10ml double cream (Tesco brand, UK) and flavouring, prepared as an emulsion. Each meal, prepared immediately beforehand, was consumed within one min. [1,1,1 $^{13}C_3$]triolein (150 mg) was mixed with the third meal, consumed at -120 min to investigate the acute effect of the IMP on TAG absorption from the meal. An intravenous bolus of [$^{2}H_5$]glycerol (0.75µmol/kg) was administered at 0 min. Blood samples were taken at regular intervals until 480 min for measurement of the isotopic enrichment of CM and VLDL-TAG with [$^{2}H_5$]glycerol and [^{13}C]oleate (**Supplementary Figure 2**).

Study Two - postprandial glucose kinetics

One indwelling cannula was inserted into an antecubital vein of each arm. Fasting blood was sampled for insulin, glucose, cholesterol, TAG and non-esterified fatty acids (NEFA) concentrations. A primed, intravenous (iv) infusion of [6,6-$^{2}H_2$]glucose (6mg/kg; 0.06mg/kg/min) was administered from -120 min to 0 min, followed by a variable infusion to 360 minutes, to measure endogenous glucose production (EGP) (23).

Subcutaneous IMP was administered at -30 min. At 0 min a liquid mixed-meal (64% carbohydrate, 14.4% protein, 21.6% fat. 500 kcal in total) containing [U-^{13}C] glucose (1.7 g) was given to measure meal-derived glucose appearance. The [6, 6-$^{2}H_2$] glucose infusion rate was adjusted at predetermined intervals to mimic the (expected) EGP (**Supplementary Figure 3**). Blood samples were taken at regular intervals for 360 min for measurement of glucose enrichment and concentration of glucose, TAG, NEFA and insulin. Participants were asked to void prior to the meal. During the study and on study termination, urinary volume and glucose concentration were measured as necessary. At 0 min, 1000mg acetaminophen dissolved in 30 ml water was given immediately prior to the meal to measure gastric emptying.

Determination of glucose enrichment in plasma. Samples were deproteinized and dried under oxygen-free nitrogen (OFN). Glucose in the sample was derivatized to form a methoxime-trimethylsilane (TMS) derivative and enrichment was measured by gas chromatography mass spectrometry (GCMS) (Agilent 5975C) in the electron ionisation (EI) mode. Ions monitored were at *m/z* 319.2, *m/z* 321.2 (m+2), *m/z* 322.2 (m+3) and m/z 323.2 (m+4) (24). Intra-assay CVs for determination of isotopic

enrichment of glucose in *m/z* 321.2/319.2 at three enrichment levels (low, middle and high) were 0.33%, 0.33% and 0.25%, and for *m/z* 323.2/319.2 were 1.99%, 0.3% and 0.2%, respectively.

Lipoprotein separation and isolation. TRL particles (sf >20) were isolated from plasma overlaid by saline containing 0.1% EDTA (w/v) by floatation ultracentrifugation in a fixed angle rotor 50.4Ti (Beckman) using a LE80-k ultracentrifuge (Beckman Coulter Optima) at 125000 *g*, for 16h at 4^{o}C.
VLDL and CM particles were isolated from the TRL samples by a sequential immunoaffinity binding method as previously described (12,22). Three monoclonal antibodies to apoB100, 4G3, 5E11and 16BSol (Heart Institute, University of Ottawa, Ca.) coupled separately to protein G Sepharose 4 Fast flow (Amersham, U.K.) were used sequentially. The bound apoB100 containing VLDL fractions from the sequential affinity chromatography were combined, and the unbound apoB48 containing CM fractions were also combined.

Determination of plasma free glycerol and TAG glycerol enrichment.
For analysis of plasma free glycerol, samples were deproteinized and further purified by ion-exchange chromatography. Freeze-dried glycerol was derivatized with MTBSTFA to form TBDMS glycerol and enrichment was measured by GCMS in EI ionization mode. Ions monitored were *m/z* 377.4 and *m/z* 382.4 (m+5) (25). TAG in VLDL and CM fractions was extracted and hydrolysed in the presence of 3% hydrochloric acid:methanol to glycerol and methylesters of fatty acids (FAME). Glycerol from TAG was then purified by ion-exchange, freeze dried, and enrichment was measured by GCMS (Agilent 5975) in EI mode following derivatisation to

TBDMS glycerol. Intra-assay CVs for plasma glycerol low, middle and high were 8.8%, 8.0% and 5.1%, respectively. Intra-assay CVs for TAG glycerol low, middle and high were 12.6%, 3.7% and 4.6%, respectively.

Determination of oleate enrichment of VLDL and CM-TAG and TAG concentration. Enrichment of VLDL and CM-TAG with [^{13}C]oleate was measured with a Trace GC Ultra with autosampler, coupled to an isotope ratio mass spectrometer (IRMS) Delta Plus XP via an oxidation reactor, reactor temperature 960^{o}C, and a combustion interface III.

An internal standard, heptadecanoic acid, added to the TAG samples at the acid-hydrolysis and esterification step, was used to measure concentration of VLDL- and CM-TAG. ApoB48 concentration was measured using a commercially available kit (Shibayagi Co Ltd, Shibukawa, Japan).

Measurement of lipid and hormone concentration. Concentrations of fasting and fed plasma NEFA, TAG, total cholesterol, HDL-cholesterol and TRL-TAG and TRL-cholesterol, were measured by enzymatic assay (ABX, Chicksands, Shefford, U.K.) using Cobas MIRA (Roche, Welwyn Garden City, U.K.). Insulin concentrations were measured by radioimmunoassay (Millipore Corporation, Billerica, MA. USA).

Data analysis. All tracer enrichments were expressed as tracer/tracee ratio (TTR). Glucose fluxes were calculated from TTR and native glucose using the Mari model (26) implemented within the Bayesian parameter estimation framework (23).

Using compartmental modelling (**Supplementary Figure 4**), chylomicron-TAG and VLDL-TAG clearance and production rates were analysed (27). A single-pool model

was used to describe CM-TAG and VLDL-TAG kinetics with plasma glycerol as precursor pool using the SAAM II program (SAAM Institute, Seattle, WA). The model represents the kinetics of the tracer-to-tracee ratio (TTR) profiles which change as labelled glycerol is removed from plasma and incorporated into the TAG fractions. Plasma glycerol kinetics were described by a sum of three exponentials representing a three compartment model. A five-compartment chain described a time delay due to synthesis and secretion of VLDL-TAG or CM-TAG.

The model assumes steady-state of native (unlabelled) glycerol throughout the experimental period, i.e. a constant appearance, disappearance, and incorporation of native glycerol into the TAG fractions. The incorporation of glycerol into VLDL by the liver and by the intestine is subject to a delay. VLDL-TAG and CM-TAG production rates were calculated as the product of VLDL-TAG fractional clearance rate (FCR) and CM-TAG FCR and their respective TAG pools. VLDL and CM-TAG pools were calculated from VLDL and CM-TAG concentration and plasma volume which was determined by the method of Pearson *et al* (28).

CM [^{13}C]oleate concentration was calculated by multiplying CM oleate TTR by CM-TAG concentration. Homeostasis model assessments of insulin resistance (HOMA2-IR) was calculated using the HOMA calculator version 2.2 (29). The Matsuda index of insulin sensitivity was calculated from the single mixed-meal in study one (30).

Statistical Methods

A general linear mixed model with repeated measures was used, employing SAS PROC MIXED, with explanatory variables period and treatment. The variance covariance matrix used for the repeated measure time course measurements was SP(POW). Participant was a random effect. For measurements such as weight, which

were measured twice for each period, the difference at each period was analysed in a two-period cross-over model with period and treatment as independent variables and participant as a random effect. An analysis was conducted with the period-by-treatment interaction included as an explanatory variable. Significance was accepted at 5% level, without use of multiplicity adjustment. Results are mean ± SEM.

Determination of sample size

Sample size was based on the primary endpoint of total rate of glucose appearance following the breakfast meal. This was calculated using data from a study measuring the effect of exenatide on postprandial total glucose Ra in type 2 diabetes (16). In this study, the total glucose Ra AUC was 23.7±3.0 μmol/kg/min (mean±SD) in participants before treatment and 14.3±3.5 μmol/kg/min, after two weeks treatment. The correlation between measurements in the same person was unknown and so was assumed to be zero (the worst case scenario). Completing the study in 12 participants would have 80% power to detect a difference of 20%.

The power calculation was recalculated based on 8 participants finishing the trial, based on the primary endpoint of total glucose Ra AUC following the meal (as described above). Completing the study in 8 participants had 80% power to detect a difference of 26%. The correlation between measurements in the same person is unknown so this was assumed to be zero (the worst case scenario). This is therefore a conservative estimate; Cersosimo *et al* (2011) found a 40% difference of glucose appearance AUC after two weeks treatment (16).

RESULTS

Demographic and biochemistry measurements.

Eight males, age 57.3±1.9 years were studied. Recruitment was from May 2014 to January 2016. The participant flow diagram is in **Supplementary Figure 5**. There were no serious adverse events. Six of the participants were taking HMG Co-A reductase inhibitors. There were no differences between, or within, lixisenatide and placebo treatment phases at baseline or at the end of treatment, for all demographic and fasting biochemistry measurements (**Table 1)**. After 4 weeks of IMP the HOMA2-IR was not significantly different with lixisenatide *vs* placebo. The Matsuda index was higher with lixisenatide (4.4±2.0 vs 3.5±2.5; *P*=0.011).

Lipid metabolic study (Study One)

No differences were seen in plasma TAG or TRL-TAG concentration at baseline (-240 min), or mean steady-state concentrations between treatments (**Figure 1A & B**), although both were slower to rise from baseline with lixisenatide compared to placebo. A steady-state in apoB48 concentration was achieved in both the lixisenatide (mean 8.70mg/L; 95% CI 7.11-10.29 mg/L; *P*=0.510) and placebo study (mean 12.12mg/L; 95% CI 9.59-14.65mg/L; *P*=0.957). VLDL-TAG pool size and mean postprandial VLDL-TAG concentration were not different between treatments although postprandial VLDL-TAG was higher at 4 time points with lixisenatide **(Figure 1C; Supplementary Table 1)**. Mean postprandial CM-TAG concentration and pool size were lower with lixisenatide compared to placebo (*P*=0.043 and *P*=0.047, respectively), (**Figure 1D).**

CM-[1-^{13}C]oleate concentration $AUC_{60\text{-}480}$ (**Figure 2A**) was reduced after lixisenatide compared to placebo (*P*=0.048). CM-TAG FCR a measure of clearance, was significantly greater with lixisenatide compared to placebo (*P*=0.044; **Figure 2B**). CM-TAG PR was not different between treatments (**Figure 2C**). VLDL-TAG FCR and PR were not different between treatments (**Figure 2B & 2C**).

Glucose metabolic study (Study Two)

Glucose concentration in response to a mixed-meal consumed at 0 min is shown in **Figure 3A**. Fasting glucose concentration at time 0 min and $AUC_{0\text{-}240}$ were significantly lower following lixisenatide (*P*=0.020, *P*=0.004 respectively). Glucagon concentration was not different between treatments (**Figure 3B**). Plasma TAG $AUC_{0\text{-}180}$, corrected for baseline, was significantly lower with lixisenatide than placebo (**Figure 3C**; *P*=0.021) but NEFA AUC was not significantly different (**Figure 3D**). Acetaminophen $AUC_{0\text{-}360}$ was lower with lixisenatide than placebo (**Figure 3E**; *P*=0.006). Insulin concentration $AUC_{0\text{-}180}$ and $AUC_{0\text{-}240}$, were significantly lower with lixisenatide than placebo (**Figure 3F**; *P*=0.024 and *P*=0.045 respectively) although $AUC_{0\text{-}360}$ was not different.

Following the meal, total glucose Ra $AUC_{0\text{-}240}$ was lower with lixisenatide (*P*=0.002; **Figure 4A**). The rate of glucose appearance from the meal (Ra_{meal}), $AUC_{0\text{-}240}$, was lower with lixisenatide than placebo (*P*=0.013; **Figure 4B**).

Baseline EGP was not different between treatments. EGP decreased from baseline following the consumption of the mixed meal, with no difference between treatments (**Figure 4C**). However, EGP $AUC_{0\text{-}180}$ and $AUC_{0\text{-}240}$, corrected for baseline, were

lower with placebo (p=0.032 and p=0.049). EGP AUC_{0-360} was not different with or without correction for baseline.

Baseline glucose disposal (glucose Rd) was not different between treatments. Glucose Rd was higher following placebo between 60-180min and lower at 270min (*P*=0.039) (**Figure 4D**). Glucose Rd AUC_{0-240} was lower with lixisenatide (*P*=0.005). Baseline metabolic glucose clearance rate (MCR) was higher with lixisenatide than placebo (*P*=0.013). Glucose MCR AUC_{0-360} was also greater with lixisenatide (*P*=0.008), although not significant once corrected for baseline values (data not shown).

DISCUSSION

The novel finding of this study was that the chronic effect of lixisenatide (assessed with a 12hr repeated feeding protocol) was to reduce CM TAG concentration through increased clearance. However, acutely, following a single meal, dietary TAG appearance was reduced (as shown by the delayed appearance of [^{13}C]oleate in CM TAG).

Our experimental protocol was designed to examine postprandial TAG metabolism by a) the appearance of [^{13}C]oleate in plasma TAG after a high-fat 80kcal single meal (labelled with [^{13}C]triolein), given 150 minutes after injection of the IMP (study one); b) hourly feeding of high-fat 80kcal single meals for twelve hours to achieve a steady-state in plasma TAG (study one) and measurement of CM and VLDL TAG kinetics, and c) the plasma TAG response to a standard 500 kcal liquid meal, in study two.

Addition of ^{13}C triolein to the third meal, in study one, demonstrated reduced fatty acid absorption with lixisenatide. Similarly it was evident from study two that lixisenatide

led to a near abolition of the postprandial TAG increment – a similar effect to that reported previously with exenatide (5) (7).

The hourly meal-feeding design addressed whether lixisenatide has a chronic effect on VLDL and CM metabolism, while eliminating any appetite dependent effects of GLP-1 on plasma TAGs. On a Western diet, people have elevated TAGs throughout the day, so repeated meal feeding may approximate a usual physiological state (31).

Our study is the first report that a GLP-1 RA improves postprandial TAG and TRL-TAG concentrations by a reduction of circulating CM-TAG concentration, via an increase in CM-TAG FCR. No previous study has measured the effect on CM-TAG kinetics. The uniqueness of this study was the ability to separate CM-TAG from VLDL-TAG using immunoaffinity. It has been reported that GLP-1 reduces postprandial CM particle production (15,32), but with no effect on CM particle clearance (15). In the study by Xiao *et al* (15), a steady-state in TRL-apoB48 concentrations was not achieved meaning that the steady-state modelling employed carried a degree if imprecision. We found no change in apoB48 concentration during the steady-state in this study.

In the repeated feeding study the IMP was administered during consumption of the first meal at -270 minutes. Drug concentrations of lixisenatide peak after 1–2 hours and it has a half-life of 2–4 hours (33) (34). Although the high affinity of lixisenatide for the GLP-1 receptor may allow for a more persistent metabolic response (35), a waning drug effect could explain the discrepancy between the lack of effect of lixisenatide on CM production rate (measured during the repeated feeding study), and the reduction in CM ^{13}C-oleate after a single meal at -120 minutes.

The effect of lixisenatide to increase CM clearance may be due to an increase in lipoprotein lipase (LPL) activity. The control of LPL expression and action is complex and is tissue specific, but includes regulation by insulin and glucagon (36) both of which can be affected by GLP-1 (37,38). We did not show significant differences in these hormones. LPL is also negatively regulated by apo-C-III and it has been shown that a single injection of exenatide can reduce the postprandial elevation of apo-C-III (7). Microvascular recruitment may also facilitate the clearance of CM-TAG (38,39) and this mechanism requires further study.

We hypothesized that lixisenatide would decrease VLDL-TAG production, due to improved insulin sensitivity. After four weeks of lixisenatide, the Matsuda Index (a measure of whole-body insulin sensitivity) had improved significantly. However, HOMA2-IR (considered to represent hepatic sensitivity) did not change which may explain the lack of effect on hepatic VLDL-TAG production (40).

At the time of study inception, it was postulated that lixisenatide had a multimodal reduction of postprandial glucose (41). However, we found that the predominant mechanism by which lixisenatide reduces postprandial glycaemia was through a reduction of glucose Ra_{meal} - itself a consequence of delayed gastric emptying. We found that lixisenatide had no additional suppressive effect on EGP from that observed with placebo. Our data therefore confirm that slowing gastric emptying is the primary mechanism for prandial glucose regulation with lixisenatide (19,42).

The experimental protocol was not designed to match insulin and glucagon concentrations between study arms. As with other GLP-1 RAs, lixisenatide stimulates

glucose-dependent insulin secretion by pancreatic β-cells (37). In our study, lixisenatide ameliorated postprandial hyperglycaemia despite a lower increment in insulin concentrations than placebo. Delayed gastric emptying would result in diminished nutrient appearance in the circulation, thereby reducing β-cell stimulation (37). Even so, the insulin response (relative to the glycemic stimulus) following lixisenatide was considerable.

Glucagon secretion can be suppressed by GLP-1 RAs (including lixisenatide) (37) but our data suggest that, in the context of delayed gastric emptying, inhibition of glucagon secretion contributes little to suppression of endogenous glucose Ra with lixisenatide. This is commensurate with data from a pancreatic clamp protocol whereby GLP-1 infusion inhibited EGP independently of insulin and glucagon (43).

The paracetamol profiles indicated marked slowing of gastric emptying with lixisenatide for up to three hours post-meal. Short-acting GLP-1 RAs, such as lixisenatide, have a more pronounced inhibitory effect on gastric emptying than longer acting GLP-1 RAs, with the latter also showing tachyphylaxis with prolonged use (19,44). Once-daily (morning) lixisenatide achieved better post-prandial glucose (PPG) control at breakfast compared with liraglutide (21) but with progressively less differentiation of PPG control over a day (45). Hypothetically, progressive lessening of the inhibitory action of lixisenatide on gastric emptying may be responsible for this pattern.

The increase in baseline (fasting) glucose MCR with lixisenatide potentially relates to improved peripheral insulin sensitivity. Whether GLP-1 RAs have a direct action on

skeletal muscle, to improve glucose clearance, is uncertain; this has been suggested to occur with exenatide and liraglutide in studies using myotubes (46), but no effect was seen in a murine model (47), or when using a pancreatic clamp with GLP-1 infusion in patients with type 2 diabetes (48). In healthy subjects, GLP-1 induces vasodilation in adipose tissue and skeletal muscle (38), so it is possible that microvascular recruitment facilitates clearance.

In conclusion we have shown that the short-acting GLP-1 RA, lixisenatide, ameliorated postprandial TAG and glucose, following a single meal, through delayed gastric emptying. However a chronic effect of lixisenatide to reduce CM-TAG was also observed which was due to increased clearance demonstrating that lixisenatide is mediating metabolic effects independently of an effect on gastric emptying.

ACKNOWLEDGEMENTS

The authors are grateful to Sarah Close, Beverley Tuthill and Roselle Herring at the CEDAR center, Royal Surrey County Hospital for their assistance with the study and Lucy Coppin, Daniel Curtis and Naomi Jordan at the Department of Nutritional Sciences, University of Surrey for their technical support.

CONFLICT OF INTEREST STATEMENT

MBW, F.S-M, SS, NJ, BF, RH, JM, DRJ, AMU have no conflict of interest.

FUNDING

This work was an investigator-led study, funded by Sanofi

CONTRIBUTION STATEMENT

F.S.-M. and M.B.W. performed the clinical studies. F.S.-M and S.S. performed the laboratory work. R.H. developed the model and advised on the modeling. J.M. provided statistical analysis. M.B.W., F.S.-M., B.F., N.J., D.RJ., and A.M.U. analyzed all the data and wrote the manuscript. M.B.W. was the lead writer. All authors reviewed the manuscript. A.M.U. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure legends

Figure 1. A) Plasma TAG, B) TRL-TAG, C) VLDL-TAG and D) CM-TAG concentration (mmol/L) at fasting (-240min) and following hourly meal drinks (-240 to 480 min) at the end of 4 weeks treatment with either lixisenatide (o) or placebo (•) (study one). Lixisenatide/placebo injection was self-administered at -270 min. Results are mean ± SEM. * $P<0.05$.

Figure 2. A) CM ^{13}C-oleate concentration (µmol/L) at fasting (-240min) and following hourly meal drinks (-240 to 480 min) at the end of 4 weeks treatment with either lixisenatide (o) or placebo (•) (study one). ^{13}C oleate was mixed with the third meal (-120 min). Lixisenatide/placebo injection was self-administered at -270 min. B) Fractional clearance rate (FCR) of VLDL TAG and CM TAG (pools/day), C) Production rate (PR) of VLDL TAG and CM TAG (mg/day/kg BW). Results are mean ± SEM. * $P<0.05$.

Figure 3. A) Plasma glucose, B) glucagon, C) TAG, D) NEFA, E) acetaminophen and F) insulin (pmol/L) at fasting (-120 to 0 min) and in response to a mixed meal and acetaminophen (consumed at 0 min) at the end of 4 weeks treatment with either lixisenatide (o) or placebo (•) (study two). Lixisenatide/placebo injection was self-administered at -30 min. Results are mean ± SEM. * $P<0.05$.

Figure 4. A) Total glucose Ra, B) Meal glucose Ra, C) EGP and D) Rd (µmol/kg/min) at fasting (-40 to 0 min) and in response to a mixed meal (consumed at 0 min) at the end of 4 weeks treatment with either lixisenatide (o) or placebo (•) (study two). Lixisenatide/placebo injection was self-administered at -30 min. Results are mean ± SEM. * $P<0.05$.

Table 1. Participant characteristics at the baseline and at the end of treatment with either lixisenatide or placebo.
There were no significant differences within or between treatments.

	Lixisenatide		**Placebo**	
	Baseline	**End**	**Baseline**	**End**
BMI (kg/m^2)	30.0 ± 1.2	29.2 ± 1.3	30.1 ± 0.9	29.6 ± 1.1
Body weight (kg)	91.8 ± 3.4	89.4 ± 3.3	92.2 ± 2.9	90.7 ± 3.1
Fat-free mass (kg)	66.3 ± 2.1	65.3 ± 1.9	66.0 ± 2.1	65.9 ± 2.0
Amylase (U/L)	46.4 ± 5.9	45.8 ± 5.4	45.4 ± 4.9	42.3 ± 5.2
ALT (iU/L)	44.5 ± 7.9	41.8 ± 6.8	41.6 ± 6.3	43.9 ± 9.0
Total plasma cholesterol (mmol/L)	3.9 ± 0.3	3.5 ± 0.3	4.1 ± 0.4	4.0 ± 0.3
Fasting plasma TAG (mmol/L)	1.68 ± 0.3	1.69 ± 0.2	1.72 ± 0.2	1.59 ± 0.2
HbA1c mmol/mol, (%)	61.5 ± 3.2 (7.8 ± 0.3)	55.1 ± 3.0 (7.2 ± 0.3)	63.4 ± 3.5 (8.0 ± 0.3)	62.6 ± 4.0 (7.9 ± 0.3)
Lipase (U/L)	34.5 ± 2.6	40.6 ± 4.3	36.8 ± 4.3	37.1 ± 3.2
Calcitonin (ng/L)	4.3 ± 0.4	2.9 ± 0.7	3.12 ± 0.5	2.4 ± 0.5

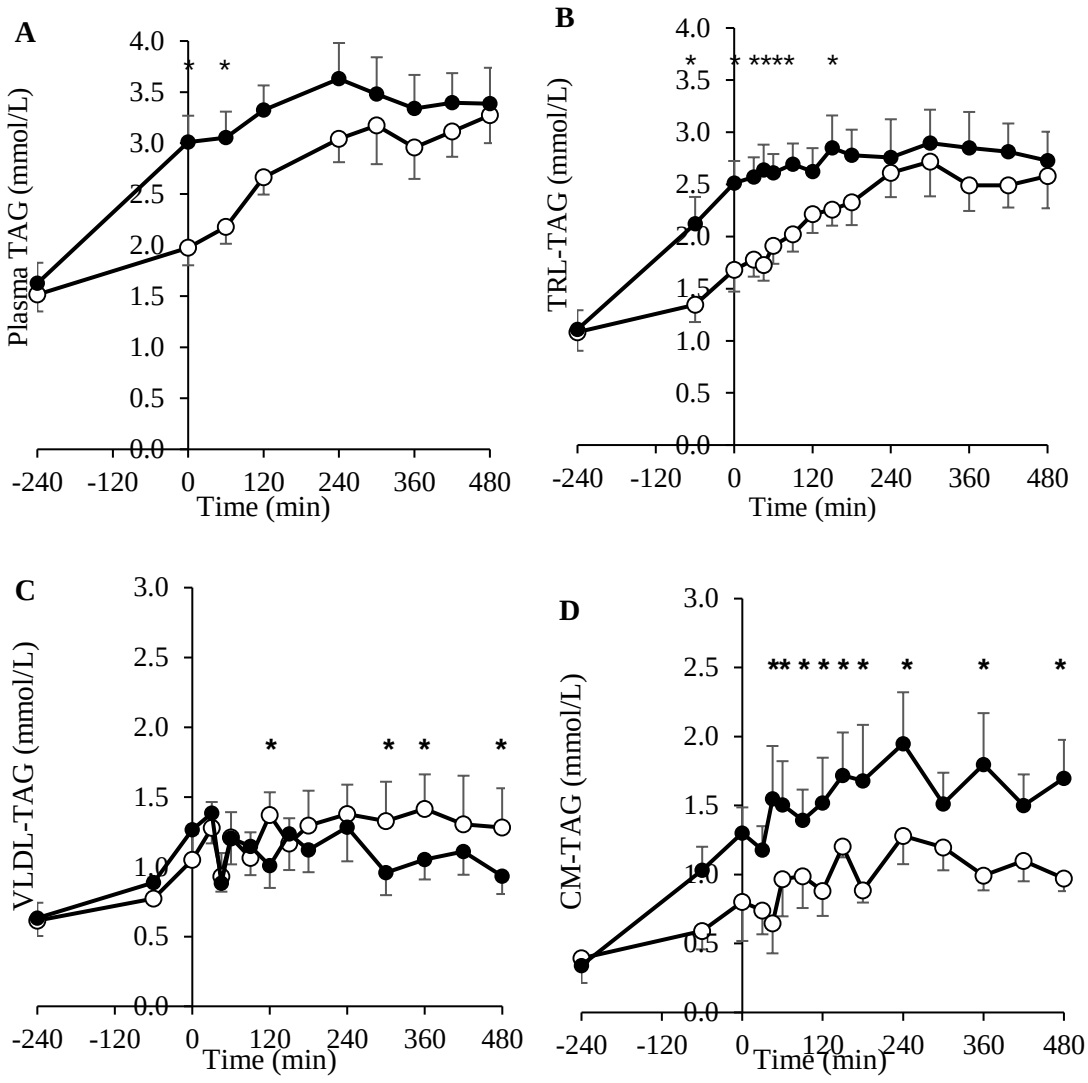
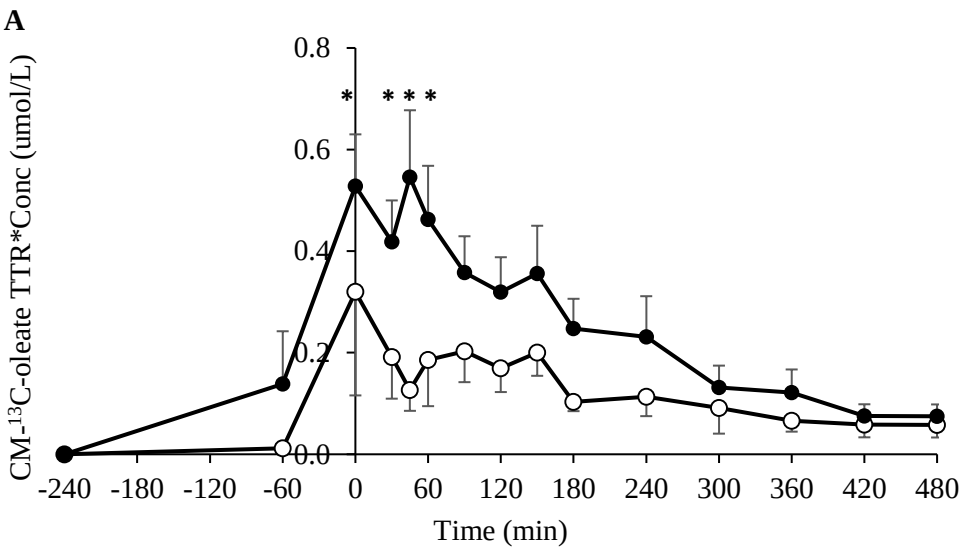

A
Plasma TAG (mmol/L)
Time (min)
B
TRL-TAG (mmol/L)
Time (min)
C
VLDL-TAG (mmol/L)
Time (min)
D
CM-TAG (mmol/L)
Time (min)

Figure 1


A
CM-13C-oleate TTR*Conc (umol/L)
0.8
0.6
0.4
0.2
0.0
* * * *
-240 -180 -120 -60 0 60 120 180 240 300 360 420 480
Time (min)

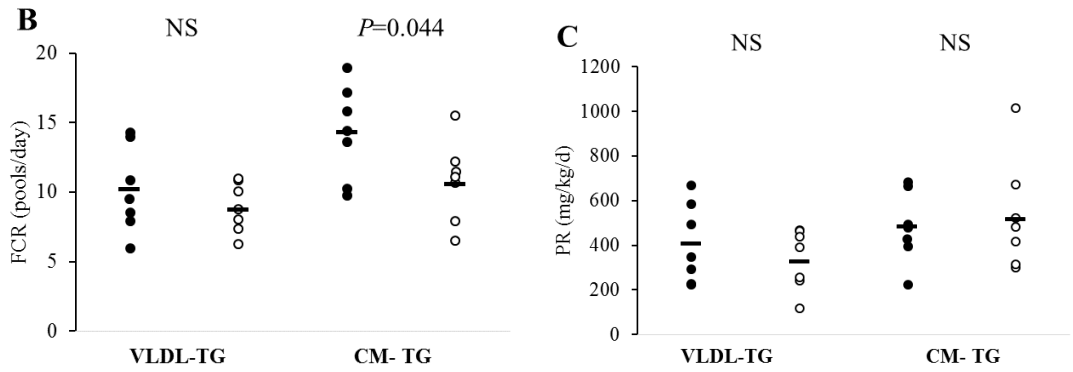

B
NS
P=0.044
FCR (pools/day)
20
15
10
5
0
VLDL-TG
CM- TG
C
NS
NS
PR (mg/kg/d)
1200
1000
800
600
400
200
0
VLDL-TG
CM- TG

Figure 2

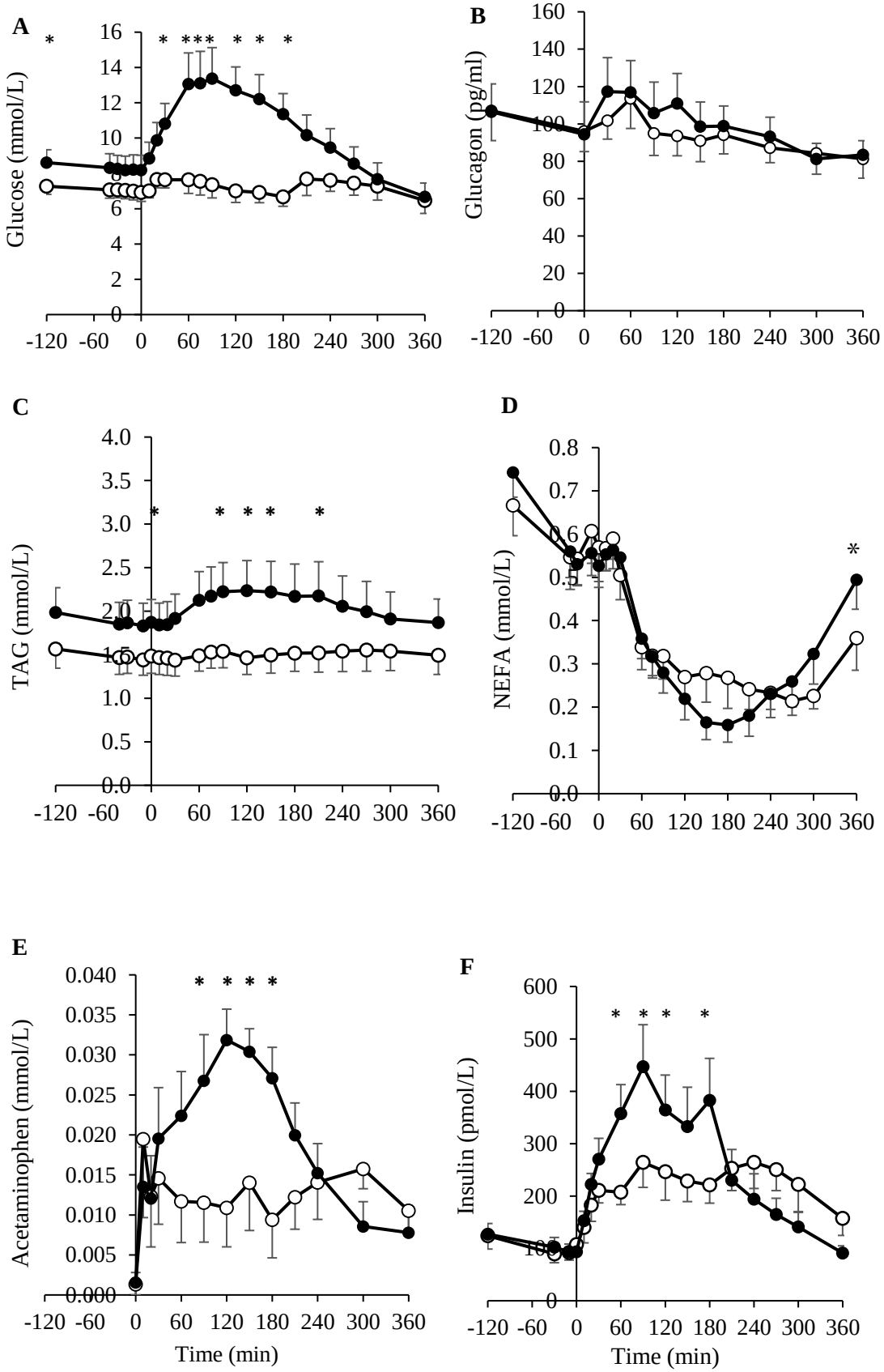

A
Glucose (mmol/L)
B
Glucagon (pg/ml)
C
TAG (mmol/L)
D
NEFA (mmol/L)
E
Acetaminophen (mmol/L)
F
Insulin (pmol/L)
Time (min)
Time (min)

Figure 3

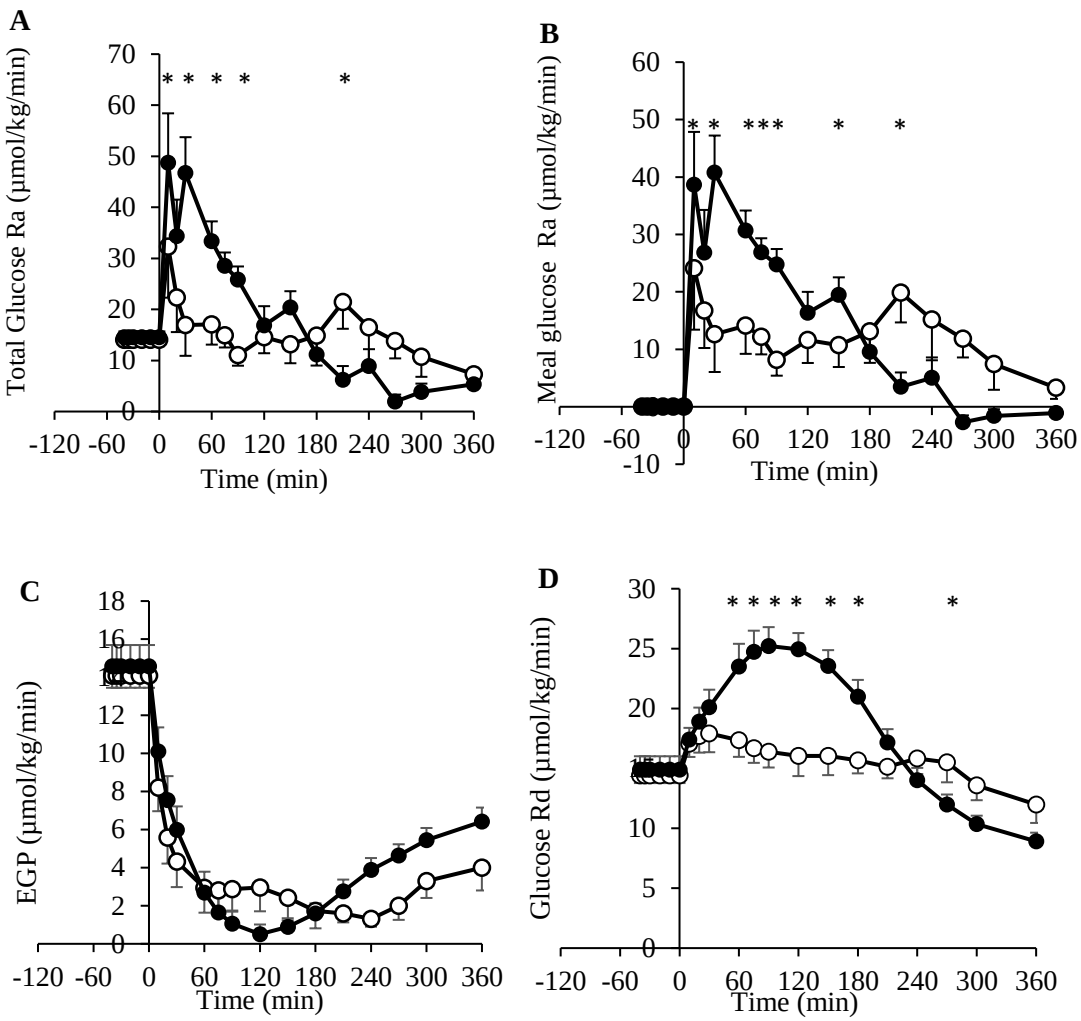

A
Total Glucose Ra (µmol/kg/min)
Time (min)
B
Meal glucose Ra (µmol/kg/min)
Time (min)
C
EGP (µmol/kg/min)
Time (min)
D
Glucose Rd (µmol/kg/min)
Time (min)

Figure 4