

## Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Greenfield JR, Miller JW, Keogh JM, et al. Modulation of blood pressure by central melanocortinergic pathways. *N Engl J Med* 2009;360:44-52. DOI: 10.1056/NEJMoa0803085.

## SUPPLEMENTARY INFORMATION

### A) Supplementary Tables

**Supplementary Table 1 – Functional characterization of MC4R mutations in patients studied.**

<b>Mutation</b>	<b>No. of subjects</b>	<b>Complete loss of function measured by cAMP production as reported previously</b>
A insertion codon 112	3	Yeo et al, Hum Mol Gen 2003;12; 561-574
CTCT deletion codon 211	4	Yeo et al, Hum Mol Gen 2003;12; 561-574
N97D	5	Yeo et al, Hum Mol Gen 2003;12; 561-574
L106P	3	Yeo et al, Hum Mol Gen 2003;12; 561-574
C271Y	11	Yeo et al, Hum Mol Gen 2003;12; 561-574
I125K	7	Yeo et al, Hum Mol Gen 2003;12; 561-574
Y35X;D37V	8	Farooqi et al, NEJM 2003; 348:1085-95
GT insertion codon 279	4	Farooqi et al, NEJM 2003; 348:1085-95
Y287X	1	Farooqi et al, NEJM 2003; 348:1085-95

**Supplementary Table 2. Metabolic and cardiovascular measurements in MC4R deficient subjects and obese controls undergoing detailed studies of autonomic function.**

	MC4R deficient subjects ( <i>n</i> = 8)	Control subjects ( <i>n</i> = 8)
Gender (M/F)	4/4	5/3
Age (yrs)	31.8 ± 14.6	36.7 ± 10.4
Body Mass Index (kg/m <sup>2</sup> )	43 ± 8.4	39.2 ± 2.9
Waist circumference (cm)	129 ± 18	120 ± 5
Waist-to-hip ratio	0.97 ± 0.10	1.01 ± 0.05
Fasting blood glucose (mmol/L)	4.7 ± 0.4	4.8 ± 0.6
Fasting plasma insulin (pmol/L)	119 (70 – 202)	103 (66 – 163)
Fasting total cholesterol (mmo/L)	4.7 ± 0.6	5.1 ± 1.4
Fasting LDL cholesterol (mmo/L)	3.1 ± 0.5	3.3 ± 1.1
Fasting HDL cholesterol (mmol/L)	0.9 ± 0.3	1.1 ± 0.3
Fasting triglycerides (mmol/L)	1.4 (0.8 – 2.3)	1.4 (0.8 – 2.5)
Fasting NEFAs (μmol/L)	390 ± 121	347 ± 61
Systolic blood pressure (mmHg)	123 ± 17	130 ± 11
Diastolic blood pressure (mmHg)	71 ± 8	75 ± 9

Data are mean ± SD or geometric mean (1 SD range). NEFAs = non-esterified fatty acids.

**Supplementary Table 3. Characteristics of overweight/obese volunteers in pharmacological study**

<b>Parameter</b>	<b>Mean (SD)</b>
<b>Cohort A</b>	<b>(N=9)</b>
Age (years)	44.0 (14.90)
Body weight (kg)	82.4 (14.79)
Height (cm)	165.4 (10.33)
Body mass index (kg/m <sup>2</sup> )	29.88 (2.317)
Waist circumference (cm)	100.2 (13.20)
<b>Cohort B</b>	<b>(N=10)</b>
Age (years)	43.4 (15.71)
Body weight (kg)	88.2 (17.57)
Height (cm)	172.0 (11.90)
Body mass index (kg/m <sup>2</sup> )	29.52 (2.716)
Waist circumference (cm)	100.7 (11.23)
<b>Cohort C</b>	<b>(N=9)</b>
Age (years)	39.1 (14.50)
Body weight (kg)	91.8 (10.65)
Height (cm)	173.8 (7.68)
Body mass index (kg/m <sup>2</sup> )	30.39 (3.342)
Waist circumference (cm)	102.9 (8.72)

**Supplementary Table 4. Body Composition and Fat Distribution in MC4R deficient subjects and obese controls undergoing detailed studies of autonomic function.**

	<b>MC4R deficient subjects (n = 8)</b>	<b>Control subjects (n = 8)</b>
<b><i>DEXA</i></b>		
<b>Total body fat (kg)</b>	<b>46.7 ± 11.5</b>	<b>48.1 ± 6.8</b>
<b>Total body fat (%)</b>	<b>41.8 ± 7.4</b>	<b>42.4 ± 6.5</b>
<b>Trunk fat (kg)</b>	<b>25 ± 5.3</b>	<b>26.1 ± 4.1</b>
<b>Trunk fat (%)</b>	<b>44.6 ± 6.2</b>	<b>45.9 ± 5.5</b>
<b>Central fat (kg)</b>	<b>4 ± 0.8</b>	<b>4 ± 0.7</b>
<b>Central fat (%)</b>	<b>46.6 ± 4.3</b>	<b>46.7 ± 5.3</b>
<b>Fat-free mass (kg)</b>	<b>65.2 ± 13.5</b>	<b>65.9 ± 11.6</b>
<b>Fat-free mass (%)</b>	<b>58.1 ± 7.5</b>	<b>57.6 ± 6.5</b>
<b><i>MRI</i></b>		
<b>IAAT (cm<sup>2</sup>)</b>	<b>148 ± 58</b>	<b>149 ± 57</b>
<b>SAAT (cm<sup>2</sup>)</b>	<b>566 ± 150</b>	<b>506 ± 107</b>
<b>IAAT/SAAT ratio</b>	<b>0.28 ± 0.13</b>	<b>0.32 ± 0.17</b>
<b>Liver fat (%)</b>	<b>11 ± 2</b>	<b>13 ± 7</b>

**Data are mean ± SD. IAAT = intra-abdominal adipose tissue. SAAT = subcutaneous abdominal adipose tissue.**

**Supplementary Table 5.** Number of subjects reporting most frequent adverse events during infusion of LY2112688.

Adverse event	LY2112688	Placebo
<b>General symptoms</b>		
headache	31	4
asthenia	17	4
hot flush	9	3
myalgia	3	0
dizziness	3	0
<b>Gastrointestinal symptoms</b>		
nausea	19	0
vomiting	6	0
abdominal pain	7	4
abdominal pain – upper/lower	6	1
diarrhoea	13	3
decreased appetite	4	0
eructation	3	0
<b>Melanocortinerbic responses</b>		
musculoskeletal stiffness	18	3
yawning	15	4
libido/erection increased	9	0

**Legend:** Spontaneous adverse events reported more commonly during LY2112688 vs. control infusion periods, regardless of assigned causality, are tabulated. Yawning, muscular stiffness and libido/erectile effects were considered to be expected with agonism of the melanocortinerbic system. Headache, asthenia and gastrointestinal symptoms were dose-dependent and 1 mg/day was considered to be the maximum well-tolerated dose. One subject with a mild baseline elevation of alanine aminotransferase experienced a marked elevation of ALT and AST without hyperbilirubinemia which resolved without sequelae. During the 7 day infusion, the need to stretch/yawn was significantly increased at all doses (0.15-1.0 mg/day). A significant increase in male erectile function was observed at the 0.45 and 1.0 mg/day doses only.

**Supplementary Table 6.** Change in fasting blood pressure and heart rate compared to placebo control on Days 2-7 of LY2112688 infusion.

Treatment	SBP (mmHg)	p-value	DBP (mmHg)	p-value	Heart Rate (bpm)	p-value
0.15 mg/day	6.08	<0.01	4.02	<0.01	2.91	0.01
0.45 mg/day	2.82	0.04	1.26	0.12	0.8	0.47
1.0 mg/day	7.09	<0.01	4.9	<0.01	2.85	0.01

**Legend:** Blood pressure and heart rate were measured each morning from Day 2 (24hr) to Day 7 (168hr). Change from time-matched placebo control measures were calculated for each of the 6 days and averaged to provide an estimate of hemodynamic effects during the extended infusions.

**Supplementary Table 7.** Mean plasma insulin, glucose and urinary norepinephrine (NE) levels in overweight/obese volunteers treated with placebo (PL) vs the melanocortin agonist LY2112688 at different doses.

	PL	0.05mg	0.15mg	0.45mg	1.0 mg	2.0mg	p-value
Insulin ( $\mu$ U/mL))	7.29	6.46	7.76	7.07	7.02	6.87	0.580
Glucose (mmol/L)	4.82	4.87	4.91	4.71	4.98	5.25	0.133
Urinary NE (ug/24hr)	26.91	30.26	28.77	30.34	30.24	29.66	0.489

## **B) Supplementary Methods**

### **Methods for studies in MC4R deficiency**

All subjects in these studies satisfied the following inclusion criteria: no historical or biochemical evidence of diabetes, renal, liver or thyroid disease, average alcohol intake <2 units/day, not participating in an organised exercise program, not treated with anorectic agents, medications known to affect carbohydrate and/or lipid metabolism or anti-hypertensive medication.

### **Measurement of blood pressure and autonomic nervous system activation**

Blood pressure was measured after a 30 minute rest period using an automated brachial blood pressure monitor (GE PRO 300V2/400 DINAMAP ®, GE Medical Systems Information Technologies, Tampa, FL) or a wrist-type blood pressure monitor (OMRON Healthcare, Hamburg, Germany). Urine samples were collected to quantify 24-hour catecholamine excretion using standard assays. Heart rate and heart rate variability were measured using a combined heart rate and movement sensor (Actiheart, Cambridge Neurotechnology, UK), which was applied to the chest wall. This digitalizes the ECG signal and stores the R-R interval time-series from which heart rate and beat-to-beat variation can be calculated (S1). These measurements were made during three distinct states; asleep (overnight from 0030 – 0530 hrs); baseline awake (30-min rest period); and during six consecutive 15-minute periods throughout the hyperinsulinemic-euglycemic clamp (starting 15 minutes after commencement of the clamp, with the last period corresponding to steady-state). During each of these time periods, we calculated median heart rate (in beats per minute, bpm), as well as time and frequency domain measures of heart rate variability. Power spectral density analysis using fast Fourier transformation, typically identifies a low frequency (LF) power component (0.04–0.15 Hz), which

reflects both sympathetic and parasympathetic effects on the sinus node, and a distinct high frequency (HF) power component (0.15–0.4 Hz), which mainly reflects parasympathetic activity. We calculated the root mean square of successive differences (RMSSD) between adjacent normal R-R intervals, a time domain measure of heart rate variability which reflects parasympathetic activity (S2). The investigator who analysed the heart rate data was blinded to genotype.

### **Body composition, abdominal and liver fat measurement**

Weight and height were measured barefoot in light clothing. Body surface area was determined using the Dubois and Dubois formula (S3). Whole-body dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy, GE Medical Systems, Madison, WI) was performed to determine total body and regional (trunk, central abdominal, arm and leg) fat mass and fat-free mass. Central abdominal fat was quantified as previously published (S4). Abdominal and liver fat estimation was performed using a whole body 1.5 Tesla clinical MRI system (GE Healthcare, Milwaukee, USA). Analysis was performed by two operators with no knowledge of patient genotype.

### **MRI measurement of subcutaneous and visceral fat**

Subjects were examined supine using an 8 channel body array receive coil. Contiguous T1 weighted MR images were obtained from the diaphragm to the pubic symphysis using 1.5 Tesla Magnetic Resonance Imaging. A large enough field of view was chosen so that the entire outline of the torso was demonstrated. From this data set, the image which was closest to the level of the umbilicus, immediately superior to the superior iliac crests, was selected for further analysis. This image was transferred to a stand-alone workstation where the images were assessed by an experienced radiologist and a technician working in consensus. By identifying and excluding muscle, bone, blood vessels and gastrointestinal structures, the

SliceOmatic® (Tomovision, Montreal, Canada), V4.3 software allowed measurement of the cross sectional area of intra-abdominal fat in the subcutaneous and intra-abdominal compartments. This technique relies on anatomical knowledge to identify non adipose structures rather than the original CT methodology which relied on attenuation values (S5).

### **MRI measurement of liver fat**

Liver fat estimation was performed using a validated imaging based method (S6). Three axial image sets were obtained at the same locations through the liver, positioned centrally in the cranio-caudal direction. Each set was acquired during a single breath-hold of 18-20 seconds and comprised four 10 mm sections spaced 1.5mm apart using a 38-42cm field of view. The first set used an in and out of phase spoiled gradient echo sequence with a 20 degree excitation flip angle and the following parameters: TR 180msec, TE 2.2 and 4.4 msec, matrix 256x128, 1 signal average. The second set used identical parameters except for a 70 degree excitation flip angle. The third set used a multi-echo gradient echo sequence acquiring 16 echoes at each location using the following parameters: TR 120msec, flip angle 20 degrees, TE in and out of phase from 2.2msec to 35.2msec, matrix 192x128, 0.5 signal averages. This final set was used to generate T2\* maps used for correction of the in-phase signal measurements. Analysis was performed using an in-house program developed in IDL (RSI Inc, Boulder, USA). Three circular regions of interest (ROIs) were positioned in the liver on each of the four images of the first data set. These were then used to obtain the relevant data from the matching locations in the second set and the T2\* maps of the third data set. The program then performed T2\* correction of the inphase signal intensities and by comparing the 20 and 70 degree data sets determined whether the fat fraction exceeded 50%. Using the T2\*

correction and the 50% check the ROI data from the 20 degree data set was then analysed to provide the mean and standard deviation for liver fat.

### **Hyperinsulinaemic-euglycaemic and hyperglycaemic clamps**

Participants were admitted to the Clinical Research Facility the morning prior to clamp studies, having been instructed to avoid strenuous physical activity and alcohol intake for 48 hours. Throughout admission, subjects were fed a standardized macronutrient intake (50% carbohydrate, 30% fat and 20% protein) according to energy requirements. Following a supervised 10-hr overnight fast, a 60 minute intravenous glucose tolerance test (IVGTT) immediately followed by a 120 minute hyperinsulinemic ( $80 \text{ mU/m}^2/\text{min}$ )-euglycemic clamp were undertaken to quantify insulin secretion and insulin sensitivity respectively on the same day, as previously described(S7). Two intravenous cannulae were inserted into large antecubital veins on opposite arms, one for infusion of glucose and insulin and the other for blood sampling. The sampling line was contained in a heating blanket throughout the clamp study to 'arterialize' venous blood samples. Following collection of 3 basal fasting blood samples (5 min apart), a 25g 25% intravenous glucose bolus was administered over 1 minute at  $t = 0$  min. Blood samples were taken for glucose and insulin at  $t = 1, 2, 3, 4, 6, 8, 10, 20, 30, 40, 50$  and 60 min. Immediately following collection of the last sample, a 120 min hyperinsulinaemic ( $80 \text{ mU/m}^2/\text{min}$ )-euglycaemic (5 mmol/L) clamp was commenced, with euglycaemia maintained by a variable-rate 20% dextrose infusion. Blood glucose levels were checked 5-minutely and the glucose infusion rate was adjusted using an iterative computer program (courtesy of TM Wallace). Clamp steady-state was defined as the final 30 minutes of the clamp. Serum non-esterified fatty acids (NEFAs) and adiponectin were measured at baseline and during clamp steady-state period. Mean intra-subject coefficient of

variation for GIR during clamp steady-state was 3% in the MC4R-deficient and 4.5% in the control group. In the hyperglycaemic clamp, hyperglycaemia (target blood glucose level 10.8 mmol/L) was initiated by a bolus of 25% dextrose and was maintained at this level by a variable-rate infusion of 20% dextrose for a total of 120 min. Indirect calorimetry was performed at baseline and during the hyperinsulinemic-euglycemic clamp steady-state period using an open-circuit, ventilated hood system (Europa Gas Exchange Monitor, Nutren Tech Ltd, Manchester, UK). Substrate oxidation rates were quantified as previously described (S8). Non-oxidative glucose disposal (storage) was calculated by subtracting carbohydrate oxidation rate from the rate of whole-body glucose utilization during clamp steady-state.

### **Analytical methods**

During the clamp, blood glucose was measured at the bedside by the glucose-oxidase method (YSI 2300, Yellow Springs Instruments, OH). Plasma glucose and immunoreactive insulin and serum lipids were measured using standard commercially available assays. Non-esterified fatty acids were measured by colorimetry using Roche Applied Science reagents adapted for use on a DAD-Behring Dimension analyzer, with an intra-assay CV of 4.8 – 7.1%. Urinary catecholamines were measured by HPLC (intra-assay CV 4%). For the purpose of analysis, samples with levels below the sensitivity of the assay were assigned a value equal to the lower limit of detection.

### **Definitions and calculations**

Hypertension was defined as systolic blood pressure  $\geq 140$  mmHg and/or diastolic blood pressure  $\geq 85$  mmHg or treatment with anti-hypertensive medication. Type 2 diabetes and impaired glucose tolerance were defined using American Diabetes

Association criteria. Insulin-stimulated whole-body glucose disposal (M value) was calculated as the mean glucose infusion rate during the last 30 minutes of the hyperinsulinemic-euglycemic clamp. Post-hepatic insulin clearance (mL/min/m<sup>2</sup>) was calculated by dividing the insulin infusion rate by the mean incremental steady-state insulin level. During the 60 min IVGTT, first-phase insulin secretion was defined as the mean of five plasma insulin levels measured every 2 min from 2–10 minutes and second phase insulin secretion as the mean of six plasma insulin levels measured every 10 min from 10–60 min. During both of these periods, we also calculated insulin area under the curve (AUC<sub>0-10 min</sub> and AUC<sub>10-60 min</sub>) using the trapezoidal method. During the hyperglycemic clamp, second-phase insulin secretion was defined as the mean of the plasma insulin levels measured every 10 min from 30–120 min. The Disposition Index (μmol/kg FFM/min) was calculated as the product of insulin sensitivity (M/I; where I is mean plasma insulin concentration during clamp steady state) and first-phase insulin secretion.

### **Agonist administration - study design**

LY2112688 (acetyl-D-arginyl-[L-cysteinyl-L-glutamyl-L-histidinyl-D-phenylalanyl-L-arginyl-L-tryptophanyl-L-cysteine]-amide) is a synthetically produced cyclic peptide containing 8 amino acid residues. LY2112688 has high affinity (K<sub>i</sub> = 0.50 nM) for the human MC4R and lower affinity for other human melanocortin receptors; MC1R (K<sub>i</sub> = 15.3 nM), MC3R (K<sub>i</sub> = 53.4 nM), and MC5R (K<sub>i</sub> = 1723 nM). In cell based assays, LY2112688 demonstrated both MC4R- and MC3R-mediated cAMP accumulation, with approximately 6-fold greater potency for MC4R vs. MC3R mediated functional response. LY2112688 produces significant weight loss in 14-day studies using the diet-induced obesity (DIO) rat model. This study was a Phase 1 dose-escalation study of LY2112688 in anticipation of clinical development for the

treatment of obesity. Based on allometric scaling from the rodent weight loss model, the minimally efficacious dose was expected to be 0.11 to 0.3 mg/d in an 80 kg human. Three cohorts of 9-10 subjects underwent five treatment periods each. In Periods 1-3 subjects received two 24hr subcutaneous infusions of LY2112688 and one of placebo, separated by a 3-day washout periods. In Periods 4 and 5, each subject received one 7-day infusion of LY2112688 and one of placebo, separated by a 7-day washout period. The cohorts were studied in a staggered parallel dose-escalation fashion. Cohort A received 0.05-0.15 mg/day. Cohort B received 0.45-1.0 mg/day. Cohort C received 0.45-2.0 mg/day. The proposed maximum dose for Cohort C was reduced after dose-limiting nausea was encountered at 2 mg/day in Periods 1-3. LY2112688 was administered by continuous subcutaneous infusion using MiniMed®, Medtronic insulin pumps.

### **Blood pressure and biochemical measurements**

Sitting blood pressure and brachial pulse rate were measured by automated device (Critikon® Dinamap PRO 100, GE Healthcare, Buckinghamshire, UK) at 0, 1, 3, 6, 12 and 24 hr during Periods 1-3 using a large cuff. The arm was elevated to the level of the heart and subjects rested for 10 minutes in a quiet environment prior to measurement. Four readings were obtained at 2 minute intervals, and the average of the latter three measures was used at each time point. Blood pressure and pulse rate were measured each morning under fasting conditions on Days 2-7 during Periods 4-5.

Fasting blood samples were obtained at the end of the 24hr infusions for insulin, glucose, and clinical safety lab tests at the end of each treatment period. Urine was collected and analyzed for 24hr norepinephrine excretion. Biochemical assays were performed at Covance and Esoterix clinical laboratories using validated assays.

Plasma concentrations of LY2112688 were assayed using a validated LC/MS/MS method. A questionnaire was used to record regarding feelings of yawning/stretching, sexual libido, and penile erections on the basis of cumulative experience during the 7-day infusion.

### **Statistical analysis**

Unless specified otherwise, normally distributed data are expressed as mean  $\pm$  SE and skewed data (insulin, Disposition Index, triglycerides, urinary epinephrine and heart rate variability parameters) as geometric mean (1 SE range). Non-normally distributed variables were analysed after logarithmic transformation. Differences between groups were examined using unpaired Student's t-tests. IVGTT-derived insulin secretion data were analysed using repeated-measures analysis of variance (ANOVA). Chi-square tests were used to compare categorical variables. Heart rate and heart rate variability responses during sleep, the baseline awake period and the hyperinsulinemic-euglycemic clamp were compared using repeated measures analysis of variance (ANOVA) with robust standard errors, adjusting for age, gender and BMI. Statistical analyses were conducted using Statview (version 5.0.1) and Stata software (version 9.2 SE). P values  $<$  0.05 (two-sided) were considered statistically significant. In the pharmacological study, a repeated-measures ANCOVA model was used on blood pressure data from all periods with time as a factor, Subject and Subject\*Period as random effects, and the pre-dose measurement as a covariate. Arithmetic mean differences from placebo ( $\pm$  SEM) are presented at each scheduled timepoint. Insulin, glucose and norepinephrine concentrations were log transformed and analysed using a repeated-measures ANOVA model with treatment as factor, and subject as random effect.

## Melanocortin agonist administration - study protocol synopsis

<b>Title of Study:</b> Safety and Tolerability of 24-hour and 7-day Subcutaneous Infusion of LY2112688 in Healthy Overweight or Obese Subjects
<b>Number of Investigator(s):</b> This single-centre study included 1 principal investigator.
<b>Study Centre:</b> The study was conducted at a single study centre: Biotrial, 7-9 rue Jean-Louis Bertrand, Technopole Atalante Villejean, 35000 Rennes, France.
<b>Objectives:</b> <u>Primary Objective:</u> To assess safety and tolerability of 24-hour and 7-day subcutaneous infusion of LY2112688 in otherwise healthy, overweight or obese subjects. <u>Secondary Objectives:</u> (i) To characterize the pharmacokinetics of LY2112688 following 24-hour and 7-day administration in healthy overweight/obese subjects; (ii) To explore potential exposure-response relationships of LY2112688 effects on caloric intake and hemodynamic parameters, (iii) Exploratory assessment of melanocortin-related endocrine parameters and urinary norepinephrine.
<b>Study Design:</b> This was a subject- and investigator-blind, placebo-controlled, dose-escalating, crossover, inpatient study with short-term (24-hour) and extended-term (7-day) continuous subcutaneous infusion of LY2112688.

**Number of Subjects Receiving Drug:**

Cohort A – 24-hour short-term (Treatment Periods 1 through 3)

0.05 mg/day, 0.15 mg/day, placebo: Male 4, Female 5, Total 9

Cohort A – 7-day extended-term (Treatment Periods 4 and 5)

0.15 mg/day, placebo: Male 4, Female 5, Total 9

Cohort B – 24-hour short-term (Treatment Periods 1 through 3)

0.45 mg/day, 1 mg/day, placebo: Male 4, Female 4, Total 8

Cohort B – 7-day extended-term (Treatment Periods 4 and 5)

1 mg/day: Male 5, Female 4, Total 9

Placebo: Male 4, Female 4, Total 8

Cohort C – 24-hour short-term (Treatment Periods 1 through 3)

1 mg/day, 2 mg/day, placebo: Male 7, Female 1, Total 8

Cohort C – 7-day extended-term (Treatment Periods 4 and 5)

0.45 mg/kg, placebo: Male 8, Female 1, Total 9

**Diagnosis and Main Criteria for Inclusion:** Overweight or obese healthy male or female (of non-childbearing potential) subjects, as determined by medical history and physical examination, between the age of 18 to 65 years, inclusive. Subjects had a BMI between 25 and 35 kg/m<sup>2</sup>, inclusive.

**Study Drug, Dose, and Mode of Administration:**

LY2112688 for injection, supplied as 12 mg white lyophilized powder in single dose vials. Dose levels were 0.05 mg/day, 0.15 mg/day, 0.45 mg/day, 1 mg/day or 2 mg/day.

**Duration of Treatment:**

Subjects were randomized to Cohorts A, B or C. Within each cohort, subjects were administered LY2112688 at two dose levels and placebo as 24-hour short-term subcutaneous infusions (Treatment Periods 1, 2 and 3) and LY2112688 at one dose level and placebo as 7-day extended-term infusions (Treatment Periods 4 and 5). After each 24-hour short-term infusion, there was a washout interval of between 3 to 6 days before starting the next treatment period. A washout interval of 7 days separated each extended-term infusion period. The study duration was approximately 7 weeks.

**Variables**

Safety: Physical examination, vital signs, electrocardiograms (ECG), clinical laboratory tests (hematology, urinalysis, clinical chemistry, serology), body weight, adverse events, troponin samples, infusion site assessments.

Pharmacokinetic: Primary parameters for analysis of LY2112688 were concentration during constant rate infusion at steady-state ( $C_{SS}$ ), time to reach  $C_{SS}$ , area under the concentration-time curve during the first 24-hour infusion period ( $AUC_{0-24}$ ), and area under the concentration-time curve during a 24-hour interval at steady-state ( $AUC_{\tau,SS}$ ).

Pharmacodynamic: Caloric intake measurement, blood pressure and heart rate measurements, psychometric measurements (Profile of Mood States (POMS™ Brief Form), Leeds Sleep Evaluation Questionnaire (LSEQ), Visual Analog Scales (VAS)), biochemical markers (alpha-MSH, ACTH, TSH, FT3, FT4, leptin, insulin, glucose and norepinephrine concentrations), symptoms questionnaire.

**Evaluation Methods:**

Safety: Safety data were summarized using descriptive methodology. QT interval data were analyzed using a repeated-measures analysis of covariance (ANCOVA) model which included RR interval as a covariate.

Bioanalytical: A validated liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was used to measure plasma concentrations of LY2112688.

Pharmacokinetic (PK): Pharmacokinetic parameters were calculated using non-compartmental analysis methods. A power model was used to assess dose proportionality of LY2112688.

Pharmacodynamic (PD): A repeated-measures analysis of variance (ANOVA) model was used to estimate treatment effects on food intake data. An ANCOVA model was used on averaged heart rate/blood pressure data. Results from the POMS, LSEQ and symptoms assessment questionnaires, as well as biochemical parameters, were analyzed using ANOVA models.

Pharmacokinetic/Pharmacodynamic: Exploratory PK/PD analysis was performed to link plasma LY2112688 concentrations with caloric intake and with hemodynamic response (blood pressure).

Contributions: For the study of subjects with MC4R deficiency; JRG, SOR and ISF designed the study, JRG, JMK, EH, SB, TKS, DJL and ISF gathered the data, JRG, SB, DJL, ISF analyzed the data, JRG and ISF vouch for the data and the analysis. For the pharmacological study; JWM, JHS, GSC designed the study, BA gathered the data, JWM, JHS, GSC and JPM analyzed the data, JWM vouches for the data and the analysis. JRG, JWM, SOR and ISF wrote the paper and all authors decided to publish the paper.

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