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Glucose-dependent Insulinotropic Polypeptide promotes lipid deposition in subcutaneous adipocytes in obese, type 2 diabetes patients: a maladaptive response

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Abstract

Glucose-dependent insulinotropic polypeptide (GIP) beyond its insulinotropic effects may regulate post-prandial lipid metabolism. While the insulinotropic action of GIP is known to be impaired in type 2 diabetes mellitus (T2DM), its adipogenic effect is unknown. We hypothesised GIP is anabolic in human subcutaneous adipose tissue (SAT) promoting triacylglycerol (TAG) deposition through re-esterification of non-esterified fatty acids (NEFA) and this effect may differ according to obesity status or glucose tolerance.

Methods: 23 subjects, categorised in four groups: normoglycaemic lean (n=6), normoglycaemic obese, (n=6), obese with impaired glucose regulation (IGR) (n=6) and obese, T2DM (n=5) participated in a double-blind, randomised, crossover study involving a hyperglycaemic clamp with a 240 minute GIP infusion (2pmol $kg^{-1}min^{-1}$) or normal saline. Insulin, NEFA, SAT-TAG content and gene expression of key lipogenic enzymes were determined before and immediately after GIP/saline infusions.

Results: GIP lowered NEFA concentrations in obese T2DM group despite diminished insulinotropic activity (mean NEFA $AUC_{0\text{-}4hr}$ ± SEM, 41992 ±9843 μmol/L/min vs 71468 ±13605 with placebo, p=0.039; 95% CI 0.31 to 0.95). Additionally, GIP increased SAT-TAG in obese T2DM (1.78 ±0.4 vs 0.86 ±0.1 fold with placebo, p=0.043; 95% CI: 0.1 to 1.8). Such effect with GIP was not observed in other three groups despite greater insulinotropic activity. Reduction in NEFA concentration with GIP correlated with adipose tissue insulin resistance for all subjects (Pearson r=0.56, p=0.005). There were no significant gene expression changes in key SAT lipid metabolism enzymes.

Conclusion: GIP appears to promote fat accretion and thus may exacerbate obesity and insulin resistance in T2DM.

Key words: GIP, type 2 diabetes, adipose tissue, lipid metabolism, NEFA

Introduction

In healthy individuals, glucose-dependent insulinotropic polypeptide (GIP) is secreted from small intestinal K cells in response to intraluminal carbohydrate, protein and most potently fat; GIP in turn stimulates (glucose-dependent) pancreatic insulin secretion. However, in patients with type 2 diabetes mellitus (T2DM), despite preserved GIP secretion (11) the insulinotropic action of GIP is severely impaired (12, 16, 35).

GIP has other important extra-pancreatic metabolic functions with receptors expressed in such tissues as bone, brain, stomach and adipose tissue, where it may modulate post-prandial lipid metabolism (7). In animal models of obesity-induced insulin resistance, genetic and chemical disruption of GIP signaling protects against the deleterious effects of high fat feeding by preventing lipid deposition, adipocyte hypertrophy and expansion of adipose tissue mass, and reducing triglyceride deposition in liver and skeletal muscle, maintaining insulin sensitivity (25, 31). Thus if GIP has a potential pro-adipogenic effect, selective GIP antagonists may be beneficial in treating obesity and type 2 diabetes mellitus (T2DM) (17).

There is evidence that plasma GIP concentrations are increased in obesity. Given that dietary fat consumption chronically stimulates the production and secretion of GIP, inducing K cell hyperplasia (8, 36), higher GIP concentrations may reflect consumption of an energy dense, high-fat diet. Early rodent studies demonstrated that a GIP infusion, during an intraduodenal lipid infusion, decreased plasma triglyceride levels (14) while GIP has been shown to enhance insulin-induced fatty acid incorporation in rat adipose tissue (9). Thus GIP, mediated through the adipocyte GIP receptor, is anabolic in adipose tissue promoting fat deposition.

It is important to distinguish between direct effects of GIP on fatty acid metabolism and indirect effects based on its insulinotropic action. Acute GIP infusion in lean healthy males (with hyperinsulinaemia and hyperglycaemia) increases adipose tissue blood flow, triacylglycerol (TAG) hydrolysis and FFA re-esterification thus promoting triglyceride deposition (5, 6). In healthy obese men, acute GIP infusion reduced expression and activity of 11β hydroxysteroid dehydrogenase type 1(11β-HSD1), a fat-specific glucocorticoid metabolism enzyme that may enhance lipolysis in subcutaneous adipose tissue (SAT) (20). In addition, it has been suggested that GIP contributes to induction of adipocyte and SAT inflammation (and thus insulin resistance), increasing production of pro-inflammatory adipokines such as monocyte chemoattractant protein-1 (MCP-1) (21), IL-6, IL-1β and osteopontin (1, 37). Thus from the available animal model and human data, GIP appears to have a key regulatory role in lipid metabolism and adipose tissue.

To date, very few studies have investigated the effects of GIP on human adipose tissue and none have involved subjects with T2DM although the reported presence of functional GIP receptors on adipocytes strongly suggests GIP modulates human adipose tissue metabolism (41). GIP has also been proposed to modulate other adipose tissue depots, and that excessive GIP secretion may underlie excessive visceral and liver fat deposition (33, 34). In support of this, results from a cross-sectional study of Danish men demonstrated an association between higher levels of GIP (during a glucose tolerance test) and a metabolically unfavourable phenotype (higher visceral: subcutaneous fat and a higher waist-hip ratio) (32).

We hypothesized that GIP would have an anabolic action in SAT promoting FFA re-esterification, which we speculated may be mediated either by enhancing lipoprotein lipase (LPL) expression/activity (a lipogenic enzyme), (15, 26) or by reducing adipose tissue

triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) expression/activity, two key lipolytic enzymes. We postulated that this effect may be different according to obesity status or glucose tolerance. Thus, we set out to determine the acute, *in-vivo* effects of intravenous GIP on i) plasma/serum insulin and NEFA concentrations, and ii) TAG content and gene expression of the key lipid regulating genes, LPL, ATGL and HSL in SAT, in obese individuals with different categories of glucose regulation (normoglycaemic, IGR and T2DM) *versus* lean, normoglycaemic controls.

Materials and methods

Subjects

We studied 23 Caucasian men, age 49 ± 12.3 years (mean ± SD). Only male subjects were studied to minimise the influence of sex steroids on lipid metabolism (e.g. considering menstrual cycle, menopause or hormone replacement therapy). Subjects with severe cardiac, renal or hepatic disease, endocrine dysfunction, major psychiatric disease, alcohol abuse, and malignancy were excluded. Subjects were sub-divided into four groups according to BMI/glucose regulation: i) lean (n=6), ii) obese (n=6), iii) obese with impaired glucose regulation [obese IGR] (n=6) and iv) obese with (treatment-naive) T2DM [obese T2DM] (n=5).

Lean and obese were defined according to a BMI ≤25 and ≥30 kg/m^2, respectively. Allocation to glucose regulation categories was based on recent medical records combined with a fasting plasma glucose concentration. Obese subjects were allocated to the obese IGR group if they had one/more of the following: fasting hyperglycaemia, impaired glucose tolerance on a 75g oral glucose tolerance test (OGTT) or HbA_1c in pre-diabetes range (6-6.5% or 42-47 mmol/mol). Obese subjects with T2DM (according to WHO diagnostic

criteria) (40), and not on pharmacological treatment for diabetes were allocated to obese T2DM group. Homeostatic model assessment (HOMA-2) was used to estimate whole body insulin resistance (23); adipose tissue insulin resistance (Adipo-IR) was calculated from fasting NEFA (mmol/L) and insulin (pmol/L) concentration (19). Baseline demographic, anthropometric and biochemical parameters of all participants are shown in Table 1.

Ethical approval Ethical approval for this project was obtained from the Northwest Research Ethics Committee, U.K (REC reference **08/H1001/20).** All subjects were studied after informed and written consent.

Study protocol

Each subject was studied on two separate occasions, 1-3 weeks apart. After overnight fasting, subjects were infused with either GIP (2 $pmol.kg.^{-1}min^{-1}$ in 0.9% saline) or placebo (0.9% saline alone). GIP was dosed based on the rate infused in previous studies (16, 35, 38) Subjects were randomly assigned to either GIP/placebo infusion on their initial visit and received the alternate infusion subsequently. Anthropometric assessments were recorded during each visit. Percentage body fat estimation was determined by whole-body bioelectrical impedance analysis (Tanita Corporation, Tokyo, Japan).

GIP infusions, hyperglycaemic clamp and blood sampling Intravenous cannulae were inserted into both antecubital fossae, for blood sampling and infusions (GIP/placebo). GIP (Polypeptide Laboratories, Strasbourg, France) was sterile-filtered and dispensed by Stockport Pharmaceuticals (Stepping Hill Hospital, Stockport, U.K). Blood glucose concentration ~8.0 mmol/l was maintained during a hyperglycaemic clamp using priming dose of 20% glucose bolus (based on weight and fasting glucose) given in the first 5 minutes followed by a variable rate infusion of 20% glucose adjusted according to whole blood

glucose levels measured every 5 minutes on a YSI blood glucose analyser (YSI U.K Ltd). Intravenous infusion of GIP/placebo was continued from 30 minutes after initiation of hyperglycaemic clamp until 240 minutes. 10 ml blood samples were taken at baseline (prior to hyperglycaemic clamp) and at 15, 30, 60, 120, 180 and 240 minutes following the initiation of GIP/placebo infusion. To minimise protein degradation, aprotinin was added to the tubes prior to sample collection. Samples were centrifuged immediately and serum was stored at -80 degree centigrade until further analysis

SAT biopsies Subcutaneous adipose tissue (SAT) biopsies were obtained at baseline and after 240 min of the GIP/placebo infusion on the contralateral site. Under local anaesthesia (1% lidocaine, adrenaline 1:200,000), a small incision was made through the skin and fascia 10cm lateral to the umbilicus. Adipose tissue samples (50-150 mg wet weight) were collected and snap frozen in liquid nitrogen and stored at -80^{o} C until further analysis.

Laboratory analysis

Biochemical analysis Plasma glucose concentration, lipid profile, liver function parameters and HbA1c were measured using a Cobas 8000 modular analyser (Roche diagnostics, USA). Blood glucose concentrations during hyperglycaemic clamp were measured using YSI 2300 STAT glucose analyser (YSI U.K Ltd, Fleet, Hampshire, U.K). Serum insulin was measured by ELISA method (Invitrogen, Fisher Scientific Ltd Loughborough, U.K). Non-Esterified Fatty Acids (NEFAs) were measured from plasma by Randox kit on a Biostat BSD 570 analyser (Randox laboratories Ltd, London). Intact GIP was measured at the University of Copenhagen, Denmark: the assay is specific for the intact N-terminus of GIP (biologically active peptide) (13).

Subcutaneous Adipose Tissue (SAT) analysis

SAT lipid content. Lysates were prepared by homogenization of fat biopsies in a buffer containing: 50mM TrisHCL pH=7.5, 150mM NaCl, 1% Triton X-100, and standard protease inhibitor cocktail (Complete Mini protease inhibitor cocktail, Roche Diagnostics, Germany). Triacylglycerol (TAG) was quantified by measuring free glycerol output following overnight lipase treatment at 37ºC (Sigma). The values were normalized according to protein content.

SAT gene expression Gene expression of LPL, ATGL and HSL were quantified through RNA extraction and real time quantitative PCR. Total RNA was isolated using RNeasy Lipid Tissue Mini Kit (QIAgen). Real-time quantitative PCR was conducted in triplicate using a BIORAD CFX-connect real time PCR instrument (BioRAD laboratories) using pre-validated TaqMan probes (Life Technologies) as follows: endogenous control β-actin (Hs99999903_m1) and target genes: lipoprotein lipase (lpl, Hs00173425_m1) ATGL (*pnpla2,* Hs00386101_m1), hormone sensitive lipase (lipe. Hs00193510_m1). Relative quantification was carried out using the ΔΔCt method with β-actin gene expression as an internal control.

Statistical analysis

Participant demographics, baseline biochemical parameters and blood glucose concentrations during hyperglycaemic clamp are expressed as mean ± SD; all other results are expressed as mean ± SEM. One-way analysis of variance (ANOVA) and Tukey's t- tests were performed to compare participant demographics and baseline biochemical parameters between the four groups in this study. Area under the curve for insulin and NEFA concentrations over 4 hour period of infusion ($AUC_{0\text{-}4hr}$) were calculated by trapezoidal rule using GraphPad Prism software. Paired t-tests were performed on changes in gene expression and lipid content (SAT-TAG) parameters to explore whether the change over the two time points differed

between GIP and placebo. P value of < 0.05 (two-tailed) was considered to be significant. A Pearson product-moment correlation coefficient was computed to assess the relationship between degree of NEFA reduction and other variables (fasting plasma glucose and Adipose tissue insulin resistance (Adipo-IR).

A linear mixed-effects model was also used to model insulin secretion and NEFA concentrations using three time points (baseline, 120 minutes and 240 minutes). Main effects for the four different groups are included along with a two-way interaction between treatment and group. This allows that the overall effect of GIP infusion in comparison to the placebo infusion can be assessed individually for different groups. Results are expressed in estimated average unit changes in insulin and NEFAs during GIP *vs.* placebo infusion.

Results

Baseline characteristics (Table 1)

Patient demographics

Twenty three individuals completed the study protocol in four sub-groups: lean (n=6), obese (n=6), obese IGR (n=6) and obese T2DM (n=5). Waist circumference and percentage body fat mass were significantly higher in obese, obese IGR, obese T2DM compared to the lean group. The duration of diabetes in obese T2DM group was 7 ± 5.5 months (mean ± SD), mean HbA1c of 54 ± 8.5 mmol/mol (7.1 ± 0.8 %) and all participants were naive to oral or injectable diabetes medications.

Baseline biochemistry

Plasma glucose and insulin concentrations

As expected, mean fasting glucose was higher in obese IGR and obese T2DM groups compared to the two other groups. Fasting insulin and HOMA-IR were significantly higher in

obese, obese IGR and obese T2DM groups *vs.* the lean group. Adipo-IR was significantly higher in obese T2DM group *vs.* lean and obese groups but not *vs*. obese IGR group (Table 1)

Metabolic parameters

All subjects in obese IGR and obese T2DM groups had metabolic syndrome based on International Diabetes Federation 2006 criteria (2) with most consequently treated for hypertension and dyslipidemia: ACE inhibitors or angiotensin receptor blockers (three subjects in obese IGR group, five subjects in obese T2DM group), beta-blockers (two obese IGR, 2 obese T2DM) and calcium channel blocker (one obese T2DM). Three subjects in each of the above two groups were on statins. Two subjects in the obese group had metabolic syndrome (one on ACE inhibitors and one a fibrate). [Table 1].

Biochemistry changes during infusions

Blood glucose. The blood glucose concentrations were maintained at ~8.0 mmol/l during the hyperglycaemic clamp with both GIP and placebo infusions in all four groups (Figure 1A-D). The whole blood glucose concentrations (mean ± SEM) from measurements at 15 minute intervals during 4 hour hyperglycaemic clamp in the four groups were: lean, 8.02 ± 0.02 (GIP) *vs*.8.17 ± 0.14 mmol/l (placebo); obese, 8.0 ± 0.07 (GIP) *vs.* 8.17 ± 0.07 mmol/l (placebo); obese IGR group, 8.08 ± 0.11 (GIP) *vs.* 8.11 ± 0.06 mmol/l (placebo) in and obese T2DM group, 8.35 ± 0.15 (GIP) *vs.* 8.46 ± 0.18 mmol/l (placebo).

The volume of 20% glucose (mean ± SEM) infused to maintain the hyperglycaemic clamp during GIP *vs*. placebo infusions in the four groups were: lean, 1124 ± 155 mls (GIP) *vs.* 631 ± 152 mls (placebo); obese, 926 ± 150 (GIP) *vs.* 462 ± 106 mls (placebo) obese IGR group, 725 ± 139 (GIP) *vs.* 398 ± 34 mmol/l (placebo) in and obese T2DM group, 508 ± 72 (GIP) *vs.* 323 ± 14 mls (placebo).

Plasma GIP Fasting plasma GIP concentrations were similar across the four groups for both visits with higher GIP concentrations achieved during GIP infusions. Plasma GIP (mean ± SEM) at baseline, 120 and 240 minutes in the four groups are as follows: lean (12.8 ± 1.1, 30.5 ± 4.6, 23.2 ± 2.6 pmol/l with GIP *vs*. 13.7 ± 2.2, 8.3 ±1.9, 9.7 ± 2.8 pmol/l with placebo, obese (15.2 ± 2.9, 38.8 ± 6.9, 21.8 ± 5.3 pmol/l with GIP *vs.* 13.0 ± 2, 15 ±3.4, 15.2 ± 5pmol/l with placebo), obese IGR (14.2 ± 3.7, 38.2 ± 7, 26.7 ± 4.7 pmol/l with GIP *vs.* 12.2 ± 2.9, 13.5 ± 2.5, 12.8 ±1.6 pmol/l with placebo), obese T2DM (14.2 ± 2, 51.6 ± 7.2, 26 ± 7.2 pmol/l with GIP *vs.* 14.4 ± 2, 23 ± 9.8, 17.8 ± 6.5 pmol/l with placebo).

Serum insulin The insulin concentrations (mean ± SEM) during GIP and placebo infusions along with hyperglycaemic clamp are shown in Figure 2 A-D. Mean $AUC_{0\text{-}4hr}$ of insulin concentrations (μIU/ml/min) was higher with GIP infusion compared to placebo in the following groups: Lean (49317 ± 6009 *vs.* 22670 ± 4361; p= 0.01), obese (71956 ± 8860 *vs.* 45921 ± 10065; p=0.1) and obese IGR groups (61884 ± 6653 *vs.* 20061 ± 3140; p=0.001) respectively. In T2DM group, the $AUC_{0\text{-}4hr}$ of insulin during GIP infusion was not different from placebo (25151 ± 4103 *vs.* 20913 ± 5514; p= 0.28) [Figure 2 E].

The change in insulin concentration over 240 minutes, compared to baseline values, differed by 63, 70 and 121 μIU/ml with GIP infusion *vs.* placebo in lean, obese and obese IGR groups respectively. In obese T2DM group, there was only a 9 μIU/ml increase in insulin concentration with GIP *vs.* placebo infusion (Figure 2F)

Plasma Non-Esterified Fatty Acids (NEFAs) Circulating NEFAs (mean ± SEM) reduced from baseline during both GIP and placebo infusions in all four groups under hyperglycaemic clamp conditions (Figure 3A-D). Mean $AUC_{0\text{-}4hr}$ for NEFAs were not different with GIP *vs.* placebo in lean and obese groups (15234 ± 1610 *vs.*15520 ± 1884; p= 0.9 in lean group and

22345 ± 4644 vs. 28770 ± 6057; p= 0.42 in obese group respectively) [Figure 3E]. NEFAs in obese IGR group appear to be lower with GIP (Figure 3C), but the mean $AUC_{0\text{-}4hr}$ (21119 ± 1882 *vs.* 32573 ± 3638; p=0.055; 95% CI 0.42 to 1.01) and reductions on a linear mixed model were not statistically significant (Figure 3 E, F). Whereas in obese T2DM group the mean $AUC_{0\text{-}4hr}$ of NEFAs (µmol/L/min) was significantly lower with GIP infusion compared to placebo (41992 ± 9843 *vs.* 71468 ± 13605; p= 0.039; 95% CI 0.31 to 0.95) and there was 82.6 µmol/L reduction in NEFAs from baseline to 240 minutes with GIP infusion compared to placebo (95% CI, -139, -26; p = 0.004) [Figure 3 E, F].

The degree of reduction in NEFA (ΔNEFA) with GIP infusion across all subjects (n=23) correlated positively with fasting plasma glucose (Pearson r = 0.44, p = 0.03) and Adipo-IR (Pearson r = 0.56, p = 0.005) (Figure 4).

Serum triacylglycerol concentration There were no significant alterations in serum triacylglycerol (TAG) concentrations with either GIP or placebo in any of the four groups (data not shown).

Subcutaneous Adipose Tissue (SAT) changes

SAT triacylglycerol (TAG) content The changes in lipid content after 240 minutes of GIP *vs.* placebo infusion relative to respective baselines on each visit are shown in Figure 5. In the obese T2DM group, the SAT-TAG content increased 1.78 ± 0.4 fold (mean ± SEM) from baseline with GIP infusion compared to 0.86 ± 0.1 fold with placebo (95% CI:0.1,1.8; p=0.043). The changes in TAG content in the other three groups were not statistically significant (data shown in Figure 5)

Gene expression of enzymes involved in lipid metabolism. The changes in mRNA expression (LPL, ATGL and HSL) in SAT after 240 minutes of GIP *vs.* placebo infusion relative to respective baselines on each visit are shown in Figure 6.

LPL, The LPL mRNA expression in the T2DM group was 1.25 fold higher from baseline with GIP infusion compared to 0.94 fold change with placebo but this was not statistically significant (p=0.27). In the other three groups the changes in LPL mRNA expression with GIP and placebo were comparable (Figure 6A).

ATGL In the T2DM group, ATGL mRNA expression was higher with GIP infusion compared to placebo (1.5 vs. 1.1 fold; p=0.12) but this was not statistically significant. In the other three groups the changes in ATGL gene expression with GIP versus placebo were comparable (Figure 6B).

HSL The changes in HSL gene expression with GIP did not differ significantly compared to placebo in all four groups (Figure 6C). Fold change data for the three enzymes in all four groups is shown in Figure 6D.

Discussion

We demonstrate that acute GIP infusion, during fasting, under hyperglycaemic conditions, reduced serum/plasma NEFAs, concomitantly increasing SAT triacylglycerol (TAG) content in obese patients with T2DM. This anabolic effect was not observed in the lean, obese or obese patients with IGR. In contrast, while GIP was able to stimulate insulin secretion in the lean, obese or obese patients with IGR, its insulinotropic action was not observed in obese patients with T2DM. Thus, in obese patients with T2DM, there is a dissociation of the effects

on GIP on beta cells and adipocytes, with blunted insulinotropic but preserved lipogenic actions respectively.

Expression of the GIP receptor (GIPR) is somehow glucose dependent and down regulated in response to hyperglycaemia (24). In patients with T2DM the blunted incretin effect (involving both incretin hormones, GLP-1 and GIP) may in part be due to reduced islet cell expression of GIP receptors (GIPR) secondary to chronic hyperglycemia (16, 29, 35, 39). The physiological role of GIP in adipose tissue in T2DM remains unclear although adipose GIPR expression may be similarly down regulated in insulin resistant human subjects and may represent a compensatory mechanism to reduce fat storage in insulin resistance, considering the interference of NEFAs on insulin signal transduction (10, 22). However, energy dense, high fat diets in obese individuals with T2DM could result in exaggerated fat storage (through exaggerated GIP release) even in the absence of adequate insulin secretion. Although we did not measure GIPR, the lipogenic action of GIP at the adipocyte appears to be more pronounced in T2DM (Figure 5). Studies in patients with NAFLD suggests elevated GIP secretion is also associated with intra-hepatocellular lipid deposition (33).

Several factors may explain the differential ability of GIP to increase NEFA re-esterification in SAT in obese T2DM subjects versus other groups. In lean, obese and obese individuals with IGR, where insulin secretion is potently stimulated and adipose tissue insulin sensitivity is preserved (lower Adipo-IR), insulin independently suppressed lipolysis, lowering NEFAs perhaps leaving GIP's effects trivial. However, in T2DM when insulin secretion is impaired and adipose tissue is insulin resistant (high Adipo-IR), the effect of GIP assumes greater importance, promoting lipid accumulation in adipocytes. This is consistent with animal data. GIP does not promote fat accumulation in adipocytes with normal insulin sensitivity, with

361 $GIPR^{-/-}$ mice showing similar adiposity to wild-type on control diet (31). However, under
362 conditions of diminished insulin action, using IRS1 deficient mice, when the effects of GIP
363 are examined (by disrupting GIP signaling, $GIP^{-/-}$ vs. $GIPR^{+/+}$) GIP was shown to promote
364 SAT and VAT expansion and decrease fat oxidation with greater SAT and VAT mass and
365 lower fat oxidation in IRS-$1^{-/-}GIPR^{-/-}$ vs. IRS-$1^{-/-}GIPR^{+/+}$ mice (42).

366

367 A few human studies examined the metabolic effect of an acute GIP infusion in lean and
368 obese individuals but none reported in people with T2DM. In studies to date, the effects of
369 GIP have been examined under different experimental conditions to those here, for example
370 during concomitant intralipid infusion and/or with hyperinsulinaemic-hyperglycaemic clamp
371 conditions and measuring arteriovenous concentrations of metabolites. These data
372 demonstrated that in lean people, GIP in combination with hyperinsulinaemia and
373 hyperglycemia, increased adipose tissue blood flow, glucose uptake, and FFA re-
374 esterification, thus resulting in increased abdominal SAT-TAG deposition (4-6). The same
375 group showed that in obese and IGR subjects GIP infusion did not have the same effect on
376 adipose tissue blood flow or TAG deposition in adipose tissue (3). However, the independent
377 contributions of insulin *vs.* GIP to these metabolic effects are difficult to dissect although GIP
378 *per se* appeared to have little effect on human subcutaneous adipose tissue in lean insulin
379 sensitive subjects, with an effect only apparent when GIP was co-administered with insulin
380 during hyperglycemia. Thus it would appear that there are direct and indirect effects of GIP.

381

382 During nutrient excess, lipogenesis is stimulated via lipoprotein lipase (LPL), hydrolysing
383 circulating lipoprotein-derived triglycerides and promoting NEFA esterification into TAG
384 and storage within lipid droplets of adipose tissue. During periods of fasting, mobilisation of
385 NEFAs from fat depots relies on the activity of key hydrolases, including hormone-sensitive

lipase (HSL) and adipose triglyceride lipase (ATGL). In SAT, insulin stimulates NEFA esterification by enhancing lipoprotein lipase (LPL), and inhibits lipolytic process (18). The majority of the animal studies have shown that GIP potentiates the role of insulin in regulation of LPL, and NEFA incorporation into adipose tissue (9, 15, 27, 31). GIP enhanced LPL gene expression in cultured subcutaneous human adipocytes through pathways involving protein kinase B and AMP-activated protein kinase (26, 28). Trying to determine the molecular mechanism by which SAT-TAG content changed, we measured SAT mRNA expression of LPL, ATGL and HSL; surprisingly, we observed no significant changes in expression to account for altered serum NEFAs or SAT-TAG content. This may represent a time-course phenomenon (changes in gene expression with GIP in human adipose tissue may occur over a longer interval). This speculation is consistent with the slow temporal onset of the molecular responses in adipose tissue in animal studies. GIP infusion may affect enzyme activity rather than gene expression and therefore results may differ if activity/phosphorylation was measured. To better appreciate the physiological effects of GIP administration on human SAT, stable isotope studies to determine dynamic changes in fat metabolism with serial tissue biopsies are required.

All studies were performed under hyperglycaemic clamp conditions to achieve comparable hyperglycaemia and to mimic post-prandial increases in GIP and insulin. The peak GIP concentrations achieved in our study during GIP infusions were comparable to levels achieved elsewhere (3). We believe the changes in NEFAs and SAT lipid content in our obese T2DM are more likely due to the effect of GIP, particularly in the absence of excess insulin secretion. Reductions in NEFA correlated positively with fasting glucose and Adipo-IR in all the subjects across the four groups suggesting the effects of GIP are more pronounced in hyperglycaemic and insulin resistant states. We recognise that higher ΔNEFA

411 would be expected in subjects with higher fasting NEFA levels however correlation with
412 Adipo-IR was only seen with GIP but not with placebo infusion (Figure 4).
413
414 Studying four distinct groups (with differing BMI and glucose tolerance) facilitates
415 evaluation of the differential effects of GIP in insulin sensitive and resistant individuals.
416 However, we acknowledge limitations including small group sizes and the degree of obesity:
417 there was limited pilot data in humans prior to initiation of this study and subsequently
418 published human studies on GIP infusion had small number of subjects (3-5). Findings from
419 our study may differ in less severely obese individuals. Lean subjects were younger
420 compared to others and may have increased insulinotropic activity to GIP (30) but there was
421 no significant difference in Insulin AUC between the groups except in obese T2DM.
422 Unrecognised interactions between anti-hypertensive or lipid modifying medication and
423 effects of GIP cannot be excluded.
424
425 In conclusion, we demonstrate that in obese patients with T2DM, acute GIP infusion in a
426 fasting state, during hyperglycaemia, lowers serum NEFA and increases the SAT lipid
427 content despite reduced insulinotropic activity. In lean, obese and obese with IGR, despite the
428 intact insulinotropic response to GIP no lipogenic effect was observed. This anabolic effect of
429 GIP further exacerbates obesity and insulin resistance.

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Disclosure summary

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Figure legends

Figure 1: Study protocol showing the duration of hyperglycaemic clamp and the time point for the start of GIP / placebo infusions. The of blood glucose concentrations (Mean ± SEM) at 15 minute intervals for the duration of hyperglycemic clamp during placebo and GIP visits are shown in **A** lean individuals, **B** obese, individuals, **C** obese individuals with IGR, **D** obese individuals with T2DM.

Figure 2: Serum insulin concentrations (mean ± SEM) during 4 hour infusions of GIP *vs.* placebo (with hyperglycaemic clamp) are shown in **A** lean individuals, **B** obese, individuals, **C** obese individuals with IGR, **D** obese individuals with T2DM. The time points for baseline blood sampling* and start of GIP/placebo infusions are shown on the X axis**. E** $AUC_{0\text{-}4hr}$ for insulin concentrations during the 4-hour infusion of GIP versus placebo for the above four groups (p values: *0.01; ** 0.001). **F** Linear mixed model analysis showing the increase in insulin concentrations with GIP compared to placebo infusion over 240 minutes, confidence intervals (CI) and p values

Figure 3: Plasma NEFA concentrations (mean ± SEM), during 4 hour infusions of GIP vs. placebo (with hyperglycaemic clamp) are shown in **A** lean individuals, **B** obese individuals, **C** obese individuals with IGR, **D** obese individuals with T2DM. The time points for baseline blood sampling* and start of GIP/placebo infusions are shown on the X axis**. E** $AUC_{0\text{-}4hr}$ for NEFA concentrations during the 4-hour infusion of GIP versus placebo for the above four groups (p values: * <0.05). **F** Linear mixed model analysis showing the decrease in NEFA concentrations with GIP compared to placebo infusion over 240 minutes, confidence intervals (CI) and p values.

Figure 4: **A, B** The correlation between plasma fasting glucose and changes in NEFA at 240 minutes from baseline (Δ NEFA $_{0\text{-}240\ min}$) during placebo and GIP infusions. **C, D** The correlation between Adipo-IR and changes in NEFA at 240 minutes from baseline (Δ NEFA $_{0\text{-}240\ min}$) during placebo and GIP infusions. Pearson's r is represented as r and p value (two tailed) with statistical significance * (<0.05) and ** (<0.01)

Figure 5: A Fold changes (mean ± SEM) in subcutaneous adipose tissue (SAT) triacylglycerol (TAG) content after 240 min GIP *vs*. placebo infusion relative to the baseline on the same day in lean individuals, obese individuals, obese individuals with IGR and obese individuals with T2DM. **B** Fold change values, confidence intervals (CI) and p values

Figure 6: Fold changes (mean ± SEM) in SAT gene expression of **A** LPL **B** ATGL and **C** HSL after 240 min of GIP *vs*. placebo infusion relative to baseline on the same day in lean individuals, obese individuals, obese individuals with IGR and obese individuals with T2DM, **D** Fold change values, confidence intervals (CI) and p values.

Figure 7: In healthy people, GIP acts on its receptors on beta cells and adipocytes to promote insulin secretion (insulinotropic action) and lipid deposition (adipogenic action) *(left figure).* In obesity, with consumption of an energy-dense, higher fat diet, there is enhanced insulin secretion (which may help overcome peripheral insulin resistance) and increased lipid deposition (which will further enhance fat storage) *(middle figure).* In T2DM, the effects of GIP on beta cell are impaired with reduced insulin secretion; the effects on the adipocyte seem to be preserved further promoting lipid deposition *(right figure).*

Table 1 Baseline demographic, anthropometric and biochemical parameters (mean ± SD).

	Lean (N=6)	**Obese (N=6)**	**Obese IGR (N=6)**	**Obese T2DM (N=5)**
Age (years)	35 ± 7	47 ± 12	57 ± 8*	57 ± 8 *
BMI (kg/m^2)	24 ± 1	40 ± 8**	37 ± 5*	45 ± 13***
Waist Circumference (cm)	94 ± 5	129 ± 19**	124 ± 14**	140 ± 17***
Body fat mass (%)	18 ± 3	38 ± 6****	31± 16****	46 ± 6****
Systolic BP (mmHg)	131 ± 15	136 ± 14	141 ± 3	135 ±12
Diastolic BP (mmHg)	78 ± 8	73 ± 5	72 ± 6	76 ± 14
Alanine transaminase (U/L)	21 ± 6	27 ± 21	30 ± 17	24 ± 11
Fasting cholesterol (mmol/l)	5.2 ± 0.7	5.0 ± 0.3	3.9 ± 0.6*	4.3 ± 1.0
HDL (mmol/L)	1.3 ± 0.3	1.1 ± 0.1	0.9 ± 0.2*	0.8 ± 0.1*
LDL (mmol/L)	3.4 ± 0.9	3.2 ± 0.5	2.5 ± 0.8	2.8 ± 0.9
Triglycerides (mmol/l)	1.1 ± 0.1	1.5 ± 0.3	1.9 ± 1.5	1.5 ± 0.5
Fasting plasma glucose (mmol/l)	5.3 ± 0.3	5.1 ± 0.9	6.0 ± 0.7	6.8 ± 1.1* $^{\Delta}$
Fasting Insulin (µIU/ml)	11.9 ±2.6	30.5 ±14.4*	38.3 ± 12.5**	36.9 ± 9.1**
Fasting NEFAs$^{\neq}$ (µmol/L)	352 ± 118	312± 123	421± 115	494 ± 150
HOMA-IR$^{¥}$	1.6 ± 0.3	3.8 ± 1.8*	4.8 ± 1.4**	4.9 ± 1.2**
Adipo-IR§ (mmol/L/pmol/L)	24.5 ± 8.1	54 ± 23.7	95.9 ±37.8**	115.7 ± 51.2**$^{\Delta}$
HbA1c (mmol/mol)	-	-	44 ± 2.3	54 ± 8.5

P value for statistically significant difference vs. Lean group is indicated as * (<0.05); ** (<0.01); *** (<0.001); **** (<0.0001) and p value for significant difference vs. obese group is indicated as $^{\Delta}$ (<0.05). $^{\neq}$ Non Esterified Fatty Acids (NEFA), $^{¥}$ Homeostasis Model Assessment-Insulin resistance (HOMA-IR), §Adipose tissue insulin resistance (Adipo-IR)

Figure 1

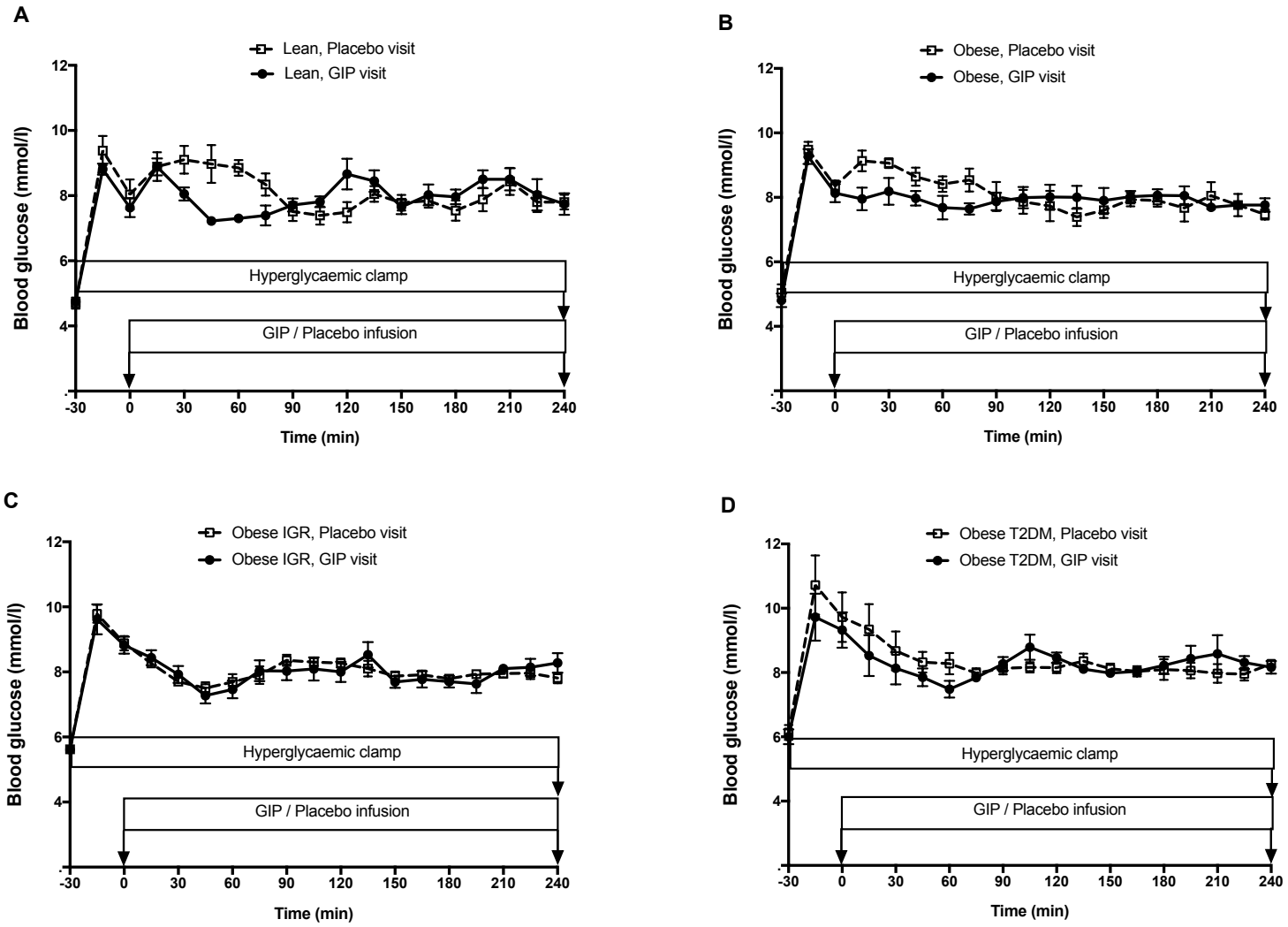
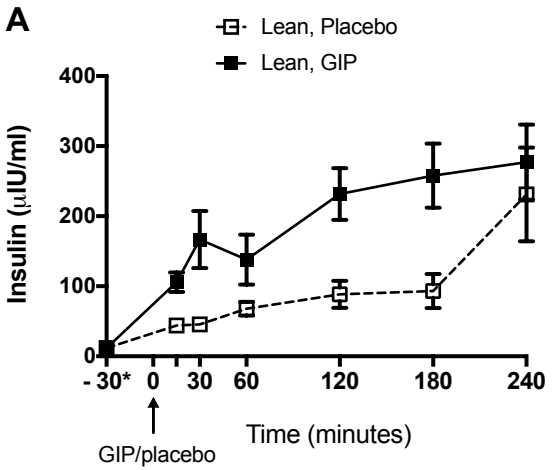
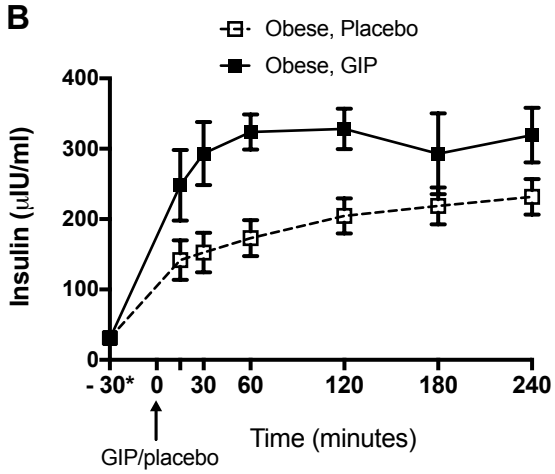
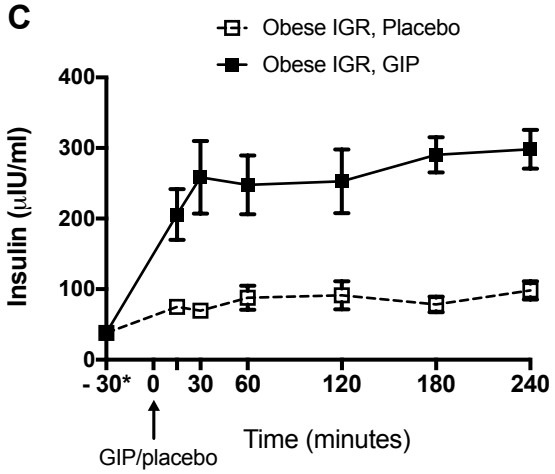
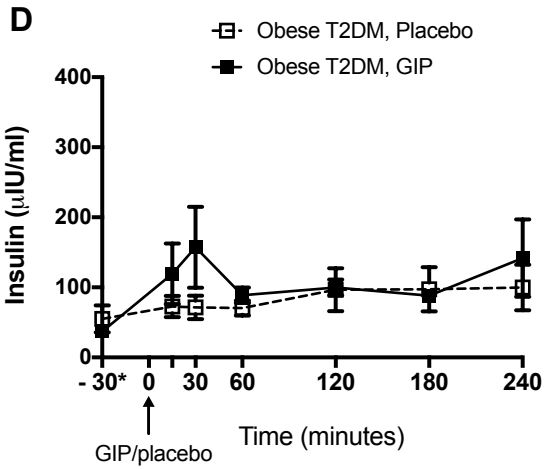

A
Lean, Placebo visit
Lean, GIP visit
Blood glucose (mmol/l)
Hyperglycaemic clamp
GIP / Placebo infusion
Time (min)
B
Obese, Placebo visit
Obese, GIP visit
Blood glucose (mmol/l)
Hyperglycaemic clamp
GIP / Placebo infusion
Time (min)
C
Obese IGR, Placebo visit
Obese IGR, GIP visit
Blood glucose (mmol/l)
Hyperglycaemic clamp
GIP / Placebo infusion
Time (min)
D
Obese T2DM, Placebo visit
Obese T2DM, GIP visit
Blood glucose (mmol/l)
Hyperglycaemic clamp
GIP / Placebo infusion
Time (min)

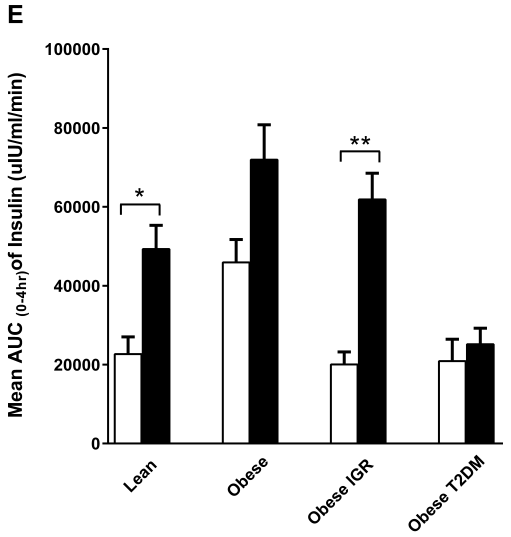
Figure 2







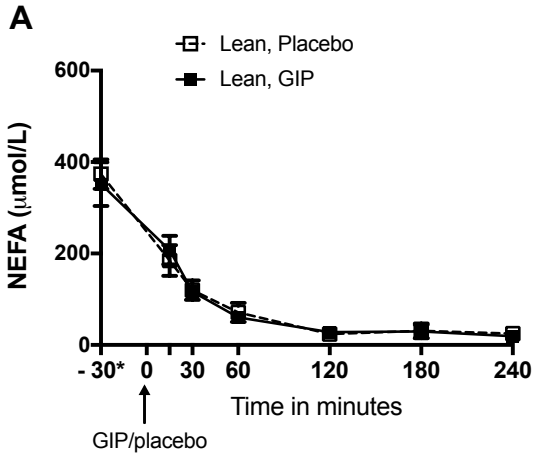


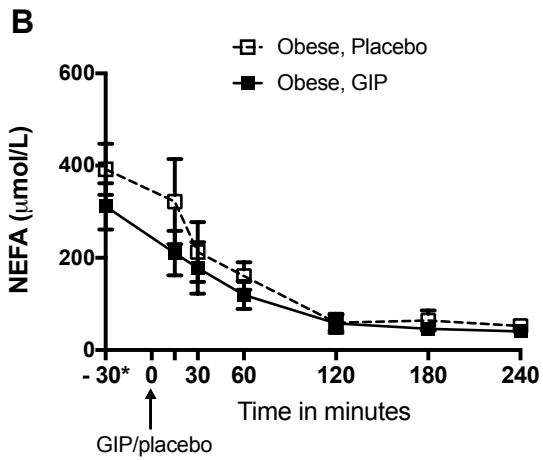


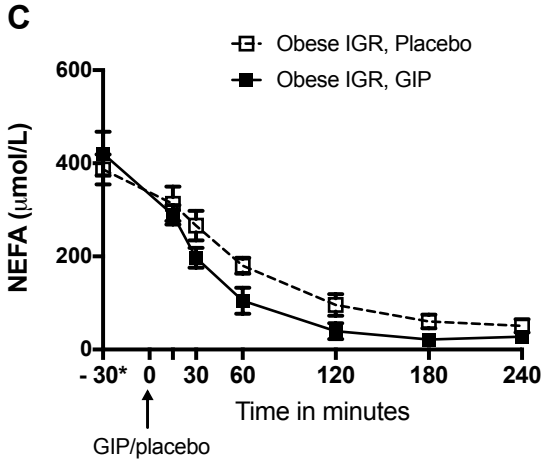
F

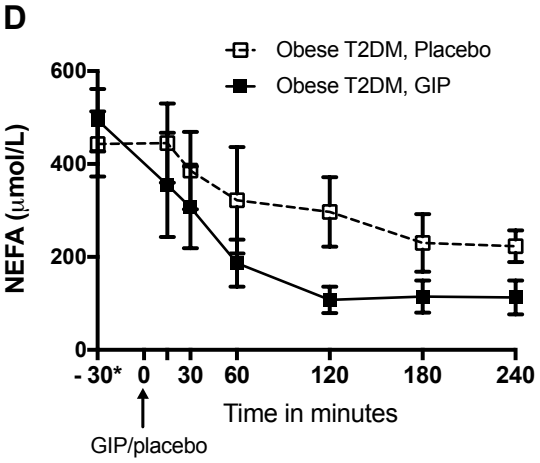
Increase in insulin concentration (μIU/ml) GIP *vs.* placebo		95% CI	p-value
Lean	63	(10, 115)	**0.019**
Obese	70	(18, 12)	**0.009**
Obese IGR	121	(68, 173)	**<0.001**
Obese T2DM	9	(- 49, 67)	0.76

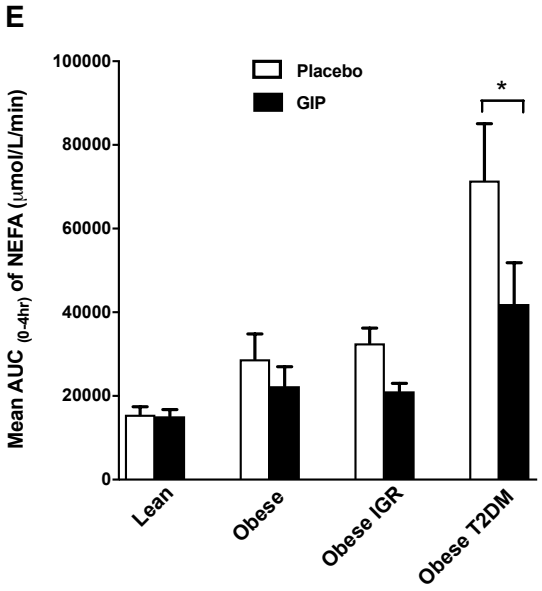
Figure 3











F

Decrease in NEFA Concentrations (μmol/l) GIP *vs.* Placebo		95% CI	p value
Lean	7.9	(-59, 44)	0.763
Obese	31.2	(-82, 20)	0.234
Obese IGR	11.4	(-63., 41)	0.668
Obese T2DM	82.6	(-139, -26)	**0.004**

Figure 4

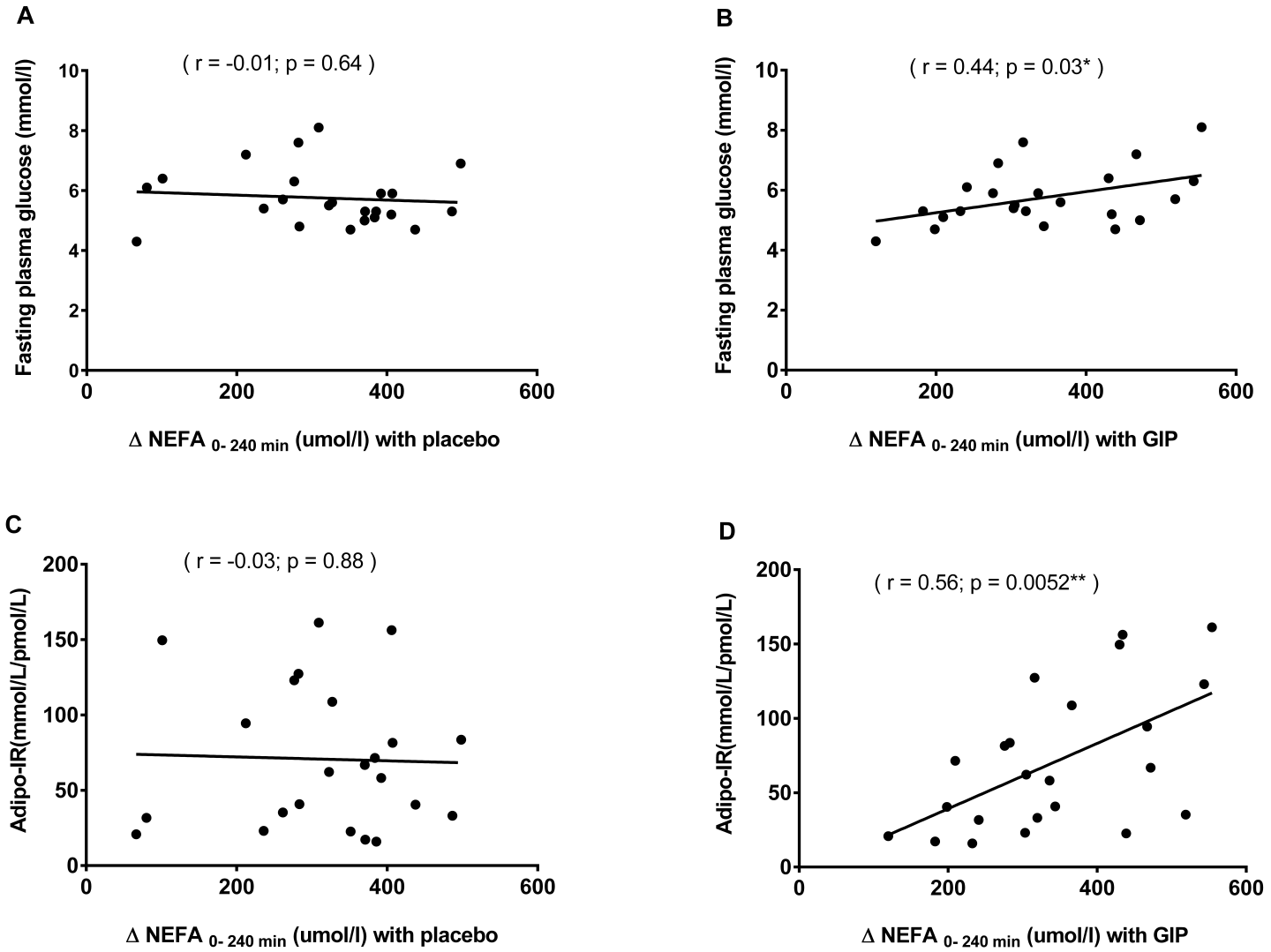
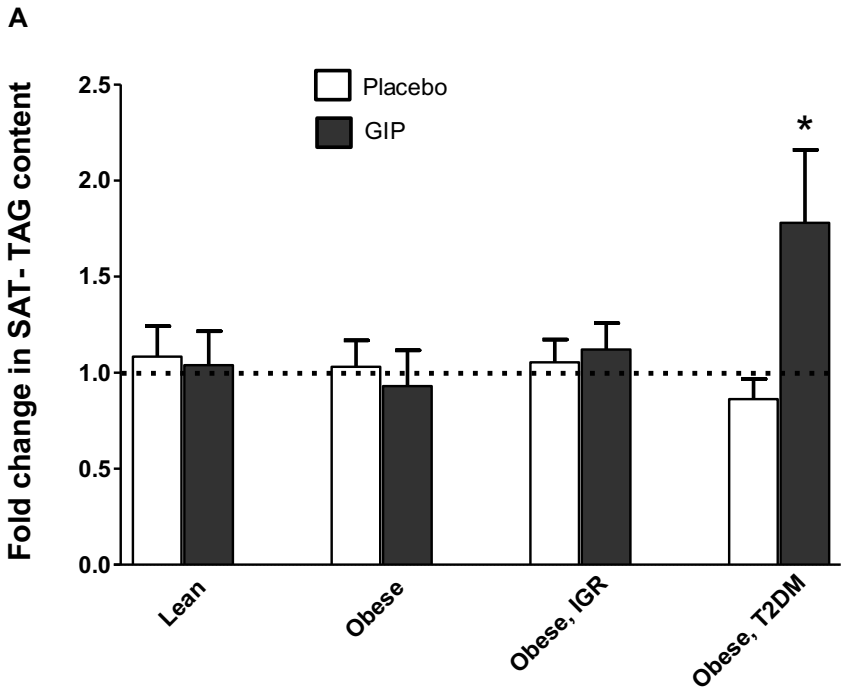

A
(r = -0.01; p = 0.64)
Fasting plasma glucose (mmol/l)
Δ NEFA 0- 240 min (umol/l) with placebo
B
(r = 0.44; p = 0.03*)
Fasting plasma glucose (mmol/l)
Δ NEFA 0- 240 min (umol/l) with GIP
C
(r = -0.03; p = 0.88)
Adipo-IR(mmol/L/pmol/L)
Δ NEFA 0- 240 min (umol/l) with placebo
D
(r = 0.56; p = 0.0052**)
Adipo-IR(mmol/L/pmol/L)
Δ NEFA 0- 240 min (umol/l) with GIP

Figure 5

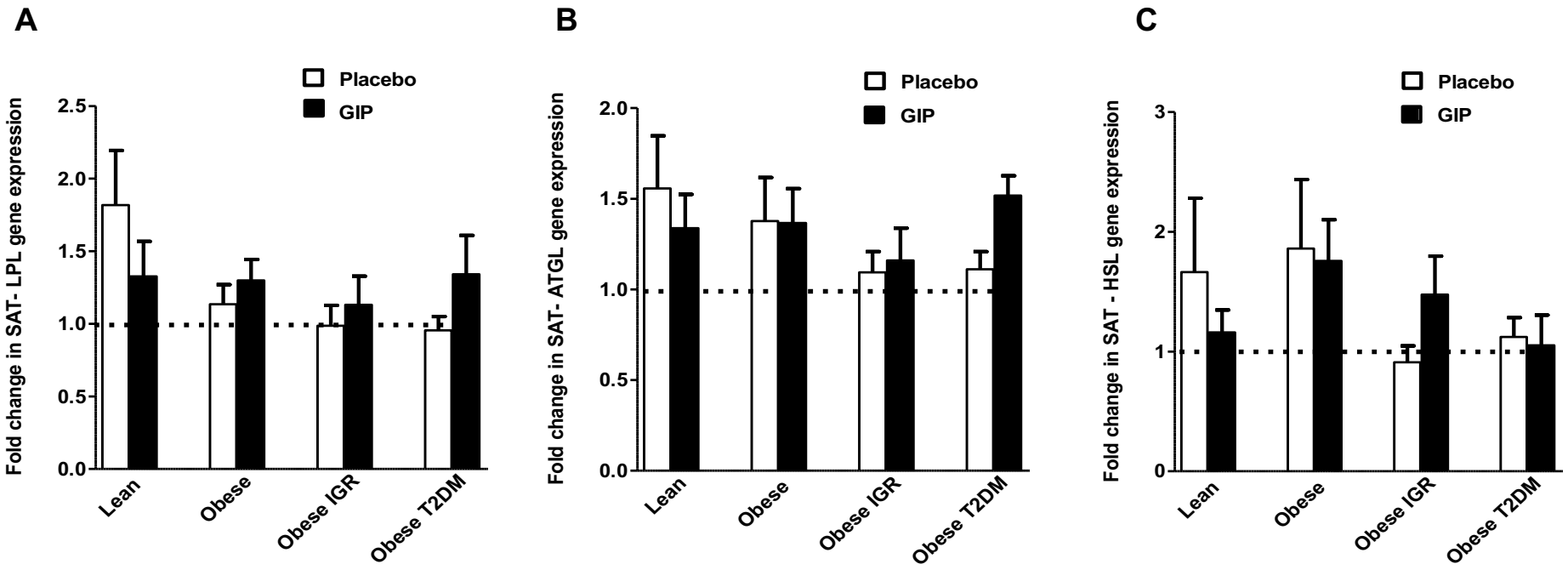
A



B

Groups	Fold change (mean ± SEM) in SAT-TAG content relative to baseline		95% CI	p-value
	Placebo	**GIP**		
Lean	1.08 ± 0.16	1.03 ± 0.18	(-0.5, 0.6)	0.84
Obese	1.03 ± 0.14	0.93 ± 0.19	(-0.43,0.62)	0.65
Obese IGR	1.05 ± 0.12	1.12 ± 0.14	(-0.56,0.4)	0.73
Obese T2DM	0.86 ± 0.1	1.78 ± 0.38	(0.1,1.8)	**0.043***

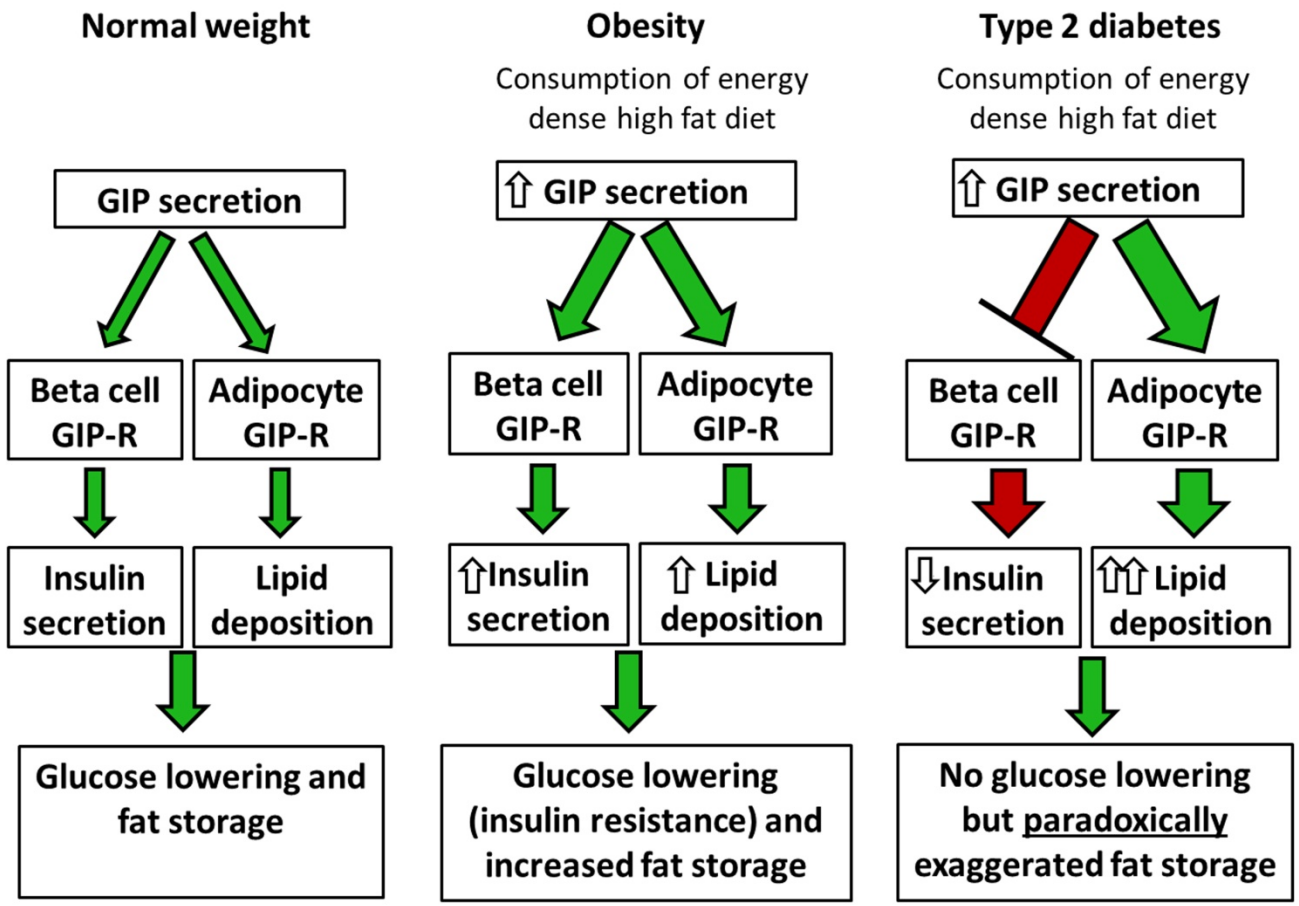
Figure 6



D

Fold change (mean ± SEM) in SAT gene expression relative to respective baselines on each visit									
	LPL			ATGL			HSL		
Groups	Placebo	GIP	P value	Placebo	GIP	P value	Placebo	GIP	P value
Lean	1.8 ± 0.4	1.2 ± 0.2	0.38	1.6 ± 0.3	1.3 ± 0.2	0.71	1.7 ± 0.6	1.2 ± 0.2	0.42
Obese	1.2 ± 0.1	1.3 ± 0.1	0.49	1.3 ± 0.2	1.3 ± 0.2	0.96	1.9 ± 0.6	1.8 ± 0.3	0.93
Obese IGR	0.9 ± 0.1	1.1 ± 0.2	0.64	1.1 ± 0.1	1.2 ± 0.2	0.90	0.9 ± 0.1	1.5 ± 0.3	0.16
Obese T2DM	0.9 ± 0.1	1.4 ± 0.2	0.27	1.1 + 0.1	1.5 ± 0.1	0.12	1.1 ± 0.2	1.0 ± 0.2	0.62

Figure 7


Normal weight
GIP secretion
Beta cell GIP-R
Adipocyte GIP-R
Insulin secretion
Lipid deposition
Glucose lowering and fat storage
Obesity
Consumption of energy dense high fat diet
⇧ GIP secretion
Beta cell GIP-R
Adipocyte GIP-R
⇧Insulin secretion
⇧ Lipid deposition
Glucose lowering (insulin resistance) and increased fat storage
Type 2 diabetes
Consumption of energy dense high fat diet
⇧ GIP secretion
Beta cell GIP-R
Adipocyte GIP-R
⇩Insulin secretion
⇧⇧ Lipid deposition
No glucose lowering but paradoxically exaggerated fat storage