

## Team Players or Opponents: Coadministration of Selective Glucagon and GLP-1 Receptor Agonists in Obese Diabetic Monkeys

Ralf Elvert,<sup>1</sup> Martin Bossart,<sup>1</sup> Andreas W. Herling,<sup>1</sup> Tilo Weiss,<sup>1</sup> Baohong Zhang,<sup>2</sup> Aimo Kannt,<sup>1,3</sup> Michael Wagner,<sup>1</sup> Torsten Haack,<sup>1</sup> Andreas Evers,<sup>1</sup> Angela Dudda,<sup>1</sup> Stefanie Keil,<sup>1</sup> Martin Lorenz,<sup>1</sup> Katrin Lorenz,<sup>1</sup> Michela Riz,<sup>1</sup> Wolfgang Hennerici,<sup>1</sup> and Philip J. Larsen<sup>1</sup>

<sup>1</sup>Sanofi-Aventis Deutschland GmbH, Industriepark Höchst, 65926 Frankfurt, German; <sup>2</sup>Sanofi Asia Pacific R&D, Shanghai 200040, China; and <sup>3</sup>Institute of Experimental and Clinical Pharmacology and Toxicology, Medical Faculty Mannheim, Heidelberg University, 68167 Mannheim, Germany

We assessed the therapeutic contribution of the individual components of glucagon-like peptide-1 receptor (GLP-1R) and glucagon receptor (GCGR) agonists alone and in combination upon energy homeostasis and glycemic control in diet-induced obese, diabetic nonhuman primates. The pharmacological active dose ranges of selective agonists were established through a dose-finding study, followed by a 6-week chronic study. Repeated subcutaneous administration of a selective GCGR agonist (30  $\mu$ g/kg once daily) did not affect food intake or body weight, whereas the selective GLP-1R agonist (3  $\mu$ g/kg once daily) alone decreased energy intake by 18% and body weight by 3.8%  $\pm$  0.9%. Combination of both agonists reduced significantly cumulative food intake by 27% and body weight by 6.6%  $\pm$  0.9%. Fasting plasma glucose (FPG) was improved by GLP-1R agonist (baseline vs end of study, 176.7  $\pm$  34.0 vs 115.9  $\pm$  16.1 mg/dL). In contrast, groups exposed to GCGR agonist experienced nonsignificant elevations of FPG. More accurate assessment of therapeutic interventions on glucose homeostasis was tested by an IV glucose tolerance test. Glucose excursion was significantly elevated by chronic GCGR agonist administration, whereas it was significantly decreased in GLP-1R agonist-treated monkeys. In the combination group, a nonsignificant increase of glucose excursion was seen, concomitantly with significantly increased insulin secretion. We conclude that chronic glucagon agonism does not affect energy homeostasis in nonhuman primates. In combination with GLP-1R agonism, glucagon agonism synergistically enhances negative energy balance with resulting larger body weight loss. However, adding GCGR to GLP-1R agonism diminishes glycemic control in diabetic monkeys. Therefore, long-term therapeutic implications of using GLP-1R/GCGR coagonists for weight management in diabetes warrants further scrutiny. (*Endocrinology* 159: 3105–3119, 2018)

**B**ariatric surgery is an effective therapeutic intervention for weight management of patients with obesity, as well as a tentative cure of type 2 diabetes mellitus. In people with type 2 diabetes mellitus, Roux-en-Y gastric bypass surgery (RYGB) leads to complete resolution of diabetes in ~80% of cases (1, 2). For this

reason, pharmacotherapies mimicking the effects of bariatric surgery on body weight homeostasis are currently pursued as a relevant and well-tolerated alternative to effective weight management.

Bariatric surgery decreases body weight through mechanisms beyond mechanical restriction. After either

ISSN Online 1945-7170

Copyright © 2018 Endocrine Society

Received 26 April 2018. Accepted 3 July 2018.

First Published Online 9 July 2018

Abbreviations: AUC, area under the curve; DIO, diet-induced obese; DPP-IV, dipeptidyl peptidase-IV; FGF21, fibroblast growth factor 21; FPG, fasting plasma glucose; FPI, fasting plasma insulin; G6Pase, glucose-6-phosphatase; GCGR, glucagon receptor; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IVGTT, IV glucose tolerance test; KBI, Kunming Biomed International; NEFA, nonesterified fatty acid; PCK1, phosphoenolpyruvate carboxykinase 1; PK, pharmacokinetic; qd, once daily; RYGB, Roux-en-Y gastric bypass surgery; s.c., subcutaneous; TEI, total energy intake; t-ketones, total ketones.

RYGB or sleeve gastrectomy, nutrients more readily access the upper intestine after ingestion, leading to exaggerated postprandial secretion of gut hormones (3, 4). Rapid resolution of type 2 diabetes mellitus in subjects undergoing RYGB is mediated by an early postoperative improvement of hepatic glucose clearance and a far slower increase in peripheral glucose uptake (5, 6). Although postprandial plasma glucose shows earlier peak levels, 2-hour glucose tolerance is dramatically improved after RYGB surgery as a result of improved  $\beta$  cell glucose responsiveness and hepatic glucose uptake (7, 8). The acute improvements of glycemia after RYGB are predominantly due to surgery-induced exaggeration of postprandial glucagon-like peptide-1 (GLP-1) secretion (9–11).

However, the weight loss experienced by patients undergoing RYGB is not due solely to these postprandial changes in plasma GLP-1 levels. Several endocrine factors released from enteroendocrine L cells display a changed postprandial release pattern after RYGB. Oxyntomodulin is of particular interest, as its prandial release is elevated >10-fold as a result of RYGB surgery (12). It is an endogenous dual GLP-1 and glucagon receptor (GCGR) agonist whereas each single component displays oppositely directed effects on glucose homeostasis yet exhibits synergism on energy homeostasis (13, 14). Early clinical experience with oxyntomodulin provided convincing evidence that thrice daily subcutaneous (s.c.) administration through 4 weeks provides robust weight loss to obese otherwise healthy subjects (15, 16).

Oxyntomodulin has poor pharmacological properties, as it is subject to both proteolytic cleavage by dipeptidyl-peptidase-4 and fast renal clearance (17). Therefore, different strategies aiming at inventing stable oxyntomodulin mimetics have been pursued during the past decade. Common for these many approaches has been to design peptides with agonistic properties on GLP-1 receptors (GLP-1Rs) as well as GCGRs (so-called GLP-1R/GCGR dual agonists). They are structurally derived from oxyntomodulin (14, 18), glucagon (19, 20), or exendin-4 (21).

In mice, chronic administration of GLP-1R/GCGR dual agonists reduces body weight and glycemia with higher efficacy than seen for selective GLP-1 agonists (22, 23). Interestingly, when selective GLP-1 and glucagon agonists are coadministered to diet-induced obese (DIO) mice they synergistically reduce body weight (24, 25), suggesting that GLP-1R/GCGR dual agonists will mimic oxyntomodulin as a superior weight loss agent vs GLP-1R agonist. In mice, glucagon administration with or without concomitant GLP-1 agonism also elevates energy expenditure (22). Similarly, short-lasting coinfusion of subanorectic doses of GLP-1 and glucagon to healthy overweight volunteers significantly reduces food intake

(26), whereas the glucagon exerted effects on energy expenditure do not always translate from mice to humans (27, 28).

Several of the GLP-1R/GCGR dual agonist activities have been taken into clinical development, but without including strategies to demonstrate distinct contribution of the individual pharmacologies on the energy homeostatic end points in a clinically relevant population. Furthermore, none of the published nonhuman primate data with dual GLP-1R/GCGR coagonists could conclude that the GCGR systems were activated at the explored dose range (23). Therefore, we chose obese diabetic nonhuman monkeys as a translationally relevant preclinical model to assess metabolic effects of GLP-1 and glucagon separately as well as in combination at relevant pharmacological exposures.

## Methods

### DIO monkeys (animals and housing conditions)

The monkey study was performed at Kunming Biomed International (KBI), located in Yunnan Province, China. KBI adhered to the *Guide for the Care and Use of Laboratory Animals* (29), guidelines for the care and use of animals for scientific purposes established by the Chinese National Advisory Committee for Laboratory Animal Research, and safety and quality assurance guidelines documented in the Guideline for Experiments Document of Kunming Biomed International (KBI-01-GE v2.0). This study was approved by the Institutional Animal Care and Use Committee of KBI.

Cynomolgus monkey (*Macaca fascicularis*) was selected as the test species of choice for more meaningful results that were translatable to humans. More than 50 monkeys were trained to identify 10 monkeys for the dose-finding study and 32 monkeys for the repeat-dose chronic study. They all fulfilled the following metabolic criteria: weight at least 8 to 16 kg and a body fat content of >25%, age 12 to 20 years, fasting glucose >110 mg/dL, and fasting insulin >70  $\mu$ U/mL (in comparison lean monkeys of same age range: weight 5 to 8 kg, fasting glucose  $\sim$ 70 mg/dL, and fasting insulin  $\sim$ 25  $\mu$ U/mL). The monkeys were individually housed in species- and size-appropriate metabolic stainless steel caging with *ad libitum* access to water and under controlled environmental conditions with room temperature of 18°C to 29°C, relative humidity of 30% to 70%, and a minimum of 10 air changes per hour. A time-controlled lighting system provided a regular 12-hour light/12-hour dark diurnal cycle. Cages were cleaned at regular intervals. The monkeys had three meals per day with a daily energy intake of  $\sim$ 680 kcal ( $\sim$ 2.85 MJ). All food was withdrawn at 5:00 PM so that monkeys were fasted overnight. Monkeys were provided with enrichment toys or devices at all times. Animals were randomized by block stratification into three homogeneous groups according body weight, fasted plasma glucose, and %HbA<sub>1c</sub>.

The three daily meals consisted of  $\sim$ 50 g of standard monkey formula feed [extruded pellets, 3.1 kcal/g (12.98 kJ/g): protein 24%, fat 15%, carbohydrate 61%] in the morning (9:00 AM to 10:00 AM), one apple [150 g,  $\sim$ 80 kcal (33 kJ)] in the afternoon (2:00 PM to 3:00 PM), and 100 g of KBI proprietary

high-fat diet feed in the evening [extruded pellets, 3.47 kcal/g (14.5 kJ/g): protein 14%, fat (porcine) 34%, carbohydrate 52%, sucrose 35%, 4:00 PM to 5:00 PM]. The total offered amount of daily energy was ~680 kcal/d (2.85 MJ/d). All of the food was withdrawn at 5:00 PM; hence, all monkeys were always fasted overnight. After each feeding time period, all the remaining food was withdrawn and intake was determined by weighing the leftover food. Each batch of monkey chow was delivered with an accompanying certificate of analysis detailing nutritional composition and levels of specified contaminants (e.g., heavy metals, aflatoxin, and insecticides). *Ad libitum* access to water through the main system was suspended on days in which water intake was quantified.

### Preparation of the selective receptor agonists

Both the GLP-1R and GCGR selective agonists were prepared via Fmoc solid-phase synthesis, purified by reverse-phase high-performance liquid chromatography using water/acetonitrile (0.1% trifluoroacetic acid) gradients and transformed into their acetate salts. The purified peptides were characterized by electrospray mass spectrometry.

The rational design of novel peptides with dual activity on the GLP-1 and GCGRs structurally derived from exendin-4 has been described previously (20, 22, 25). Following similar design criteria, structural variants of exendin-4, carrying an unnatural amino acid in position 2 to block dipeptidyl peptidase-IV (DPP-IV) cleavage, and a fatty acid side chain in position 14 to extend half-life via albumin binding, which showed selective agonism either at the GLP-1R or the GCGR, were obtained (see Fig. 1).

The activity of the compounds at the GLP-1R and GCGR was determined using functional assays that measure cAMP response in HEK293 cell lines stably expressing mouse or monkey GLP-1R or GCGR (Table 1). The cAMP content in the cells was determined using a kit (Cisbio Corp.; catalog no. 62 AM4PEJ) following the manufacturer's instructions.

### Dose-finding study

The purpose of the dose-finding study was to identify the pharmacologically active dose range of selective GCGR and GLP-1R agonists to be used for the subsequent repeat-dose chronic study. Fifteen obese diabetic monkeys (*M. fascicularis*) were selected with >8 kg of body weight and >8 years of age and screened for their metabolic profiles. Monkeys were trained for 4 weeks to habituate to blood draw procedure and for presentation of the subscapular/cervical region for s.c. injection of test substances with minimal physical restraint. Ten monkeys (n = 5 per group) were selected for the 2-week dosing period, which was performed in dose-escalation mode to find the maximal tolerated dose. Dosing (s.c. injection) was performed between 10:30 and 11:00 AM, following breakfast. The GCGR

agonist-treated group started with vehicle treatment from day 1 to 3, 3 µg/kg from day 4 to 6, 10 µg/kg from day 7 to 9, 30 µg/kg from day 10 to 12, and 100 µg/kg from day 13 to 15. The same dosing pattern was followed for the GLP-1R agonist. GLP-1R agonist dosing in monkeys was increased from vehicle to 0.3 µg/kg to 1 µg/kg to 3 µg/kg and to a maximal dose of 10 µg/kg. All monkeys were continuously monitored until end of a 2-week washout period. Daily food intake measurement was conducted and total energy intake (TEI) was calculated for the entire duration of the study. Body weight measurements were performed and recorded once weekly during the training and washout periods and every 3 days during the dosing period. On the first day of each dose step for the GCGR agonist and on the third day of each dose step for the GLP-1R agonist, a glucose profile was measured with blood sampling time points of *t* = 0 (predose) and 1, 2, 3, 4, 6, 8, and 24 hours postdose. The glucose profiling was performed under *ad libitum* fed conditions and was shifted to day 3 for the GLP-1R agonist to avoid confounding effects of the instantaneous and acute food-suppressing impact of GLP-1R agonism.

### Chronic dosing study

Pharmacologically active doses were then investigated in a repeat-dose chronic study. A metabolic profile of >50 monkeys of >8 kg of body weight, >8 years of age, fasting glucose of >110 mg/dL, and fasting insulin of >70 µU/mL was performed. There was a run-in period during which monkeys were injected subcutaneously with vehicle once daily (qd). Food intake (calculated as TEI) and water intake were measured daily along with twice-weekly body weight evaluation and baseline value determination for metabolic biomarkers, safety biomarkers, IV glucose tolerance test (IVGTT), liver biopsy for gene expression analysis, and profile for glucose, insulin, fibroblast growth factor 21 (FGF21), and ketone bodies. Thirty-two trained monkeys (n = 8 per group) were selected for the chronic dosing period and stratified for body weight, fasting plasma glucose (FPG), and fasting plasma insulin (FPI) and insulin response from baseline during the IVGTT.

All treatments were administered subcutaneously qd for 6 weeks (4 weeks evaluation plus 2 weeks run-out). The dosing period was designed as a four-arm, dose-ramping study, and monkeys were treated with either vehicle, the selective GCGR agonist, the selective GLP-1R agonist, or a combination of both GLP-1R and GCGR agonists. The initial dose was administered daily from days 1 to 3; then the dose was increased on days 4 to 6, with a further increase to the maintenance dose from day 7 to the run-out period. The selective GCGR agonist was initially administered at 3 µg/kg, then 10 µg/kg, with a maintenance dose of 30 µg/kg. The GLP-1R agonist was increased from 0.3 µg/kg to 1 µg/kg to the maintenance dose of 3 µg/kg. The

No	1	5	10	15	20	25	30	35																																		
glucagon	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T													
exendin-4	H	G	E	G	T	F	T	S	D	L	S	K	Q	M	E	E	E	A	V	R	L	F	I	E	W	L	K	N	G	G	P	S	S	G	A	P	P	P	S	NH2		
GLP-1R agonist	H	s	E	G	T	F	T	S	D	V	S	K	Q	K(yGlu-C16)	E	K	R	A	A	Aib	E	F	I	E	W	L	K	N	T	G	P	S	S	G	A	P	P	P	S	NH2		
GCGR agonist	T	z	a	s	Q	G	T	F	T	S	D	Y	S	K	Q	K(yGlu-C16)	E	S	R	R	A	Q	E	F	I	E	W	L	L	A	G	G	P	E	S	G	A	P	P	P	S	NH2

**Figure 1.** Amino acid sequences of glucagon, exendin-4, and the selective GLP-1R and GCGR agonists. Amino acids that are identical between glucagon and exendin-4 are colored green, residues unique to glucagon are shown in yellow, and residues unique to exendin-4 are colored gray. Additional modifications that have been introduced to enhance selectivity or metabolic stability are shown in orange. Residue 14 in both GLP-1R and GCGR agonists is modified by addition of a C16 fatty acid (palmitic acid) at the ε-amino group of lysine using a γ-glutamic acid spacer.

**Table 1. *In Vitro* Receptor Agonist Potencies (cAMP Production) in HEK-293 Cell Lines Stably Expressing Mouse or Monkey GLP-1 or Glucagon Receptor**

Peptide	Mouse Receptors		Monkey Receptors	
	GLP-1R [EC <sub>50</sub> (pM)]	GCGR [EC <sub>50</sub> (pM)]	GLP-1R [EC <sub>50</sub> (pM)]	GCGR [EC <sub>50</sub> (pM)]
Exendin-4	0.4	>10 <sup>8</sup>	0.6	>10 <sup>8</sup>
Glucagon	43.9	0.4	30.8	1.5
GLP-1R agonist	1.0	54,600	0.6	>10 <sup>8</sup>
GCGR agonist	396	1.3	484	3.4

combination treatment was administered as two separate injections of the GCGR and GLP-1R agonist: 3 and 0.3 µg/kg, followed by 10 and 1 µg/kg, and then increased to 30 and 3 µg/kg. TEI and water intake were monitored daily and body weight was measured every 3 to 4 days. Metabolic profiling for glucose and insulin were performed during the dosing period on days 10 and 42, whereas IVGTT was performed on day 31 of the chronic treatment.

After the dosing period, monkeys followed a 2-week run-out period with continued s.c. treatment of vehicle or test articles (administered at the maintenance dose). The run-out period was performed to investigate treatment-related impact on glucose tolerance, body composition, and liver-related gene expression without taking TEI and body weight development into account. However, TEI and water intake were measured daily and body weight twice weekly, and an IVGTT and liver biopsy were performed. A dual energy x-ray absorptiometry scan (Hologic Discovery QDR Series; Discovery Wi; Hologic, Bedford, MA) was conducted twice during the study—once during the run-in period (day –19/–18) and once during the run-out period (day +31). Plasma profiles for glucose, insulin, total ketone bodies, and FGF21 levels were measured on three occasions: day –11 (baseline), day +10 (end of dose ramping), and on day +28 (end of evaluation period). Blood samples for pharmacokinetic (PK) analysis were collected on day +28 directly before and 1, 2, 5, 8, and 22 hours after dosing.

### Liver biopsy for target gene expression for glucagon-related genes

Liver biopsy was performed from all animals under anesthesia (ketamine, 5 to 10 mg/kg IM). To avoid any impact on TEI and body weight development, the biopsy was determined once during the run-in period and during the run-out period at least 1 week after the IVGTT. Two to three liver tissue samples (at ~0.5 to 1.0 cm per sample) were obtained. Hepatic gene expression of glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase 1 (PCK1), and FGF21 were studied to test validity and feasibility of biomarkers for GCGR target engagement. The tissue punches were immediately transferred into pre-labeled 1.5-mL RNAase-free tubes containing RNAlater solution (Merck Life Sciences Co., Ltd, Shanghai, China). The tissues in RNAlater were kept at 2°C to 8°C overnight and all remaining RNAlater solution was carefully removed from the tube and tissues were stored at –80°C. Gene expression was determined by relative quantitation using the 2<sup>–ΔΔCT</sup> method to compare the fold change of treatment compared with baseline. It measures the cycle threshold difference between target gene and two housekeeping genes (GAPDH and RPL37A) and compares the cycle threshold difference values of treatment

samples to baseline samples to calculate 2<sup>–ΔΔCT</sup>. The study was finalized with a 4-week washout period with continued and close monitoring of food intake, body weight, and metabolic profiling during the recovery phase.

### IVGTT

On IVGTT treatment days (day –19/–18, baseline; and day +31, run-out) all dosing commenced 2.5 hours before IV glucose bolus, that is, IVGTT time 0 hours. A 50% glucose solution was administered (1.0 mL of solution per kg of body weight) during 30 seconds, and seven serial blood samples (1.0 mL each) were collected at 1, 3, 5, 10, 20, 40, and 60 minutes from the end of the IV glucose bolus.

### Analytical procedure for blood samples

Whole-blood samples were collected from a peripheral vein after an overnight fast according to the specified time points indicated above and transferred directly into potassium EDTA tubes. Plasma was separated by centrifugation at 2500g for 10 minutes at 4°C within 30 minutes of collection and apportioned into at least three aliquots. Two plasma aliquots were retained for glucose, insulin, total ketones (t-ketones), HO-butyrates, FFA, and FGF21 analyses. Glucose, HO-butyrates, and t-ketones were analyzed using the Roche C311 or C501 biochemical analyzer, insulin was analyzed by a Cobas e 411 immunology analyzer, and FGF21 (200 µL of plasma) was analyzed by ELISA.

### Statistical analysis

All data are presented as mean ± SEM. Depending on homogeneity of variances, analysis was performed either on raw data for homogeneous variances or on rank-transformed values in the case of heterogeneous variances. Statistical significance was considered as *P* < 0.05. A one-way ANOVA for factor treatment followed by a Dunnett multiple comparison test vs vehicle was used for the parameters change in fat mass, change in HbA<sub>1c</sub>, 2<sup>–ΔΔCT</sup> values of gene expression parameters, and body weight loss for each specific day of the study. A two-way ANOVA with repeated measures on one factor, followed by a Dunnett test vs vehicle, was used for the parameters food consumption and rank-transformed FGF21 for specific days of the study. A two-way ANOVA with repeated measures on one factor, followed by a Dunnett test vs baseline values, was applied for the parameters body weight, rank-transformed FPG, rank-transformed area under the curve (AUC) for glucose and AUC for insulin. Multiple two-way analysis for each specific treatment with repeated measures on two factors followed by a Winer analysis for factor study day were used for the parameters rank-transformed glucose

profile, insulin profile, and ketone bodies profile of the study in obese monkeys. For the dose-finding study in obese monkeys, only a descriptive statistical analysis was used. All analyses were performed using SAS (version 9.2) under HP-UX via internal interface software EverStat v6.0.12.

## Results

### Dose-finding study

The pharmacologically active doses of the GLP-1R and GCGR agonists were determined in a dose-escalation paradigm where dose was increased every 3 days with a focus on TEI and blood glucose levels. For the GCGR agonist-treated group, no clinical signs were observed. In the GLP-1R agonist-treated group, one monkey showed persistently poor food intake after dosing at the 10  $\mu\text{g}/\text{kg}$  (qd) dose during the washout period. This monkey became hypoglycemic and was therefore gavaged with blended food on day 18. Thereafter, food consumption recovered and normalized. Another monkey was observed to have watery stool in the morning of day 18 during the washout period, but spontaneously recovered without any treatment. No other abnormal clinical signs were noted in any animals during the entire observation period.

No statistically significant effect on TEI was observed in monkeys administered escalating doses of the selective GCGR agonist (3  $\mu\text{g}/\text{kg}$   $\rightarrow$  10  $\mu\text{g}/\text{kg}$   $\rightarrow$  30  $\mu\text{g}/\text{kg}$   $\rightarrow$  100  $\mu\text{g}/\text{kg}$  qd; Supplemental Fig. 1). In contrast, TEI was significantly reduced in monkeys treated with escalating doses of the selective GLP-1R agonist (0.3  $\mu\text{g}/\text{kg}$   $\rightarrow$  1  $\mu\text{g}/\text{kg}$   $\rightarrow$  3  $\mu\text{g}/\text{kg}$   $\rightarrow$  10  $\mu\text{g}/\text{kg}$ ), with TEI reduced by 47.7%  $\pm$  13.4% (mean  $\pm$  SEM) at 3  $\mu\text{g}/\text{kg}$  and 80.7%  $\pm$  6.8% at 10  $\mu\text{g}/\text{kg}$  dose levels. This high reduction of TEI excluded the highest dose of GLP-1R agonist for the chronic dosing.

Glucose profiling data revealed a dose-dependent increase from baseline in the selective GCGR agonist-treated monkeys at the two highest dose steps (30  $\mu\text{g}/\text{kg}$  and 100  $\mu\text{g}/\text{kg}$  qd; Supplemental Fig. 2). At the 30  $\mu\text{g}/\text{kg}$  dose, blood glucose increased (38.9%  $\pm$  6.5%) within 1 hour after treatment and normalized during the following hours. At the 100  $\mu\text{g}/\text{kg}$  dose, blood glucose increased (101.2%  $\pm$  17.6%) within 2 hours of treatment and stayed elevated (73.7%  $\pm$  19.4%) for 8 hours after treatment. This strong increase of blood glucose excluded the highest dose of GCGR agonist for chronic treatment.

Treatment with the GLP-1R agonist decreased plasma glucose levels in a dose-dependent manner with highest reduction observed at the 3  $\mu\text{g}/\text{kg}$  (-30.4%  $\pm$  5.0% within 2 hours) and 10  $\mu\text{g}/\text{kg}$  (-26.1%  $\pm$  6.4%) dose levels. Mean baseline FPG values were >100 mg/dL in both groups. No significant differences in FPG were observed in the GCGR agonist-treated monkeys (not

shown). In contrast, significant reduction in FPG compared with baseline was seen in GLP-1R agonist-treated monkeys at the highest two dose levels (3 and 10  $\mu\text{g}/\text{kg}$  qd). Mean glucose values were significantly lower on day 13 (118.8  $\pm$  13.5 mg/dL,  $P < 0.05$ ) after GLP-1R agonist treatment at 3  $\mu\text{g}/\text{kg}$  and on day 14 (95.8  $\pm$  7.5 mg/dL,  $P < 0.05$ ) after treatment at 10  $\mu\text{g}/\text{kg}$  compared with the baseline values (183.8  $\pm$  30.9 mg/dL).

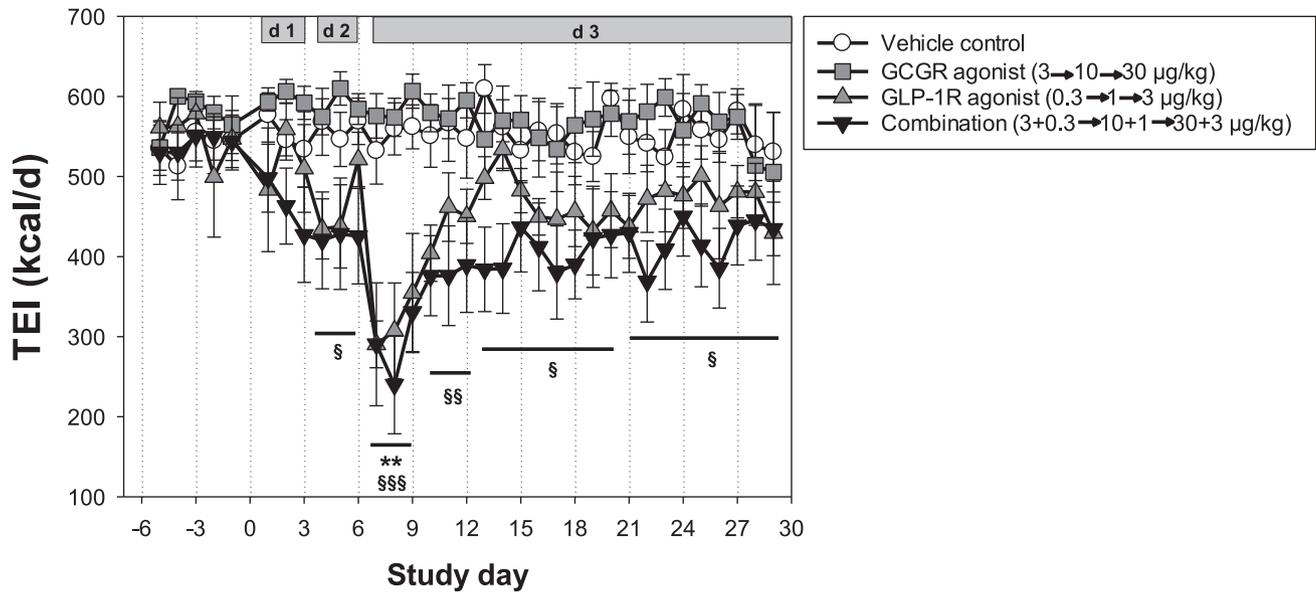
From the results of the dose-finding prestudy and observed clinical findings during the washout period, GCGR and GLP-1R agonists at dose levels of 30 and 3  $\mu\text{g}/\text{kg}$ , respectively, were selected for the subsequent repeat-dosing chronic study.

### Chronic study

The major indicator for tolerability during the multiple dose efficacy study in cynomolgus monkeys was the impact on TEI and accompanying body weight loss. Data from three monkeys that were prematurely terminated from the study were excluded [one from each group: vehicle, GCGR (predosing period), and GLP-1R].

Monkeys in the vehicle group exhibited TEI ranging from 500 to 600 kcal/d (2.09 to 2.51 MJ) throughout the study period. Increasing the dose for the GCGR agonist from 3 to 10  $\mu\text{g}/\text{kg}$  to the maintenance dose of 30  $\mu\text{g}/\text{kg}$  (s.c., qd) did not affect TEI compared with the vehicle controls (Fig. 2). The selective GLP-1R agonist significantly reduced TEI acutely during the last dose step on day +7 to day +9 from 1  $\mu\text{g}/\text{kg}$  to the maintenance dose of 3  $\mu\text{g}/\text{kg}$  ( $P < 0.001$ ) and tended to attenuate TEI during progression of the study. However, cumulative energy intake revealed an overall reduction to 13,196  $\pm$  911 kcal (55.2  $\pm$  3.8 MJ), which corresponds to 18% less energy intake compared with vehicle controls. Coadministration of 10  $\mu\text{g}/\text{kg}$  GCGR agonist and 1  $\mu\text{g}/\text{kg}$  GLP-1R agonist produced a significant reduction in TEI [420.2  $\pm$  60.6 kcal/d (1.76  $\pm$  0.25 MJ); day +4 to day +6,  $P < 0.05$ ]. This effect was amplified by the maintenance dose and TEI decreased to 239.9  $\pm$  61.2 kcal/d (1.00  $\pm$  0.26 MJ) on day +8 ( $P < 0.0001$ ). The impact of the maintenance combination doses of GLP-1 and GCGR agonists on TEI diminished only slightly during the progression of study period, showing significant differences vs vehicle controls in dosing periods day +10 to 12 ( $P < 0.01$ ), day +13 to 20 ( $P < 0.05$ ), and day +21 to 29 ( $P < 0.05$ ) and a reduction of cumulative intake to 11,680  $\pm$  1,184 kcal (48.9  $\pm$  5.0 MJ), which corresponds to a reduction of -27%. Hence, combination of both agonists showed persistent reduction of TEI throughout the study.

The body weight in vehicle-treated monkeys was stable during the study (Table 2; Fig. 3). GCGR agonist treatment did not affect body weight in the obese



**Figure 2.** TEI of obese and diabetic *M. fascicularis* (n = 7 to 8 per group) during the chronic, repeat-dose study. To facilitate chronic treatment with the maximum tolerated dose of GLP-1R agonist (3 µg/kg s.c., qd) and GCGR agonist (30 µg/kg s.c., qd), dose ramping was conducted every third day in three dose steps (d1, day +1 to +3; d2, day +4 to +6; and d3, day +7 to run-out period). Values are mean ± SEM. \*\*P < 0.001, GLP-1R agonist vs obese control; §P < 0.05, §§P < 0.01, §§§P < 0.0001, combination vs obese control.

monkeys (baseline, 11.3 ± 0.6 kg; end of dosing period, 11.2 ± 0.6 kg).

Monkeys treated with the GLP-1R agonist alone showed a modest but significant decrease in body weight with a loss of -3.8% ± 0.9% on day 29 (Fig. 3; P < 0.05 vs vehicle controls). Absolute values revealed a statistically significant reduction from day +11 until the end of the dosing period compared with baseline values on day 1 (P < 0.001).

The combination group had a relative weight change of -6.6% ± 0.9% on day 29 (Fig. 3; P < 0.0001 vs vehicle controls). In both GLP-1R agonist-containing groups, weight loss was significantly higher compared with vehicle controls, which was observed for the combination group immediately after the last dose ramping step on day +7 and for the GLP-1R agonist group on day +18. Furthermore, the combination treatment elicited a significantly greater impact on body weight reduction as compared with the GLP-1R monotherapy (P < 0.05 on day +18 to day +29).

All drug-treated groups showed a loss in fat mass compared with vehicle-treated controls, which was not statistically significant (Table 3).

Glucose values were reasonably stable during the study period in the vehicle group (Fig. 4). Similarly, insulin measurements were stable, but demonstrated an increase during the day due to feeding.

In the GCGR agonist group, glucose increased significantly 2 and 5 hours after dosing on day +28 compared with baseline (P < 0.01). Insulin measurements demonstrated an increase during the day due to feeding again without a statistically significant difference between day +28 and baseline.

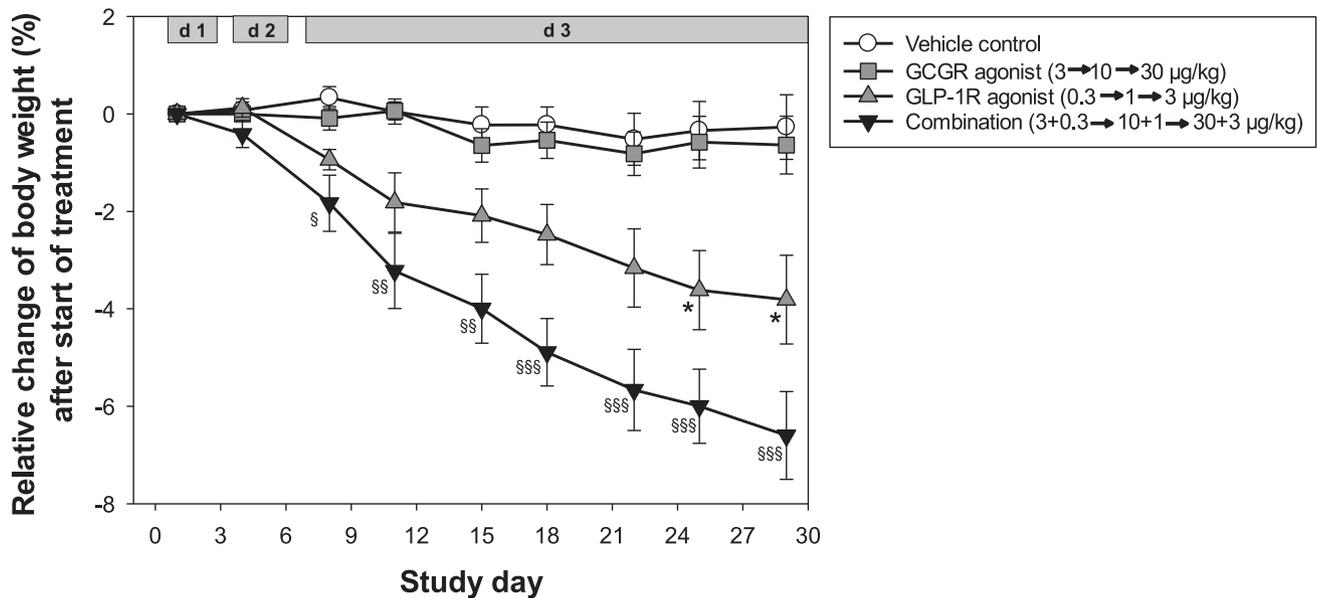
In contrast, treatment with GLP-1R agonist decreased glucose on day +10 and day +28 compared with baseline (P < 0.05 at 0 to 8 hours on day +28 compared with baseline). The GLP-1R agonist reduced glucose levels to ~90 mg/dL at 2 hours on day +28. Insulin was decreased on both days (P < 0.001 at 2 hours on day +28 vs baseline).

**Table 2. Baseline Values Obtained During the Run-in Period From Day -11 to +1 Prior Start of Treatment**

	Vehicle Control (n = 7)	GCGR Agonist (n = 7)	GLP-1R Agonist (n = 7)	Combination (n = 8)
Body weight, kg	10.8 ± 0.35	11.3 ± 0.62	10.8 ± 0.48	11.1 ± 0.5
TEI, kcal/d; MJ/d	535.0 ± 33.5; 2.24 ± 0.14	536.1 ± 28.2; 2.24 ± 0.12	561.2 ± 31.7; 2.35 ± 0.13	530.1 ± 39.8; 2.22 ± 0.17
HbA <sub>1c</sub> , mmol/mol; %	51.1 ± 10.4; 6.8 ± 0.95	46.9 ± 9.4; 6.4 ± 0.86	60.4 ± 8.7; 7.7 ± 0.8	46.8 ± 8.6; 6.4 ± 0.79
FPG, mg/dL	172.4 ± 27.9	174.1 ± 20.4	182.9 ± 25.9	182.4 ± 31.7
FPI, µU/mL	250.8 ± 74.5	329.6 ± 78.9	462.0 ± 166.5	317.5 ± 100.3
Total ketone bodies, µmol/L	63.8 ± 10.1	42.8 ± 5.1	79.0 ± 27.6	67.6 ± 14.5
Plasma FGF21, pg/mL	300.6 ± 116.2	196.2 ± 97.9	92.6 ± 25.6	113.5 ± 25.5
NEFA, mmol/L	0.27 ± 0.06	0.25 ± 0.04	0.35 ± 0.05	0.32 ± 0.05

Values are mean ± SEM.

Abbreviations: FPI, fasting plasma insulin; NEFA, nonesterified fatty acid.



**Figure 3.** Body weight development as relative change during dosing period. Individual body weights taken on day +1 (before dosing and before breakfast) were designated as the baseline values and measured values were expressed as percentage change of baseline (d1, day +1 to +3; d2, day +4 to +6; and d3, day +7 to run-out period). Values are mean  $\pm$  SEM; n = 7 to 8 per group. \* $P$  < 0.05, GLP-1R agonist vs vehicle control;  $^{\S}P$  < 0.05,  $^{\S\S}P$  < 0.01,  $^{\S\S\S}P$  < 0.001, combination vs vehicle control.

In the combination group glucose measurement showed a significantly lower value at 0 hours ( $P$  < 0.001) whereas insulin was significantly elevated 5 hours after dosing (Fig. 4;  $P$  < 0.01).

Total ketone bodies were reasonably stable in the vehicle control group. In GCGR agonist-treated monkeys, ketone bodies increased 8 and 22 hours after treatment on day +28 ( $P$  < 0.05 and  $P$  < 0.001, respectively). In GLP-1R agonist-treated monkeys, ketone bodies tended to be higher for the first 2 hours on day +10 compared with baseline, but were at almost the same levels from 5 to 22 hours on three occasions. No significant differences were measured after 28 days of chronic treatment. In the combination group total ketone bodies were elevated on days +28 at 0 and 22 hours after dosing compared with baseline (Fig. 4;  $P$  < 0.001) and even more on day +10.

In several plasma samples, levels of FGF21 were below the detection limit of the analytical assay. The average

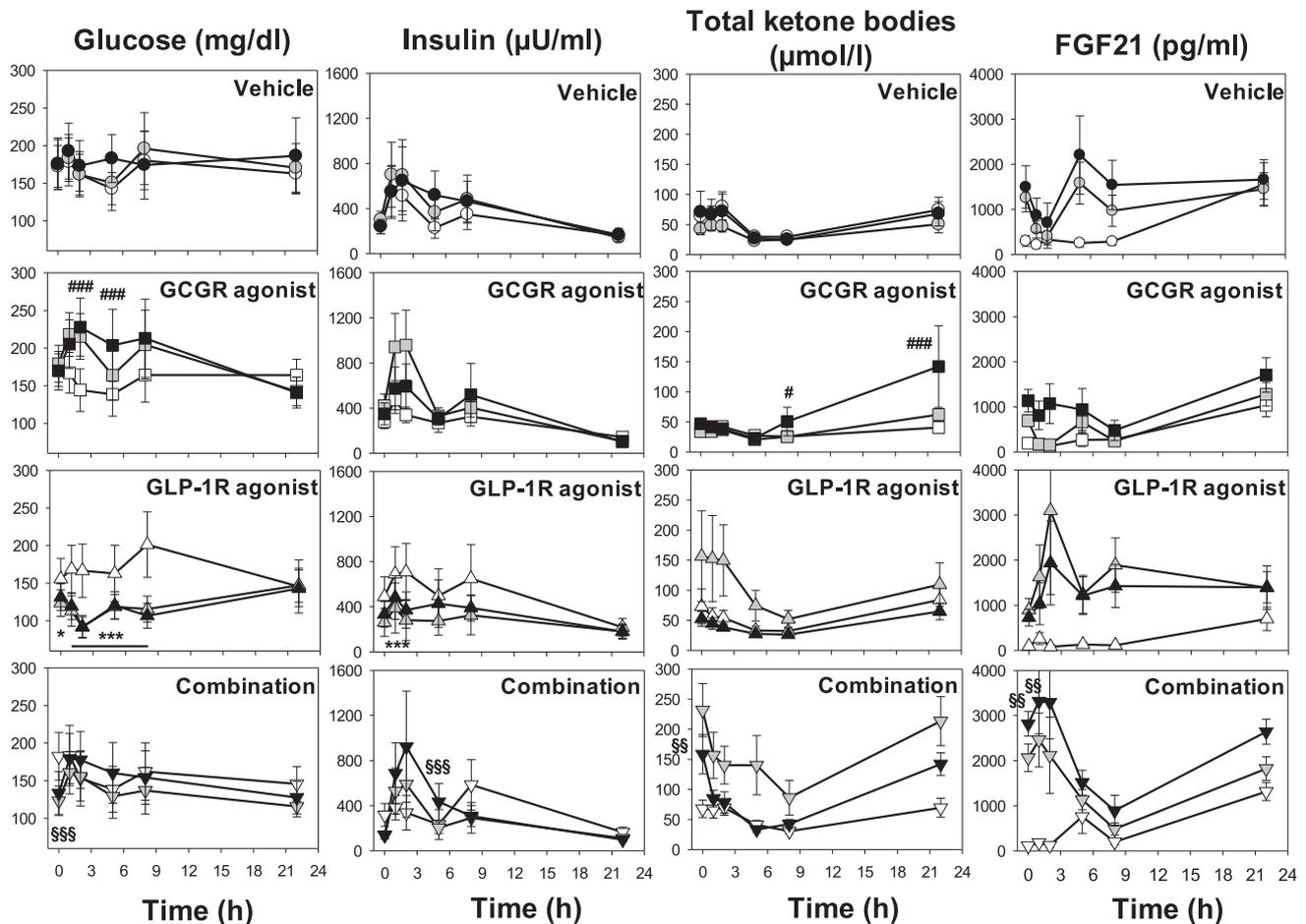
FGF21 plasma protein values on all three occasions for the vehicle and GCGR agonist-treated group were <2000 pg/mL. However, FGF21 plasma protein demonstrated some daily rhythm in the vehicle group, especially on day +10 and day +28 (Fig. 4) differently from the baseline profile. Clearly elevated FGF21 plasma protein was observed on day +10 in the GLP-1R agonist group, with highest FGF21 plasma protein (>3000 pg/mL) at 2 hours on day +10. Plasma FGF21 was also elevated on days +10 and +28 in the combination group, with highest FGF21 plasma protein (>3000 pg/mL) at 1 and 2 hours on day +28 ( $P$  < 0.001 vs vehicle at 1 and 2 hours).

Whereas mean  $\pm$  SEM baseline HbA<sub>1c</sub> levels were similar for vehicle (6.8%  $\pm$  0.9%; 51.1  $\pm$  10.4 mmol/mol), GCGR agonist (6.4%  $\pm$  0.9%; 46.9  $\pm$  9.4 mmol/mol), and combination (6.4%  $\pm$  0.8%; 46.8  $\pm$  8.6 mmol/mol) groups, it was higher for the GLP-1R agonist group (7.7%  $\pm$  0.8%; 60.4  $\pm$  8.7 mmol/mol).

**Table 3. Total Fat Mass and Relative Change on Day -19/-18 (Baseline) and on Day +31 During Run-out Period**

Group	Dose ( $\mu$ g/kg)	n	Total Fat Mass (g)		Relative Change (%)
			Day -19 / -18 (Baseline)	Day +31	
Vehicle control		7	5012 $\pm$ 341	4623 $\pm$ 264	-7.05 $\pm$ 3.00
GCGR agonist	30	7	5524 $\pm$ 212	5308 $\pm$ 262	-4.02 $\pm$ 2.03
GLP-1R agonist	3	7	4977 $\pm$ 317	4401 $\pm$ 249	-11.16 $\pm$ 2.57
Combination (GCGR agonist plus GLP-1R agonist)	30 + 3	8	4937 $\pm$ 317	4214 $\pm$ 284	-14.76 $\pm$ 1.39

Values are mean  $\pm$  SEM.



**Figure 4.** Plasma profiling for glucose, insulin, total ketone bodies, and FGF21, measured on day –11 [baseline: white circles, vehicle group; white squares, GCGR agonist; white triangles, GLP-1R agonist; white inverted triangles, combination treatment], day +10 [end of dose-ramping: gray circles, vehicle group; gray squares, GCGR agonist; gray triangles, GLP-1R agonist; gray inverted triangles, combination treatment], and on day +28 [end of dosing period: black circles, vehicle group; black squares, GCGR agonist; black triangles, GLP-1R agonist; black inverted triangles, combination treatment]. Values are mean  $\pm$  SEM;  $n = 7$  to 8 per group for glucose, insulin, and total ketone bodies;  $n = 2$  to 8 per group for FGF21. \* $P < 0.05$ , \*\*\* $P < 0.001$ , GLP-1R agonist vs baseline; # $P < 0.05$ , ### $P < 0.001$ , GCGR agonist vs baseline; \$\$\$ $P < 0.001$ , combination vs baseline.

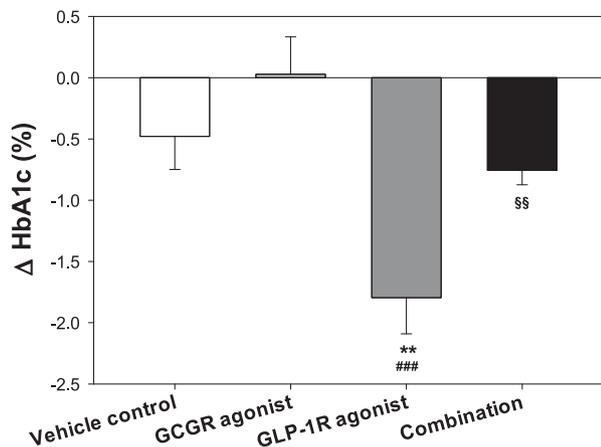
Absolute change after chronic treatment vs baseline was highest for the GLP-1R agonist-treated monkeys ( $-1.8\% \pm 0.3\%$ ,  $-19.6 \pm 3.2$  mmol/mol) shown in Fig. 5. Compared with vehicle-treated monkeys, a significant reduction was observed ( $P < 0.01$  vs vehicle), which was even more pronounced when compared with GCGR agonist-treated monkeys ( $+0.03\% \pm 0.3\%$ ,  $+46.9 \pm 9.4$  mmol/mol,  $P < 0.001$  vs GCGR).

The impact of the selective GLP-1R agonist on % HbA<sub>1c</sub> was diminished when administered in combination with the selective GCGR agonist in the combination group ( $-0.76\% \pm 0.12\%$ ,  $-8.3 \pm 1.3$  mmol/mol), showing no significant reduction compared with vehicle controls and no significant reduction compared with the GCGR agonist-treated groups ( $P = 0.0844$ ).

FPG was significantly reduced only in the GLP-1R agonist-treated monkeys compared with baseline (baseline:  $176.7 \pm 34.0$  mg/dL vs run-out:  $115.9 \pm 16.1$  mg/dL,  $P < 0.001$ ). FPG at  $t = 0$  for the GCGR

agonist-treated monkeys was significantly elevated from  $136.9 \pm 21.2$  (baseline) to  $213.7 \pm 28.2$  mg/dL (run-out) ( $P < 0.001$ ). The combination-treated monkeys showed FPG values that were not significantly elevated from  $151.6 \pm 21.3$  (baseline) to  $193.5 \pm 33.5$  mg/dL (run-out), but clearly diminished the beneficial impact of GLP-1R agonist alone.

The IVGTT data of vehicle-treated monkeys were stable during the study for both glucose and insulin measurements (Fig. 6). Glucose excursion during IVGTT was considerably increased in the GCGR agonist group compared with baseline. Calculating the AUC (AUC<sub>glucose</sub> in mg/dL·min) revealed an increase of  $+22.1\% \pm 4.3\%$  ( $P < 0.001$ ). In GLP-1R agonist-treated monkeys the AUC<sub>glucose</sub> was significantly decreased by  $-23.3\% \pm 1.1\%$  ( $P < 0.0001$ ). Plasma insulin levels were increased in both treatment groups; however, values were only significantly elevated in the GLP-1R agonist group (AUC<sub>insulin</sub>  $+88.0\% \pm 25.6\%$ ,  $P < 0.001$ ). In the combination



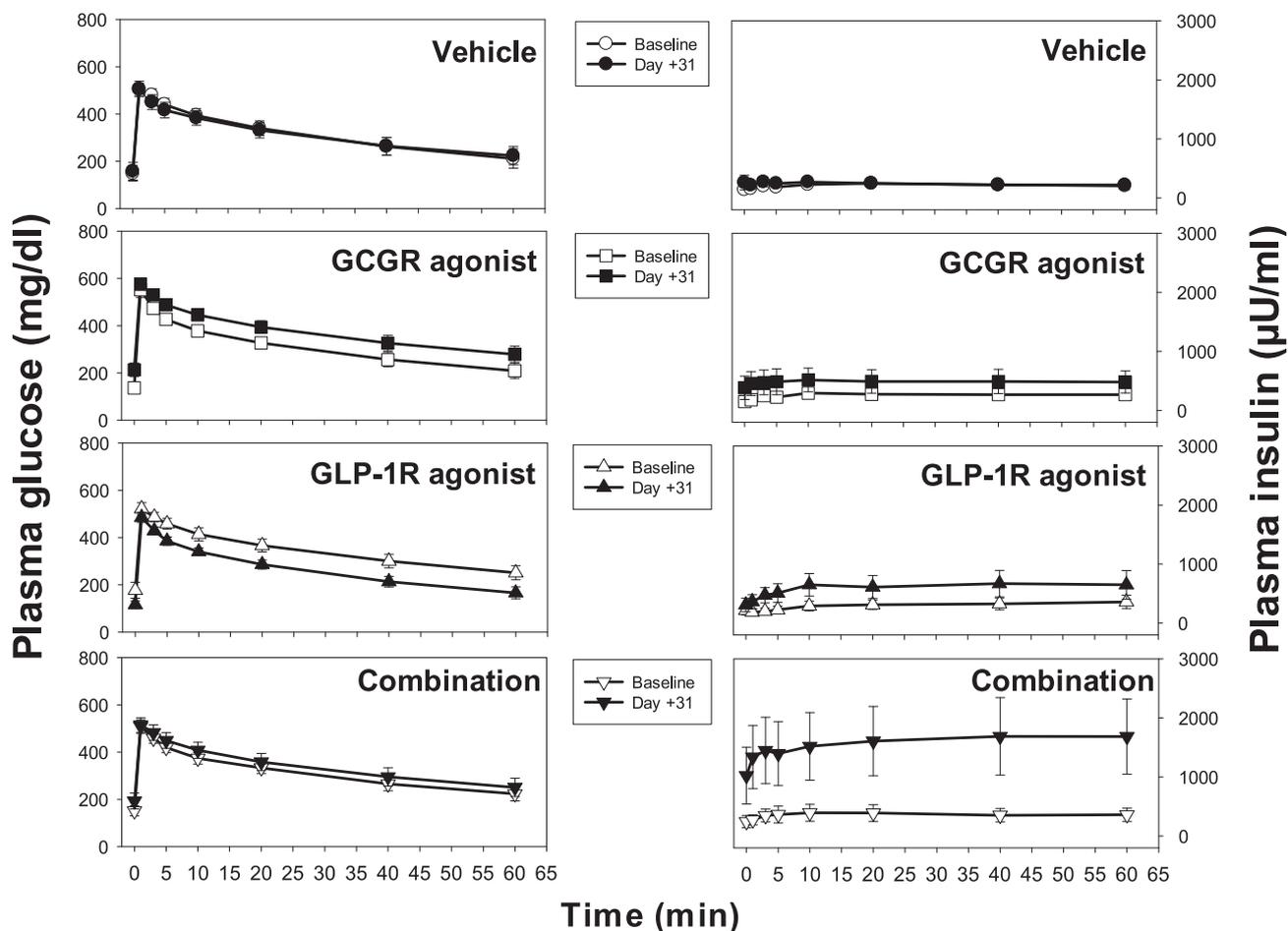
**Figure 5.** Change in HbA<sub>1c</sub> (%) values determined on day -13 (baseline) to day +29 of treatment. Absolute change after chronic treatment was calculated as  $\Delta\%$ HbA<sub>1c</sub> vs baseline. Baseline values varied between 6.4% and 7.6%. Values are mean  $\pm$  SEM;  $n = 7$  to 8 per group. \*\* $P < 0.01$ , vs vehicle; §§ $P < 0.01$ , vs GLP-1R agonist; ### $P < 0.001$ , vs GCGR agonist.

group, compared with baseline IVGTT values, a slight but statistically nonsignificant increase was observed in glucose excursion ( $AUC_{\text{glucose}} +8.6\% \pm 7.8\%$ , not significant), but insulin secretion was significantly increased

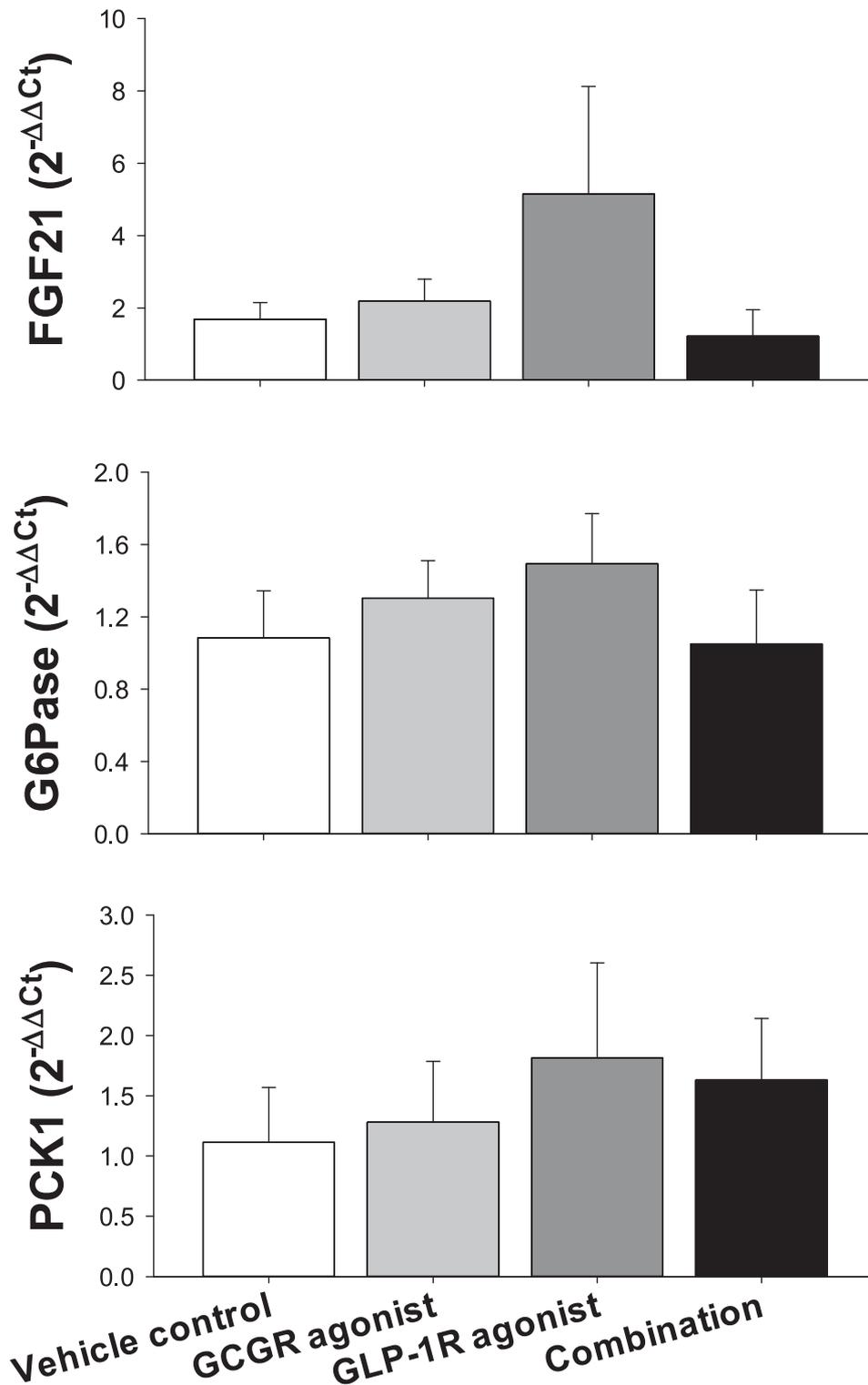
( $AUC_{\text{insulin}} +246.3\% \pm 57.6\%$ ,  $P < 0.0001$ ). For investigation of  $\beta$  cell responsivity, an acute insulin response analysis for AUC values at 0 to 10 minutes corresponding to first phase insulin secretion was performed. Only the combination group showed a significantly improved responsivity to the glucose bolus ( $P < 0.01$ ).

Hepatic gene expression fold change of *G6Pase*, *PCK1*, and *FGF21* in liver tissue samples comparing run-in and run-out periods did not differ significantly between treatment groups (Fig. 7).

Whereas the GLP-1R agonist showed similar exposure in both single- and combination-treated animals on day +28, the exposure of the GCGR agonist was somewhat higher in the combination-treated than the single-treated animals (Table 4), although combination administration was done as two single injections of the individual agonists at different injection sites. Similar observations were made on day +10 (data not shown). Although we have to encounter reports describing potential interaction between two concomitant administered peptides on their metabolism, we cannot exclude that coadministration of the GLP-1R agonist interfered with clearance of the GCGR agonist.



**Figure 6.** An IVGTT was conducted twice during the study, once during the run-in period (day -19/-18, baseline) and once during the run-out period (day +31). All dosing commenced at 2.5 hours before the end of IV glucose bolus administration, that is, IVGTT time 0 ( $t = 0$ ). Dosing began at 6:30 AM and continued in a staggered manner until all monkeys were dosed. Values are mean  $\pm$  SEM,  $n = 7$  to 8 per group.



**Figure 7.** Hepatic gene expression fold change of G6Pase, PCK1, and FGF21 obtained from liver biopsy during run-out period compared with run-in period. Values are mean  $\pm$  SEM; n = 7 to 8 per group.

## Discussion

Oxyntomodulin is an endogenous dual peptide agonist, as it exerts agonist activity on both GLP-1R and GCGR (30). The current study was undertaken to investigate the roles of the individual pharmacological components of

oxyntomodulin mimetics on whole-body energy homeostasis. Several dual GLP-1R/GCGR agonists have advanced to clinical trials, but without clear demonstration of engagement of the individual receptors. Thus, it is possible that reported data from nonhuman primates (23) as well from early clinical experimentation merely

**Table 4. PK Profile (ng/mL) on Day +28 of Treatment of GCGR and GLP-1R Agonist Administered Alone or as Double Injection at Two Injection Sites (Combination Treatment)**

Time After Dosing, h	0	1	2	5	8	22
GCGR agonist	0.9 ± 0.6	16.6 ± 4.0	21.8 ± 6.7	19.4 ± 6.3	12.3 ± 4.3	1.7 ± 1.0
GLP-1R agonist	1.5 ± 1.5	8.6 ± 1.5	11.8 ± 1.6	11.5 ± 2.6	8.2 ± 3.2	2.5 ± 2.5
GCGR agonist (combination)	5.5 ± 1.7	59.4 ± 5.4	75.5 ± 6.9	61.8 ± 9.8	41.8 ± 8.2	7.1 ± 2.2
GLP-1R agonist (combination)	0.5 ± 0.4	8.8 ± 1.2	10.8 ± 1.2	10.1 ± 0.9	7.1 ± 1.2	0.7 ± 0.5

Values are mean ± SEM; n = 6 to 8 per group.

reflect pharmacological effects expected to result from exposure to a highly potent GLP-1 agonist with efficacy on body weight similar to what has been reported for use of high-dose semaglutide (31).

The current study brings insights to how chronic exposure to pharmacological doses of selective glucagon agonists affects energy homeostasis in nonhuman primates. Contrasting to data obtained from acute glucagon exposure studies in humans (26), we found that chronic exposure to selective glucagon agonism has no detectable impact on energy homeostasis in nonhuman primates, as body weight and food consumption were maintained throughout the study. However, the resulting GCGR agonist exposure was sufficiently high to elicit diabetogenic efficacy, as seen in the GCGR-treated monkeys. Even in combination with a GLP-1R agonist its beneficial antiglycemic impact was diminished.

Earlier reports summarizing data from short-term clinical interventions with either single injections or short-term infusions have shown glucagon to negatively impact energy homeostasis, as it was both anorectic and moderately increased energy expenditure (32, 33). Despite numerous studies demonstrating short-term effects of glucagon on feeding in rodents and humans, we found that continuous glucagon agonist exposure exerts minimal anorectic effects. Rather, in line with glucagon's physiological role as a potential promotor of increased energy expenditure in rodents (34, 35), a logical counterregulatory response to chronic glucagon exposure would be increased appetite.

Continuous exposure to glucagon may lead to desensitization. Thus, short-term infusion of a moderate glucagon dose lost efficacy on splanchnic glucose production in humans irrespective of the presence of insulin (36). However, as we presently observed continuously elevated glycemia during concomitant exposure to the GCGR agonist, it seems unlikely that tachyphylaxis to glucagon signaling emerges in nonhuman primates and therefore probably cannot explain its lack of effects on body weight.

In contrast to GLP-1 agonists, glucagon, at the doses studied, impacts neither appetite nor gastric motility (27). Therefore, it seems unlikely that glucagon has a role as a

true satiety signal but may rather cause acute anorexia as a result of its impact on glycogenolysis and ketone body formation. In rats and rabbits, glucagon is more anorectic when delivered into the portal vein, and the effect is lost upon vagotomy (34, 35). Vagal afferents may respond to acute changes in glycogenolysis and this signal may impact feeding. Similarly, studies in rats show that anorectic effects of 3-OH-butyrate are dependent on intact vagal nerve (37). As glucagon minimally impacts glycogenolysis in a fed state with ample hepatic insulin exposure, its potential anorectic effects should be more pronounced during states of negative energy balance. This is in line with our current observations demonstrating a more pronounced drop in feeding of nonhuman primates when the glucagon agonist is coadministered with the GLP-1R agonist.

In mice, chronic exposure to glucagon agonists induces negative energy balance, most likely through an increase in energy expenditure. However, short-term clinical infusion studies of glucagon have shown mixed results with no or minimal elevation of whole-body energy expenditure in healthy volunteers (26–28, 33). In an overnight infusion study, continuous exposure to ~80 pmol/L glucagon elevated energy expenditure by 50 to 70 kcal/d (0.21 to 0.29 MJ) in healthy volunteers (33). Whether such supraphysiological glucagon exposure (twofold to threefold higher levels than fasting glucagon) is sufficient to contribute meaningfully to negative energy balance is unclear. As all animals in the current study were subjected to a fixed caloric diet, it seems unlikely that the applied dose of GCGR agonist dose when given alone impacted energy expenditure because no weight loss vs placebo was detected.

To our knowledge, this is the first study to clearly deconvolute chronic exposure pharmacology of individual components of dual GLP-1R/GCGR agonists in nonhuman primates. Recently data from a toxicology assessment of dual GLP-1R/GCGR agonist in lean nondiabetic monkeys was published (23). However, apart from showing a robust dose-dependent weight loss, that study neither reported 24 hour glycemic profile nor hepatic gene expression, making it impossible to qualify that the dual agonist actually engaged GCGR pathways.

In light of the negligible effects of chronic glucagon agonist exposure on body weight, it was surprising to observe a clear synergy of GLP-1 and glucagon agonist combination on energy homeostasis. Therefore, it seems fair to conclude that synergistic effects of GLP-1 and GCGR signaling on body weight are obtained by the combination of a robust anorectic agent with an agent moderately preventing the decrease in energy expenditure normally associated with persistent negative energy balance occurring with use of anorectic agents. The magnitude of the weight loss is, perhaps, surprising. Although a small reduction of food intake was seen in the cohort receiving the GLP-1R/GCGR combination vs the GLP-1R group, this is too small to explain the much larger drop in body weight observed. In the absence of reliable technology to measure energy expenditure in nonhuman primates, we propose to validate this assumption through a well-controlled clinical trial. A combination of GLP-1R and GCGR agonism seemingly exerts very favorable synergism on body weight and hepatic fuel storage.

Under conditions of ample availability of glucose, chronic glucagon exposure permanently elevates hepatic glucose production (38). The chronic presence of a glucagon agonist partially offset the glucose-lowering efficacy otherwise obtained with selective GLP-1R agonists in nonhuman primates. As many individuals struggling with obesity and excess hepatic fat deposition are dysglycemic, identification of the optimal target population for GLP-1R/GCGR dual agonists is warranted and more research is needed on the suitability of GLP-1R/GCGR dual agonists for weight management in people with type 2 diabetes mellitus. Nonhuman primates chronically exposed to dual GLP-1R/GCGR agonism maintained the same response to IV glucose but with markedly elevated insulin secretion. In contrast, the cohort treated with the selective GCGR agonist alone developed impaired glucose tolerance with minimal elevation of insulin response. Thus, an apparent hyper-responsive  $\beta$  cell population may have contributed to maintenance of glycemia with combination therapy. It is possible that chronic exposure to a GLP-1R agonist could have mediated this effect, as GLP-1R agonists have been suggested to exert trophic effects on  $\beta$  cells (39). The

currently employed peptide has comparable potency/efficacy compared with native glucagon. It is likely that GCGR agonists with lesser potency are more suitable partners for GLP-1R agonists in designing the optimal GLP-1R/GCGR dual agonists for therapeutic use in patients who are obese with type 2 diabetes mellitus.

Relative hyperglucagonemia and impaired hepatic insulin sensitivity, leading to persistently elevated nocturnal glucose production, is a common feature of type 2 diabetes mellitus (38). Glucagon's ability to persistently stimulate hepatic glucose production under nonfasting conditions may constitute a safeguard against excessive hepatic energy storage, ultimately promoting non-alcoholic fatty liver disease. Recent clinical experiences with a GCGR antagonist have confirmed that both elevated body weight and excessive hepatic lipid deposition can occur upon chronic blockade of GCGR signaling (40). Combining GCGR agonism with GLP-1R-induced anorexia promotes faster clearance of hepatic glycogen depot and resulting hepatic  $\beta$ -oxidation of fatty acids. In nonhuman primates, combination of GLP-1R and GCGR agonism led to moderate but significant ketogenesis, suggesting clearance of body lipids (see Table 5). Rodent studies have confirmed the beneficial effects of GLP-1R/GCGR dual agonism on liver lipids, leading to a resolution of nonalcoholic steatohepatitis (41).

Although mobilization of liver triglycerides can contribute to glucagon-mediated ketogenesis, the acutely preferred substrate for hepatic ketone body formation is adipocyte-derived nonesterified fatty acids (NEFAs) (42). In nonhuman primates, GCGR agonist exposure triggered peripheral lipolysis as evidenced by elevation of plasma NEFAs, but GCGR-induced ketogenesis was only observed during concomitant GLP-1R agonist administration. Thus, despite persistent GCGR agonist exposure and resulting elevated plasma levels of NEFAs, concurrent ketogenesis did not occur at neutral energy balance. Mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase is the rate-limiting enzyme promoting hepatic ketogenesis. The activity of HMG-CoA is negatively impacted by succinyl-coenzyme A levels, explaining why conditions characterized by relatively high flux of tricarboxylic cycle intermediaries are not associated with ketogenesis despite the availability

**Table 5. Free Fatty Acids as  $\delta$  Values From Baseline on Day –10 to Day +28 During Dosing Period**

	GCGR Agonist (n = 7)	GLP-1R Agonist (n = 7)	Combination (n = 8)
Change in free fatty acids, mmol/L	+0.28 $\pm$ 0.03 <sup>a,b</sup>	+0.01 $\pm$ 0.04	+0.26 $\pm$ 0.07 <sup>b,c</sup>

Values are mean  $\pm$  SEM.

<sup>a</sup>*P* < 0.001 vs baseline.

<sup>b</sup>*P* < 0.01 vs GLP-1R agonist-treated monkeys.

<sup>c</sup>*P* < 0.01 vs baseline.

of NEFA substrates (43). Unfortunately, the small liver biopsies obtained from nonhuman primates did not allow for analysis of hepatic glycogen, lipids, and tricarboxylic acid cycle intermediaries, so it was not possible to elucidate whether long-term GCGR agonist exposure at body weight neutrality leads to excess accumulation of liver lipids.

Because GCGR agonists lose efficacy on body weight, glycemia, and lipid metabolism in FGF21 knockout mice, it has been proposed that FGF21 is a constitutive requirement for glucagon to exert its physiological effects on energy homeostasis (44, 45). However, FGF21 is not necessarily a specific downstream mediator and hence biomarker of GCGR-mediated pharmacology. Liver-derived FGF21 has been proposed as a starvation signal released by hepatocytes under physiological circumstances and characterized by decreased hepatic fuel storage (46). Several conditions characterized by negative energy balance, including GLP-1R agonist-induced weight loss, have been associated with elevations of plasma FGF21 levels (47, 48). In line with this, we observed significant elevations of plasma FGF21 in both GLP-1R agonist- and in GLP-1R/GCGR agonist-treated nonhuman primates after 28 days of therapy. In contrast, 24 hour plasma profile of ketone bodies and FGF21 remained unaffected in nonhuman primates treated with the selective GCGR agonist. As judged by its effect on glycemia, the GCGR agonist dose was clearly pharmacologically active, confirming that GCGR agonism alone does not regulate FGF21. This is in line with a recent glucagon infusion study in healthy volunteers in which 13 hours of hyperglucagonemia had no impact on plasma levels of FGF21 (33). Rather, the current study showed that plasma levels of FGF21 were tightly associated with hepatic ketogenesis, supporting a key regulatory role of hepatic PPAR $\alpha$ -mediated transactivation upon hepatic FGF21 secretion (49). For these reasons, we conclude that plasma FGF21 levels are not useful as a specific biomarker of GCGR target engagement.

A decade ago, diabetologists would have considered utilization of a GCGR agonist-based therapeutic agent for weight management and diabetes therapy outright irrational. However, observations made initially from bariatric surgery-induced alterations of the enteroendocrine response to feeding and the advent of longer-acting oxyntomodulin mimetics have challenged this otherwise counterintuitive application of GCGR agonists. The current study has shown that GCGR agonism administered together with GLP-1R agonism triggers pharmacological synergies on energy homeostasis not observed when the two agonists are injected alone. To capture most of the metabolic advantages following bariatric surgery, the future challenge will be to

design new polypharmacologically active molecular entities encompassing the optimal balance between relevant enteroendocrine peptides, such as GLP-1, glucagon, glucose-dependent insulinotropic polypeptide, and neuropeptide Y.

## Acknowledgments

We appreciate the great support and performance of the monkey study at KBI, China, with special thanks to Tony Wang, Rosario M. Perez, Paul Higgins, and Shaohui (Jason) Ji. We thank Thomas Klabunde for support in data analysis. We further thank Anish Konkar and Krister Bokvist for reading the manuscript and providing valuable comments. Editorial support was provided by Nicole Draghi of Caudex and Lisa Lovelidge for Caudex and funded by Sanofi.

**Financial Support:** There was no external funding source for this study. The study was supported by Sanofi.

**Author Contributions:** R.E., A.W.H., T.W., M.B., and B.Z. contributed substantially to the concept and design of the nonhuman primate study. P.J.L., R.E., M.B., A.K., A.E., and W.H. drafted the manuscript. All authors approved the version of the manuscript to be published.

**Correspondence:** Ralf Elvert, PhD, Sanofi-Aventis Deutschland GmbH, Industriepark Höchst, 65926 Frankfurt, Germany. E-mail: [ralf.elvert@sanofi.com](mailto:ralf.elvert@sanofi.com).

**Disclosure Summary:** All authors were employed by Sanofi at the time the study was performed. Sanofi manufactures and markets pharmaceuticals related to the treatment of diabetes. Some authors hold stock in Sanofi.

## References

- Buchwald H, Estok R, Fahrback K, Banel D, Jensen MD, Pories WJ, Bantle JP, Sledge I. Weight and type 2 diabetes after bariatric surgery: systematic review and meta-analysis. *Am J Med.* 2009; **122**(3):248–256.e5.
- Perugini RA, Malkani S. Remission of type 2 diabetes mellitus following bariatric surgery: review of mechanisms and presentation of the concept of “reversibility.” *Curr Opin Endocrinol Diabetes Obes.* 2011; **18**(2):119–128.
- Braghetto I, Davanzo C, Korn O, Csendes A, Valladares H, Herrera E, Gonzalez P, Papapietro K. Scintigraphic evaluation of gastric emptying in obese patients submitted to sleeve gastrectomy compared to normal subjects. *Obes Surg.* 2009; **19**(11):1515–1521.
- Falkén Y, Hellström PM, Holst JJ, Näslund E. Changes in glucose homeostasis after Roux-en-Y gastric bypass surgery for obesity at day three, two months, and one year after surgery: role of gut peptides. *J Clin Endocrinol Metab.* 2011; **96**(7):2227–2235.
- Bojsen-Møller KN, Dirksen C, Jørgensen NB, Jacobsen SH, Hansen DL, Worm D, Naver L, Kristiansen VB, Holst JJ, Madsbad S. Increased hepatic insulin clearance after Roux-en-Y gastric bypass. *J Clin Endocrinol Metab.* 2013; **98**(6):E1066–E1071.
- Bojsen-Møller KN, Dirksen C, Svane MS, Jørgensen NB, Holst JJ, Richter EA, Madsbad S. Variable reliability of surrogate measures of insulin sensitivity after Roux-en-Y gastric bypass. *Am J Physiol Regul Integr Comp Physiol.* 2017; **312**(5):R797–R805.
- Lindqvist A, Spégel P, Ekelund M, Garcia Vaz E, Pierzynowski S, Gomez MF, Mulder H, Hedenbro J, Groop L, Wierup N. Gastric bypass improves  $\beta$ -cell function and increases  $\beta$ -cell mass in a porcine model. *Diabetes.* 2014; **63**(5):1665–1671.

8. Immonen H, Hannukainen JC, Iozzo P, Soinio M, Salminen P, Saunavaara V, Borra R, Parkkola R, Mari A, Lehtimäki T, Pham T, Laine J, Kärjä V, Pihlajamäki J, Nelimarkka L, Nuutila P. Effect of bariatric surgery on liver glucose metabolism in morbidly obese diabetic and non-diabetic patients. *J Hepatol.* 2014;**60**(2):377–383.
9. Jørgensen NB, Dirksen C, Bojsen-Møller KN, Jacobsen SH, Worm D, Hansen DL, Kristiansen VB, Naver L, Madsbad S, Holst JJ. Exaggerated glucagon-like peptide 1 response is important for improved  $\beta$ -cell function and glucose tolerance after Roux-en-Y gastric bypass in patients with type 2 diabetes. *Diabetes.* 2013;**62**(9):3044–3052.
10. Svane MS, Bojsen-Møller KN, Nielsen S, Jørgensen NB, Dirksen C, Bendtsen F, Kristiansen VB, Hartmann B, Holst JJ, Madsbad S. Effects of endogenous GLP-1 and GIP on glucose tolerance after Roux-en-Y gastric bypass surgery. *Am J Physiol Endocrinol Metab.* 2016;**310**(7):E505–E514.
11. Craig CM, Liu LF, Deacon CF, Holst JJ, McLaughlin TL. Critical role for GLP-1 in symptomatic post-bariatric hypoglycaemia. *Diabetologia.* 2017;**60**(3):531–540.
12. Wewer Albrechtsen NJ, Hornburg D, Albrechtsen R, Svendsen B, Toräng S, Jepsen SL, Kuhre RE, Hansen M, Janus C, Floyd A, Lund A, Vilsbøll T, Knop FK, Vestergaard H, Deacon CF, Meissner F, Mann M, Holst JJ, Hartmann B. Oxyntomodulin identified as a marker of type 2 diabetes and gastric bypass surgery by mass-spectrometry based profiling of human plasma. *EBioMedicine.* 2016;**7**:112–120.
13. Bataille D, Tatemoto K, Gespach C, Jörnvall H, Rosselin G, Mutt V. Isolation of glucagon-37 (bioactive enteroglucagon/oxyntomodulin) from porcine jejunum-ileum. Characterization of the peptide. *FEBS Lett.* 1982;**146**(1):79–86.
14. Druce MR, Minnion JS, Field BCT, Patel SR, Shillito JC, Tilby M, Beale KEL, Murphy KG, Ghatei MA, Bloom SR. Investigation of structure-activity relationships of oxyntomodulin (Oxm) using Oxm analogs. *Endocrinology.* 2009;**150**(4):1712–1722.
15. Wynne K, Park AJ, Small CJ, Meeran K, Ghatei MA, Frost GS, Bloom SR. Oxyntomodulin increases energy expenditure in addition to decreasing energy intake in overweight and obese humans: a randomised controlled trial. *Int J Obes.* 2006;**30**(12):1729–1736.
16. Wynne K, Park AJ, Small CJ, Patterson M, Ellis SM, Murphy KG, Wren AM, Frost GS, Meeran K, Ghatei MA, Bloom SR. Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, controlled trial. *Diabetes.* 2005;**54**(8):2390–2395.
17. Lynch AM, Pathak N, Pathak V, O'Harte FP, Flatt PR, Irwin N, Gault VA. A novel DPP IV-resistant C-terminally extended glucagon analogue exhibits weight-lowering and diabetes-protective effects in high-fat-fed mice mediated through glucagon and GLP-1 receptor activation. *Diabetologia.* 2014;**57**(9):1927–1936.
18. Pocai A, Carrington PE, Adams JR, Wright M, Eiermann G, Zhu L, Du X, Petrov A, Lassman ME, Jiang G, Liu F, Miller C, Tota LM, Zhou G, Zhang X, Sountis MM, Santoprete A, Capito' E, Chicchi GG, Thornberry N, Bianchi E, Pessi A, Marsh DJ, SinhaRoy R. Glucagon-like peptide 1/glucagon receptor dual agonism reverses obesity in mice. *Diabetes.* 2009;**58**(10):2258–2266.
19. Day JW, Ottaway N, Patterson JT, Gelfanov V, Smiley D, Gidda J, Findeisen H, Bruemmer D, Drucker DJ, Chaudhary N, Holland J, Hembree J, Abplanalp W, Grant E, Ruehl J, Wilson H, Kirchner H, Lockie SH, Hofmann S, Woods SC, Nogueiras R, Pfluger PT, Perez-Tilve D, DiMarchi R, Tschöp MH. A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. *Nat Chem Biol.* 2009;**5**(10):749–757.
20. Sánchez-Garrido MA, Brandt SJ, Clemmensen C, Müller TD, DiMarchi RD, Tschöp MH. GLP-1/glucagon receptor co-agonism for treatment of obesity. *Diabetologia.* 2017;**60**(10):1851–1861.
21. Evers A, Haack T, Lorenz M, Bossart M, Elvert R, Henkel B, Stengel S, Kurz M, Gliem M, Dudda A, Lorenz K, Kadereit D, Wagner M. Design of novel exendin-based dual glucagon-like peptide 1 (GLP-1)/glucagon receptor agonists. *J Med Chem.* 2017;**60**(10):4293–4303.
22. Finan B, Ma T, Ottaway N, Müller TD, Habegger KM, Heppner KM, Kirchner H, Holland J, Hembree J, Raver C, Lockie SH, Smiley DL, Gelfanov V, Yang B, Hofmann S, Bruemmer D, Drucker DJ, Pfluger PT, Perez-Tilve D, Gidda J, Vignati L, Zhang L, Hauptman JB, Lau M, Brecheisen M, Uhles S, Riboulet W, Hainaut E, Sebokova E, Conde-Knape K, Konkar A, DiMarchi RD, Tschöp MH. Unimolecular dual incretins maximize metabolic benefits in rodents, monkeys, and humans. *Sci Transl Med.* 2013;**5**(209):209ra151.
23. Henderson SJ, Konkar A, Hornigold DC, Trevaskis JL, Jackson R, Fritsch Fredin M, Jansson-Löfmark R, Naylor J, Rossi A, Bednarek MA, Bhagroo N, Salari H, Will S, Oldham S, Hansen G, Feigh M, Klein T, Grimsby J, Maguire S, Jeremias L, Rondinone CM, Coghlan MP. Robust anti-obesity and metabolic effects of a dual GLP-1/glucagon receptor peptide agonist in rodents and non-human primates. *Diabetes Obes Metab.* 2016;**18**(12):1176–1190.
24. Finan B, Yang B, Ottaway N, Smiley DL, Ma T, Clemmensen C, Chabenne J, Zhang L, Habegger KM, Fischer K, Campbell JE, Sandoval D, Seeley RJ, Bleicher K, Uhles S, Riboulet W, Funk J, Hertel C, Belli S, Sebokova E, Conde-Knape K, Konkar A, Drucker DJ, Gelfanov V, Pfluger PT, Müller TD, Perez-Tilve D, DiMarchi RD, Tschöp MH. A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents. *Nat Med.* 2015;**21**(1):27–36.
25. Day JW, Gelfanov V, Smiley D, Carrington PE, Eiermann G, Chicchi G, Erion MD, Gidda J, Thornberry NA, Tschöp MH, Marsh DJ, SinhaRoy R, DiMarchi R, Pocai A. Optimization of co-agonism at GLP-1 and glucagon receptors to safely maximize weight reduction in DIO-rodents. *Biopolymers.* 2012;**98**(5):443–450.
26. Cegla J, Troke RC, Jones B, Tharakan G, Kenkre J, McCullough KA, Lim CT, Parvizi N, Hussein M, Chambers ES, Minnion J, Cuenco J, Ghatei MA, Meeran K, Tan TM, Bloom SR. Coinfusion of low-dose GLP-1 and glucagon in man results in a reduction in food intake. *Diabetes.* 2014;**63**(11):3711–3720.
27. Bagger JJ, Holst JJ, Hartmann B, Andersen B, Knop FK, Vilsbøll T. Effect of oxyntomodulin, glucagon, GLP-1, and combined glucagon +GLP-1 infusion on food intake, appetite, and resting energy expenditure. *J Clin Endocrinol Metab.* 2015;**100**(12):4541–4552.
28. Tan TM, Field BC, McCullough KA, Troke RC, Chambers ES, Salem V, Gonzalez Maffe J, Baynes KC, De Silva A, Viardot A, Alsafi A, Frost GS, Ghatei MA, Bloom SR. Coadministration of glucagon-like peptide-1 during glucagon infusion in humans results in increased energy expenditure and amelioration of hyperglycemia. *Diabetes.* 2013;**62**(4):1131–1138.
29. National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals. *Guide for the Care and Use of Laboratory Animals.* 8th ed. Washington, DC: National Academies Press; 2011.
30. Holst JJ. Enteroglucagon. *Annu Rev Physiol.* 1997;**59**(1):257–271.
31. Blundell J, Finlayson G, Axelsen M, Flint A, Gibbons C, Kvist T, Hjerpested JB. Effects of once-weekly semaglutide on appetite, energy intake, control of eating, food preference and body weight in subjects with obesity. *Diabetes Obes Metab.* 2017;**19**(9):1242–1251.
32. Nair KS. Hyperglucagonemia increases resting metabolic rate in man during insulin deficiency. *J Clin Endocrinol Metab.* 1987;**64**(5):896–901.
33. Chakravarthy M, Parsons S, Lassman ME, Butterfield K, Lee AY, Chen Y, Previs S, Spond J, Yang S, Bock C, Yi F, Moon J, Wohlers-Kariesch E, Smith SR, Meyer C. Effects of 13-hour hyperglucagonemia on energy expenditure and hepatic glucose production in humans. *Diabetes.* 2017;**66**(1):36–44.
34. Vanderweele DA, Geiselman PJ, Novin D. Pancreatic glucagon, food deprivation and feeding in intact and vagotomized rabbits. *Physiol Behav.* 1979;**23**(1):155–158.

35. Geary N, Smith GP. Selective hepatic vagotomy blocks pancreatic glucagon's satiety effect. *Physiol Behav.* 1983;31(3):391–394.
36. Bomboy JD Jr, Lewis SB, Lacy WW, Sinclair-Smith BC, Liljenquist JE. Transient stimulatory effect of sustained hyperglucagonemia on splanchnic glucose production in normal and diabetic man. *Diabetes.* 1977;26(3):177–184.
37. Fisler JS, Egawa M, Bray GA. Peripheral 3-hydroxybutyrate and food intake in a model of dietary-fat induced obesity: effect of vagotomy. *Physiol Behav.* 1995;58(1):1–7.
38. Baron AD, Schaeffer L, Shragg P, Kolterman OG. Role of hyperglucagonemia in maintenance of increased rates of hepatic glucose output in type II diabetics. *Diabetes.* 1987;36(3):274–283.
39. van Raalte DH, Bunck MC, Smits MM, Hoekstra T, Corner A, Diamant M, Eliasson B, Marja R, Heine RJ, Smith U, HanneleYki-Järvinen, Mari A. Exenatide improves  $\beta$ -cell function up to 3 years of treatment in patients with type 2 diabetes: a randomised controlled trial. *Eur J Endocrinol.* 2016;175(4):345–352.
40. Engel S, Xu L, Andryuk P. Efficacy and tolerability of MK-0893, a glucagon receptor antagonist (GRA) in patients with type 2 diabetes (T2DM). *Diabetes.* 2011;60(Suppl. 1):A85.
41. Valdecantos MP, Pardo V, Ruiz L, Castro-Sánchez L, Lanzón B, Fernández-Millán E, García-Monzón C, Arroba AI, González-Rodríguez Á, Escrivá F, Álvarez C, Rupérez FJ, Barbas C, Konkar A, Naylor J, Hornigold D, Santos AD, Bednarek M, Grimsby J, Rondinone CM, Valverde AM. A novel glucagon-like peptide 1/glucagon receptor dual agonist improves steatohepatitis and liver regeneration in mice. *Hepatology.* 2017;65(3):950–968.
42. Van Harken DR, Dixon CW, Heimberg M. Hepatic lipid metabolism in experimental diabetes. V. The effect of concentration of oleate on metabolism of triglycerides and on ketogenesis. *J Biol Chem.* 1969;244(9):2278–2285.
43. Quant PA, Tubbs PK, Brand MD. Glucagon activates mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in vivo by decreasing the extent of succinylation of the enzyme. *Eur J Biochem.* 1990;187(1):169–174.
44. Habegger KM, Heppner KM, Geary N, Bartness TJ, DiMarchi R, Tschöp MH. The metabolic actions of glucagon revisited. *Nat Rev Endocrinol.* 2010;6(12):689–697.
45. Habegger KM, Stemmer K, Cheng C, Müller TD, Heppner KM, Ottaway N, Holland J, Hembree JL, Smiley D, Gelfanov V, Krishna R, Arafat AM, Konkar A, Belli S, Kapps M, Woods SC, Hofmann SM, D'Alessio D, Pfluger PT, Perez-Tilve D, Seeley RJ, Konishi M, Itoh N, Kharitonov A, Spranger J, DiMarchi RD, Tschöp MH. Fibroblast growth factor 21 mediates specific glucagon actions. *Diabetes.* 2013;62(5):1453–1463.
46. Gálman C, Lundåsen T, Kharitonov A, Bina HA, Eriksson M, Hafström I, Dahlin M, Amark P, Angelin B, Rudling M. The circulating metabolic regulator FGF21 is induced by prolonged fasting and PPAR $\alpha$  activation in man. *Cell Metab.* 2008;8(2):169–174.
47. Nonogaki K, Hazama M, Satoh N. Liraglutide suppresses obesity and hyperglycemia associated with increases in hepatic fibroblast growth factor 21 production in KKAY mice. *Biomed Res Int.* 2014;2014:751930.
48. Yang M, Zhang L, Wang C, Liu H, Boden G, Yang G, Li L. Liraglutide increases FGF-21 activity and insulin sensitivity in high fat diet and adiponectin knockdown induced insulin resistance. *PLoS One.* 2012;7(11):e48392.
49. Badman MK, Pissios P, Kennedy AR, Koukos G, Flier JS, Maratos-Flier E. Hepatic fibroblast growth factor 21 is regulated by PPAR $\alpha$  and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metab.* 2007;5(6):426–437.