SUPPLEMENTAL MATERIAL

Supplemental Methods

Human study cohorts

***Cohort 1*:** This cohort was recruited as part of clinical research functional MRI studies at Imperial College London, UK. The participants (age >18y) included both sexes and individuals across diverse BMI categories: lean (n=30, BMI $\leq$25 kg/m^2), overweight (n=33, BMI >25 to 30 kg/m^2) and obese (n=27, BMI >30 kg/m^2, including n=9 with BMI >40 kg/m^2). The cohort had a median age = 33 years [interquartile range (IQR) 23.8, 41.0] (range 19-55), BMI = 27.0 kg/m^2 [IQR 24.4, 32.1] (range 19.1-53.1], % body fat = 31.4% [IQR 19.6, 40.3] (range 7.8-58.2), with 60.0% (n=54) females, 62.2% (n=56) of European Caucasian ethnicity, and 3.3% (n=3) with Type 2 diabetes mellitus (T2DM). Exclusion criteria for these studies included: (i) vegetarian, vegan, gluten or lactose intolerant, (ii) non-Western diet (assessed by dietary record), (iii) recent change in weight (>5% change in the preceding 3 months), (iv) current smoker or alcohol excess, (v) any history of significant cardiovascular disease (other than hypertension or hyperlipidemia), untreated endocrine disease, significant neurological or psychiatric disorder including addiction, stroke or epilepsy, current major depressive disorder, (vi) current eating disorder (e.g. anorexia nervosa or bulimia nervosa), (vii) T1DM, or current use of insulin or GLP-1 analogue, (viii) pregnancy or breastfeeding. A flow chart summary of the study design is presented in Supplemental Figure 5. Results in Figure 4B-C, E-L, Supplemental Figure 6 and Supplemental Figure 7 were generated from this study cohort.

***Expanded cohort 1 (Cohort 1Ex)**:* Fasting plasma LEAP2 concentrations (but not acyl-ghrelin) were also available from an additional n=15 adults with obesity, and were added to those from

Cohort 1 to create an expanded cohort of n=105 adults, including more participants with obesity: lean (n=30, BMI ≤25.0 kg/m^2), overweight (n=33, BMI >25.0 to 30.0 kg/m^2), obese (including n=21, BMI >30 to 40 kg/m^2 and n=21, BMI >40 kg/m^2). This expanded cohort had a median age = 35 years [IQR 25,45] (range 19-63), BMI = 27.9 kg/m^2 [IQR 24.9, 37.9] (range 19.1-55.5], with 63.8% (n=67) females, 70.5% (n=74) of European Caucasian ethnicity, and 7.6% (n=8) with T2DM. Results in Figure 4A and D were generated from Cohort 1Ex.

***Cohort 2*:** The second cohort of participants was recruited as part of a previously reported fMRI study (1) at UT Southwestern Medical Center and the Veterans Administration North Texas Health Care System at Dallas, TX, USA. In the study, plasma samples were collected from either women with obesity (BMI >35 kg/m^2) or age-matched normal weight women (control; BMI <25 kg/m^2). The median age of the women was 38.5 years [IQR 28, 47.5] (range 22-59) for women with obesity and was 45 years [IQR 37, 51] (range 30-55) for normal weight women. These women were part of a study designed primarily to determine the impact of obesity on brain activation in response to food images; the imaging part of that study was published previously (1). Exclusion criteria included: (i) untreated Axis I psychiatric diagnoses, (ii) previous serious head injury, (iii) left-handedness, (iv) contraindications to MRI, (v) prior bariatric surgery, or (vi) untreated severe medical illness. Of note, although the original report included 15 normal weight women and 15 women with obesity, for the present study, fasted and fed plasma samples from the initial study session visit were available for analysis from only 12 normal weight women. Also, for this study, samples were available from 20 women with obesity, some of whom were not part of the previous fMRI study. A flow chart summary of the study design is presented in Supplemental Figure 8. Results in Figure 5A-F were generated from Cohort 2.

Cohort 2A: A subset of the n=20 women with obesity in Cohort 2 individual underwent VSG surgery ~2 weeks after the initial study session visit (n=7). At their initial visit, this subset of women with obesity had mean age = 44.7 ± 3.9 years (range 34-59) and a BMI = 42.9 ± 2.1 kg/m^2 (range 35.9-51.9). Results in Figure 6F-I were generated from Cohort 2A.

Cohort 3**:** The third cohort was recruited from bariatric clinics at Imperial Weight Centre, St. Mary's Hospital, and Chelsea and Westminster Hospital, London, UK for clinical research studies at Imperial College London. The cohort contained only participants with obesity (BMI > 35 kg/m^2). This cohort of n=20 adults had mean age = 45.8 ± 2.5 years (range 19-63), a mean BMI = 45.0 ± 1.2 kg/m^2 (range 34.9-54.8), a median % body fat = 49.8% [IQR 47.5, 53.3] (range 29.1-55.2), with 90.0% (n=18) females, 50.0% (n=10) of European Caucasian ethnicity, and 30.0% (n=6) with T2DM. Plasma acyl-ghrelin results were unavailable for Cohort 3. Results in Figure 5G-L were generated from the study Cohort 3.

Cohort 3A: A subset of n=14 adults from Cohort 3 had fasted assessments both before and at ~3 months post-RYGB surgery, while n=8 were also studied at ~2 years post-RYGB surgery. At their initial visit, these participants had a mean age = 47.9 ± 2.6 years (range 31-63), mean BMI = 44.4 ± 1.1 kg/m^2 (range 38.6-50.9), a median % body fat = 51.1% [IQR 48.2, 53.3] (range 40.4-54.8), with 92.9% (n=13) females, 64.3% (n=9) of European Caucasian ethnicity, and 35.7% (n=5) with T2DM. Results in Figure 6A-D were generated from Cohort 3A.

Cohort 3B: A subset of n=11 of the Cohort 3 humans had post-prandial assessments ~3 months [median = 15 weeks [IQR 11.6, 16.7] (range 11.0-17.4)] after RYGB surgery. Results in Figure 6E was generated from Cohort 3B.

Processing of blood samples to measure glucose, acyl-ghrelin, and LEAP2

Blood samples in mice were collected between 10:00 AM and 11:00 AM by quick superficial temporal vein bleed into EDTA-coated microtubes kept on ice. For acyl-ghrelin measurement, the collection tubes contained either the protease inhibitors p-hydroxymercuribenzoic acid (Sigma Aldrich, St. Louis, MO; final concentration 1 mM; for acyl-ghrelin measurement) or aprotinin (Sigma Aldrich; final concentration 250 KIU/mL; for LEAP2 measurement). The samples were immediately centrifuged at 4°C at 1,500 g x 15 min. For acyl-ghrelin stabilization, HCl was added (1:10) to the p-hydroxymercuribenzoic acid-treated plasma to achieve a final concentration of 0.1 N. Blood glucose in mice was measured in untreated blood at the time of the bleeds using a Bayer Contour blood glucose monitoring system (Mishawaka, IN). Blood glucose measurements in diabetic mice higher than the detection limit of 600 mg/dL of the blood glucose monitoring system were noted as 600 mg/dL for the purposes of this study.

Human blood samples collected at UT Southwestern were added to ice-cold EDTA-containing BD vacutainers (Becton-Dickinson, Franklin Lakes, NJ; Catalog # 367841), after which they were processed with p-hydroxymercuribenzoic acid and HCl, as above, to obtain stabilized samples for acyl-ghrelin estimation. Blood samples from participants to estimate LEAP2 were added to EDTA-containing vacutainers (Becton-Dickinson; Catalog #367899), which were then centrifuged at 4°C at 1,200 g x 10 min.

Human blood samples collected at Imperial College London, UK for both LEAP2 and acyl-ghrelin were collected into chilled lithium heparin polypropylene tubes, containing 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (Sigma-Aldrich, Dorset, UK;

Catalog # A8456) and aprotinin (Nordic Pharma Ltd., Reading, UK) protease inhibitors to give final concentrations of 1 mg/mL and 200 KIU/mL, respectively. Blood samples were centrifuged at 4ºC at 4000 rpm for 10 min. Aliquots of separated plasma for the acyl-ghrelin assay were immediately acidified with HCl (final concentration of 0.05 N). All samples were stored at -80ºC until assay.

Determination of plasma LEAP2 and acyl-ghrelin levels

LEAP2 was estimated in both mouse and human plasma samples using the same LEAP2 (38-77) (Human)/LEAP2 (37-76) (Mouse) enzyme-linked immunosorbent assay (ELISA) kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, Catalog # EK-075-40). For LEAP2 estimation, mouse plasma samples were diluted 4X and human plasma samples were diluted 10X in the assay buffer. LEAP2 measurements for human plasma samples collected in Imperial College London, UK were performed at UT Southwestern in a blinded fashion. Duplicate LEAP2 measurements in human plasma samples (n=363) had a mean intra-assay co-efficient of variation (95% confidence intervals) of 6.4% (5.8,6.9).

Acyl-ghrelin concentrations in mouse plasma samples were determined by commercial ELISA kits (EMD Millipore, Billerica, MD; Catalog #EZRGRA-90K). Acyl-ghrelin concentrations for human plasma samples collected at UT Southwestern Medical Center and at the Veterans Administration North Texas Health Care System at Dallas, TX, USA also were determined by a commercial ELISA kit (EMD Millipore, Billerica, MD; Catalog #EZGRA-88K). For acyl-ghrelin measurement, human plasma samples were undiluted and mouse plasma samples were diluted 2X or 4X in assay buffer. Endpoint calorimetric measurements for the ELISA assays were performed using a BioTek PowerWave XS Microplate spectrophotometer and KC4 junior

software (Winooski, VT). Human plasma samples collected at Imperial College London were assayed for acyl-ghrelin in duplicate by a two-site sandwich ELISA at University of Virginia (UVa), USA (2). Ten human samples collected at Imperial College London, UK were assayed for acyl-ghrelin using both the two-site UVa ELISA and the Millipore ELISA kit, in a blinded fashion. Acyl-ghrelin concentrations determined using the Millipore ELISA kit showed excellent correlation with those determined using the UVa acyl-ghrelin ELISA (intra-class correlation coefficient = 0.85, p=0.004), though concentrations determined using the Millipore ELISA kit were higher than those determined by the UVa assay (Supplemental Figure 10).

Measurement of other hormones and analytes in human samples

Other assays in human samples were performed using unprocessed serum or fluoride oxalate-treated plasma sent immediately to the routine clinical laboratory. Plasma glucose and serum insulin and triglycerides were measured in the Department of Clinical Biochemistry, Imperial College Healthcare NHS Trust, London, UK using either an Architect ci8200 or Axsym analyser (Abbott Diagnostics, Maidenhead, UK). Insulin resistance was assessed using HOMA-IR as [glucose (mmol/L) x insulin (mU/L)] / 22.5 (3). To convert plasma glucose in mmol/L to mg/dL values were multiplied by 18. Results for HOMA-IR and serum triglycerides were calculated as the average of both fasting time points to improve reliability, while plasma glucose concentrations are presented only from the second time point when LEAP2 and acyl-ghrelin were measured.

Electrophysiology

Brain slices were prepared from 6 to 10-week old male NPY-hrGFP mice (4) using procedures described previously (5, 6). Briefly, male mice were deeply anesthetized with i.p. injection of 7%

chloral hydrate and transcardially perfused with a modified ice-cold artificial CSF (ACSF; containing 126 mM NaCl, 2.8 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 5 mM glucose). The mice were then decapitated, and the entire brain was removed and immediately submerged in ice-cold, carbogen-saturated (95% O_2 and 5% CO_2) ACSF. Coronal sections (250 μm) were cut with a Leica VT1000S Vibratome (Leica Biosystems, Buffalo Grove, IL, USA), and then incubated in oxygenated ACSF at room temperature for at least 1 h before recording. During recording, the slices were bathed in oxygenated ACSF (32–34°C) at a flow rate of ~2 ml/min.

The pipette solution for whole-cell patch-clamp recording, modified to include an intracellular dye (0.03 mM Alexa Fluor 350 hydrazide dye; Molecular Probes, Inc., Eugene, OR, USA; Catalog # A10439), contained 120 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 5 mM EGTA, 1 mM CaCl2, 1 mM $MgCl_2$, and 2 mM MgATP (pH 7.3). Epifluorescence was briefly used to target fluorescent cells, at which time the light source was switched to infrared differential interference contrast imaging to obtain the whole-cell recording (Zeiss Axioskop FS2 Plus equipped with a fixed stage and a QuantEM:512SC electron-multiplying charge-coupled device camera). Electrophysiological signals were recorded using an Axopatch 700B amplifier (Molecular Devices, San Jose, CA, USA), low-pass filtered at 2–5 kHz, and analyzed offline on a PC with pCLAMP programs (Molecular Devices). Membrane potential was measured by whole-cell current clamp recordings from NPY neurons in brain slices. Recording electrodes had resistances of 2.5–5 MΩ when filled with the K-gluconate containing internal solution.

To measure the activity on NPY neurons, acyl-ghrelin (Rat; Innovagen, Lund, Sweden; Catalog # SP-GHRL-1) and LEAP2 (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA; Catalog #

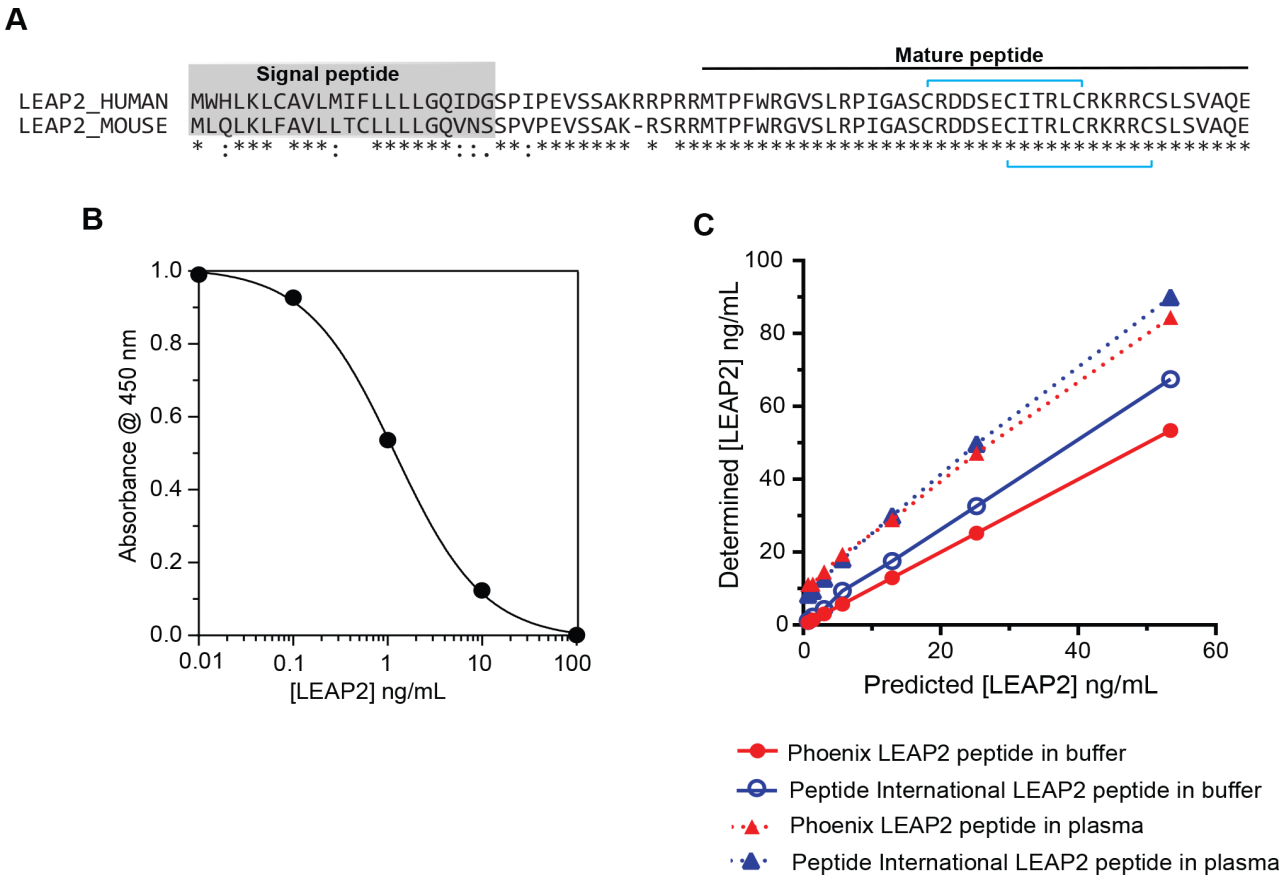
075-40) were added to the ACSF perfusing the brain sections. The peptide containing solutions were typically perfused for 5 min. A treatment effect was required to be associated temporally with peptide application, and the response had to be stable within a few minutes. A neuron was considered depolarized or hyperpolarized if a change in membrane potential was at least 2 mV in amplitude.

Quantitative PCR

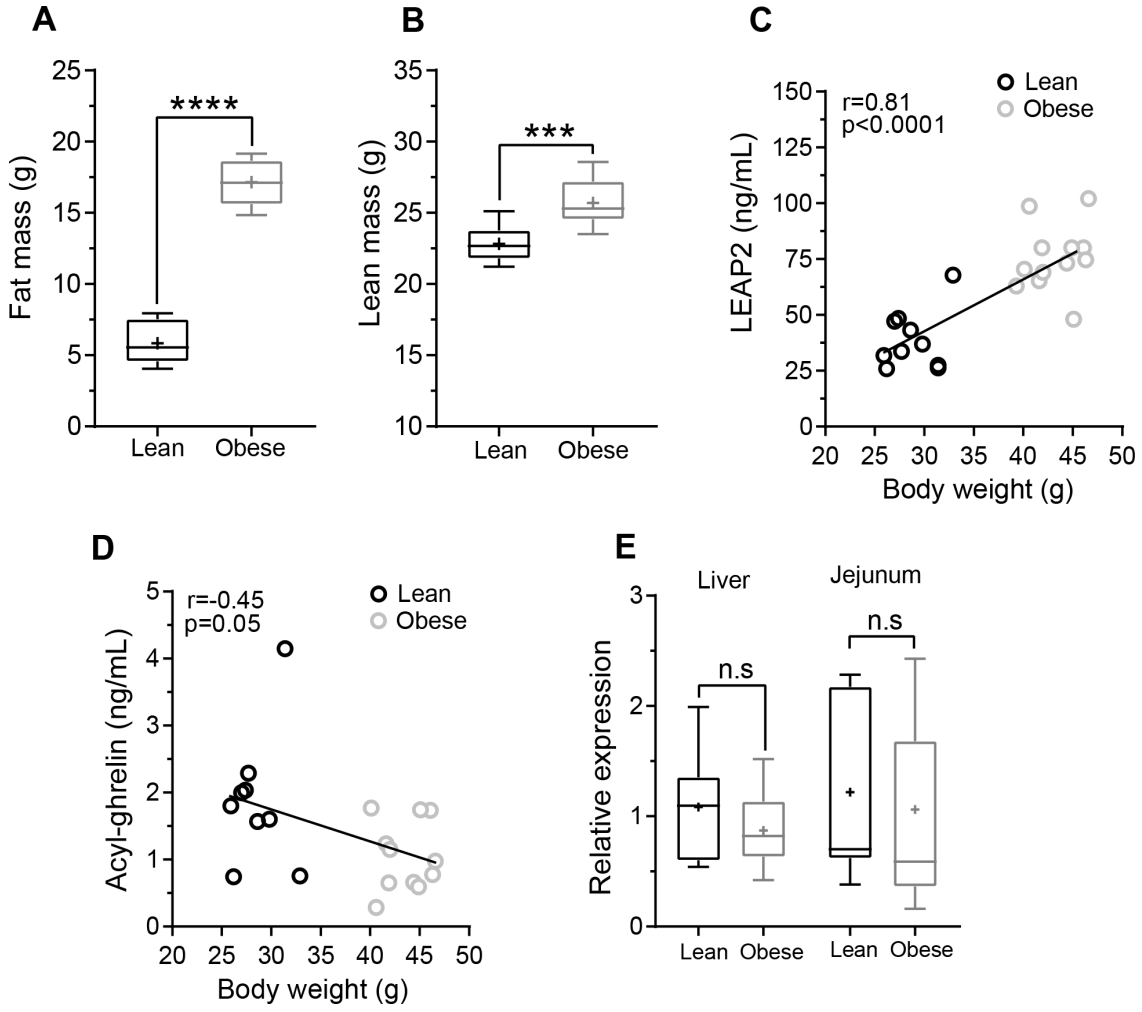
Samples of liver and jejunal mucosal cells (scraped from the middle third of the small intestine) were homogenized using 5 mm stainless steel beads in a TissueLyserII bead mill (Qiagen, Germantown, MD). Total RNA was isolated using the guanidium thiocyanate-phenol-chloroform extraction method by addition of RNA STAT-60 (Amsbio, Cambridge, MA). The isolated RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Total RNA (2 µg) was treated with ribonuclease-free deoxyribonuclease (Promega, Madison, WI, USA), and complementary DNA was synthesized by reverse transcription using SuperScript III (ThermoFisher, Grand Island, NY, USA). Primers used for LEAP2 [forward- 5'-GCTGCTGGGTCAGGTCAATAG-3', reverse- 5'-CCGGGATCTCTTTGCTGAAC-3'] were newly-designed and validated by analysis of template titration and dissociation curves. Quantitative PCR was performed using an Applied Biosystems 7900HT fast real-time system (Applied Biosystems, Foster City, CA, USA) and either SYBR green chemistry (for LEAP2) or TaqMan Gene Expression Master Mix (for 18S ribosomal RNA). The reaction mixture for qPCR contained the reverse-transcribed RNA, 150 nM of each primer or 1 µL 20X Assay on demand (AOD) and 5 µl of 2X iTaq Universal SYBR Green PCR master mix (Bio-Rad, Herculus, CA) or 2X TaqMan Gene Expression Master Mix (ThermoFisher). The mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method. They are represented relative to the invariant control gene, 18S

ribosomal RNA, and normalized to the values of the control group as indicated in the figure legends.

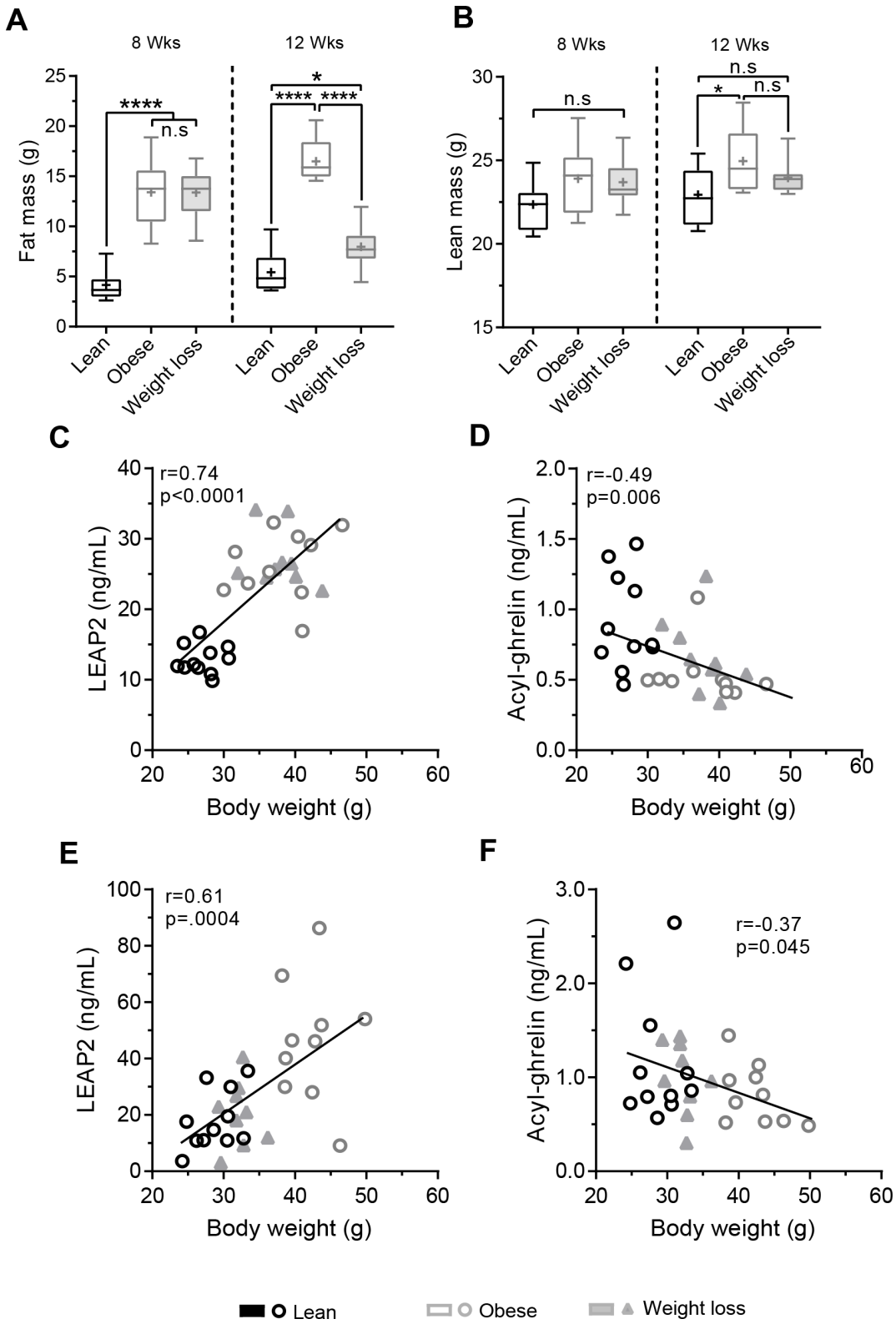
Supplemental Figures



Supplemental Figure 1: Validation of commercial LEAP2 ELISA kit to estimate plasma LEAP2 levels in humans and mice. (A) Alignment of LEAP2 peptide sequence of humans and mice using the Uniport protein alignment tool. LEAP2 is synthesized as a 77 amino acid prohormone in humans and a 76 amino acid prohormone in mice. The peptides have a classic signal peptide (gray shade). They are processed to mature peptides consisting of 40 amino acids with 2 disulfide bridges (blue lines) spanning 4 highly conserved cysteine residues. Asterisk "*" – indicates fully conserved amino acid, Colon ":" – indicates conservation between amino acids of strongly similar properties, and Period "." – indicates conservation between amino acids of weakly similar properties. Amino acids without symbols are not conserved. The mature peptide sequence is identical in mouse [LEAP2 (37-76)] and human [LEAP2 (38-77)]. (B) An example of the "competitive" LEAP2 ELISA standard curve generated with the LEAP2 (38-77) (Human)/LEAP2 (37-76) (Mouse) ELISA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, Catalog # EK-075-40), showing the dose response of the kit's LEAP2 peptide standard competing off the biotylated LEAP2 peptide, resulting in reduced absorbance at 450 nM. (C) Comparison of Phoenix Pharmaceuticals LEAP2 ELISA kit-estimated concentrations of LEAP2 peptide obtained from Phoenix Pharmaceuticals (catalog# 075-40) and LEAP2 peptide obtained Peptide International (catalog# PLP-4405-s) (Y-axis) to the corresponding known concentrations of LEAP2 in the assayed samples as determined from information provided by the manufacturers. Notably, as the plasma also contains LEAP2, adding the same known amounts of the synthetic LEAP2 peptide to mouse plasma (dotted lines), which also contains LEAP2 peptide, as to assay buffer (solid lines) results in an upward parallel shift in the dotted lines.

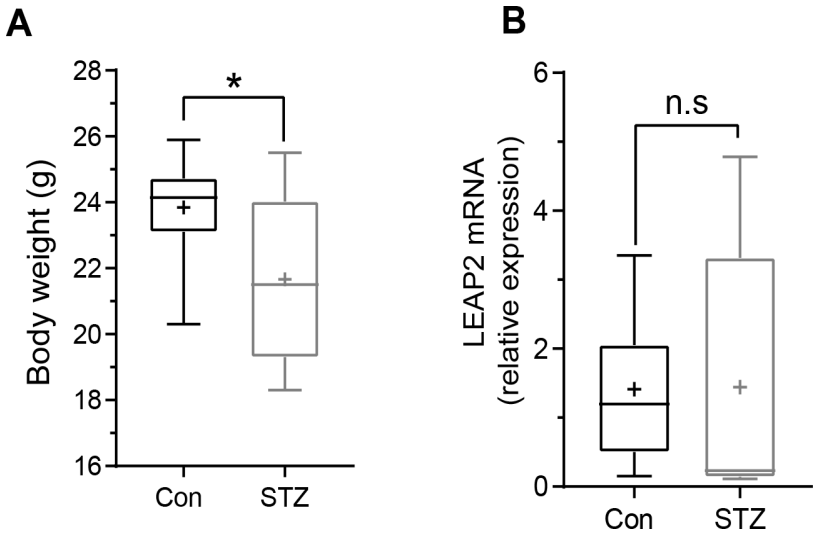


Supplemental Figure 2: Body composition, plasma LEAP2, and acyl-ghrelin responses to chronic HFD in mice. Fat mass (A) and lean mass (B) of lean and obese mice measured after 16 weeks of *ad libitum* access to standard chow or HFD, respectively. Relationships of plasma LEAP2 (C) and plasma acyl-ghrelin (D) with body weight at 16 weeks. (E) Relative LEAP2 mRNA expression in the liver and jejunum of the lean and obese mice at 16 weeks. Data were analyzed by Student's unpaired t-test (A,B,E), and Pearson's correlation co-efficient (r; C,D). n= 10-12.

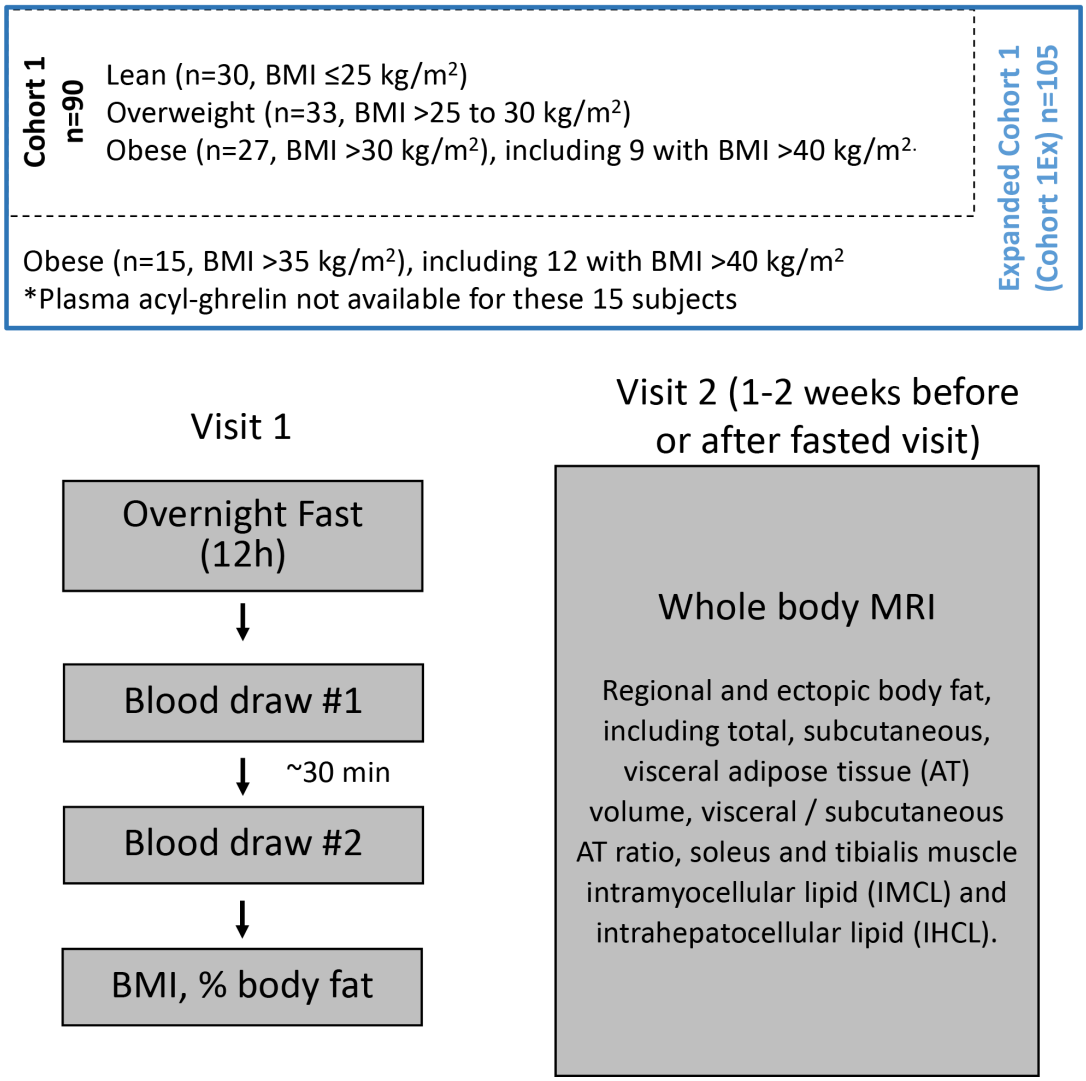


Supplemental Figure 3: Body composition, plasma LEAP2 and acyl-ghrelin responses to weight loss in DIO mice. Fat mass (A) and lean mass (B) measured at 8 weeks and 12 weeks in C57BL/6N mice fed chow for 12 weeks (lean), HFD for 12 weeks

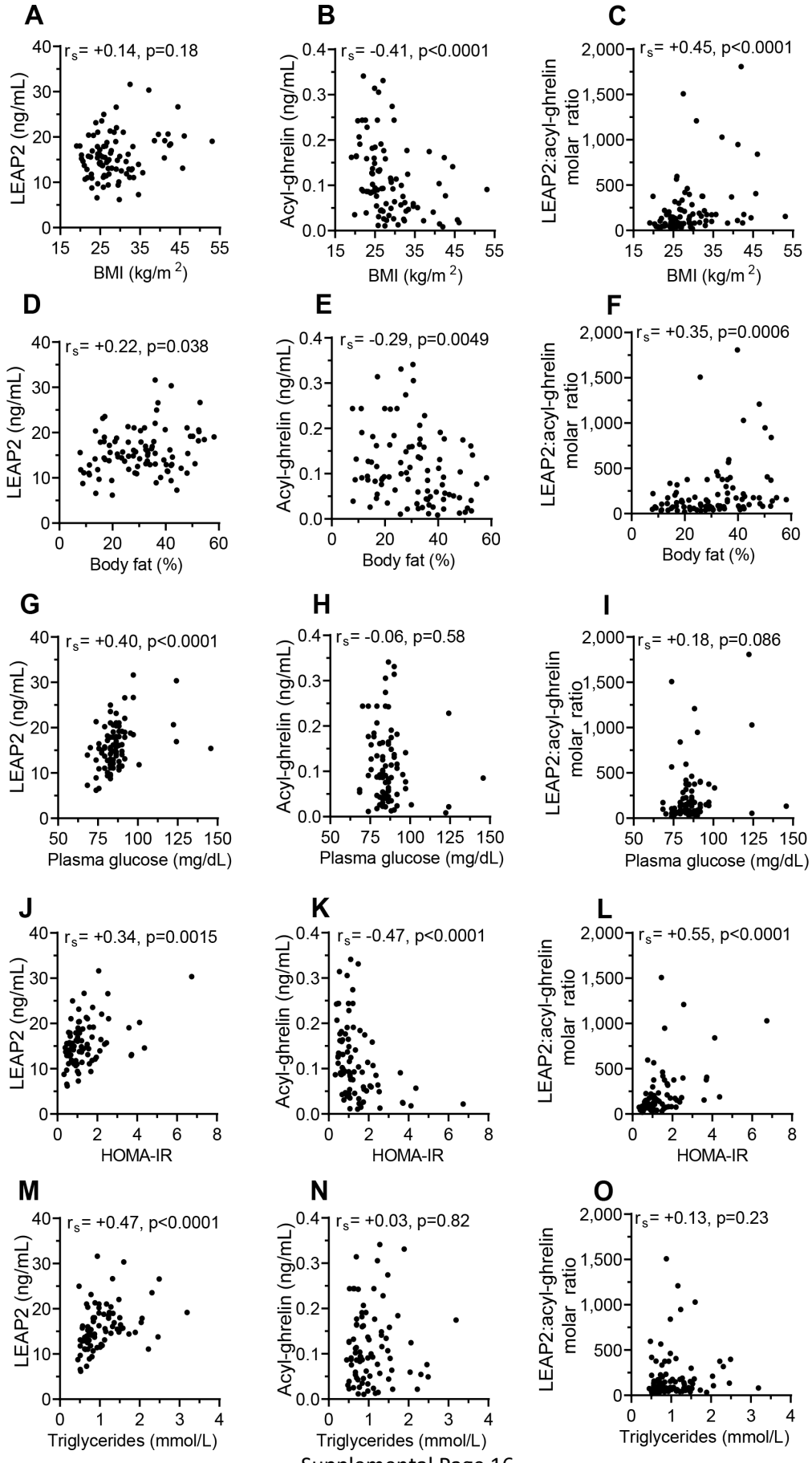
(obese), or HFD for 8 weeks to induce obesity followed by chow for 4 weeks to induce weight loss (weight loss). Relationships of plasma LEAP2 (C,E) and plasma acyl-ghrelin (D,F) with body weight of the mice, when measured at 8 weeks of the study (before the diet switch from HFD to chow in the weight loss group; panels C and D) and at 12 weeks of the study (4 weeks after the diet switch from HFD to chow in the weight loss group; panels E and F). Data were analyzed by one-way ANOVA followed by Sidak's post hoc test (A,B) or Pearson's correlation co-efficient (r; C-F). n= 9-11.



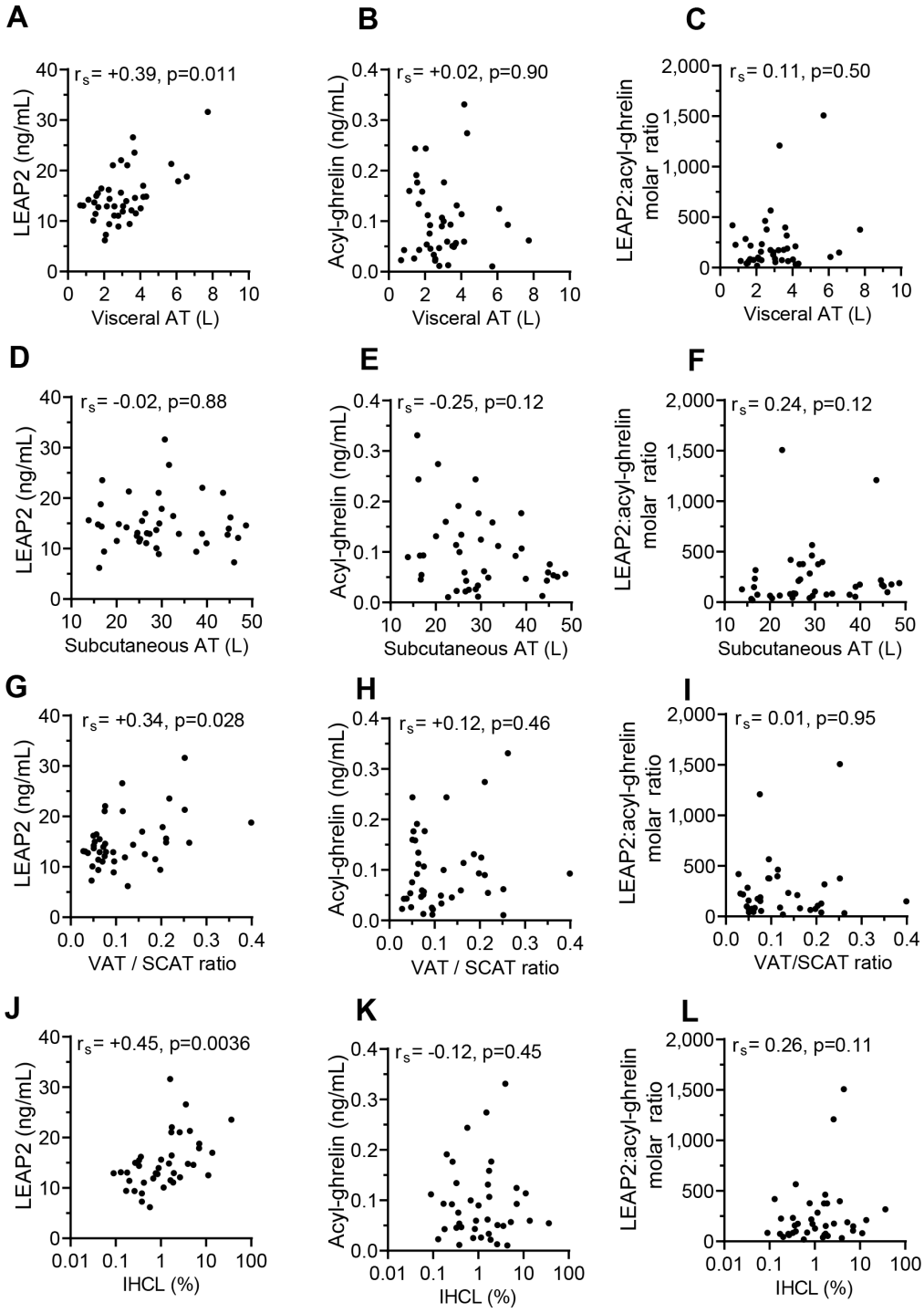
Supplemental Figure 4: Body weight and liver LEAP2 mRNA expression in a mouse STZ model of Type 1 diabetes mellitus. Body weight (A) and liver LEAP2 mRNA expression (B) in mice 6 days after STZ treatment or vehicle (Control; Con). Data were analyzed by Student's unpaired t-test. n=10-12.



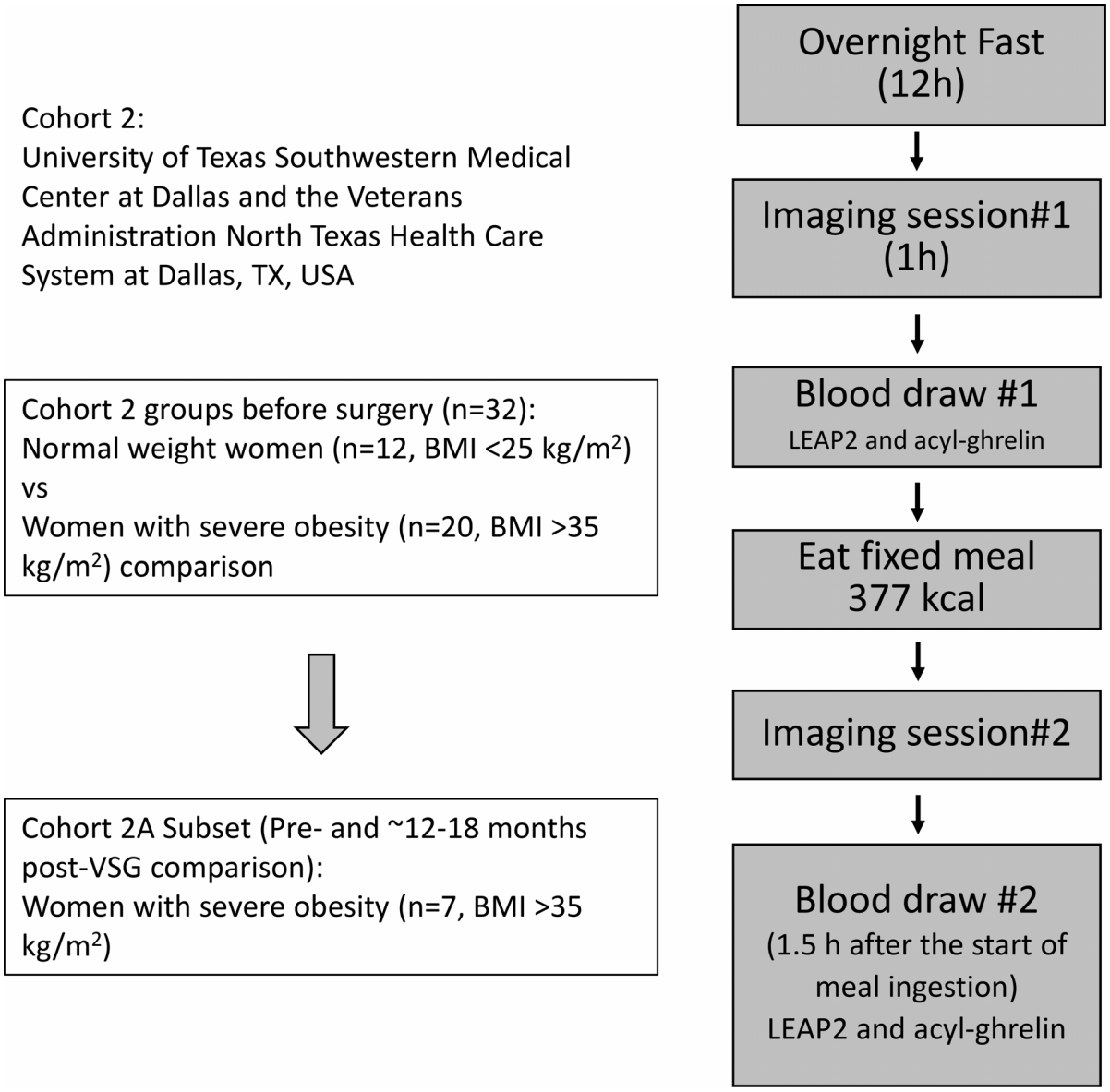
Supplemental Figure 5: Human Cohort 1 information. Characteristics of the patients with flow chart summary of the study design.


A
r_s= +0.14, p=0.18
LEAP2 (ng/mL)
BMI (kg/m^2)
B
r_s= -0.41, p<0.0001
Acyl-ghrelin (ng/mL)
BMI (kg/m^2)
C
r_s= +0.45, p<0.0001
LEAP2:acyl-ghrelin molar ratio
BMI (kg/m^2)
D
r_s= +0.22, p=0.038
LEAP2 (ng/mL)
Body fat (%)
E
r_s= -0.29, p=0.0049
Acyl-ghrelin (ng/mL)
Body fat (%)
F
r_s= +0.35, p=0.0006
LEAP2:acyl-ghrelin molar ratio
Body fat (%)
G
r_s= +0.40, p<0.0001
LEAP2 (ng/mL)
Plasma glucose (mg/dL)
H
r_s= -0.06, p=0.58
Acyl-ghrelin (ng/mL)
Plasma glucose (mg/dL)
I
r_s= +0.18, p=0.086
LEAP2:acyl-ghrelin molar ratio
Plasma glucose (mg/dL)
J
r_s= +0.34, p=0.0015
LEAP2 (ng/mL)
HOMA-IR
K
r_s= -0.47, p<0.0001
Acyl-ghrelin (ng/mL)
HOMA-IR
L
r_s= +0.55, p<0.0001
LEAP2:acyl-ghrelin molar ratio
HOMA-IR
M
r_s= +0.47, p<0.0001
LEAP2 (ng/mL)
Triglycerides (mmol/L)
N
r_s= +0.03, p=0.82
Acyl-ghrelin (ng/mL)
Triglycerides (mmol/L)
O
r_s= +0.13, p=0.23
LEAP2:acyl-ghrelin molar ratio
Triglycerides (mmol/L)

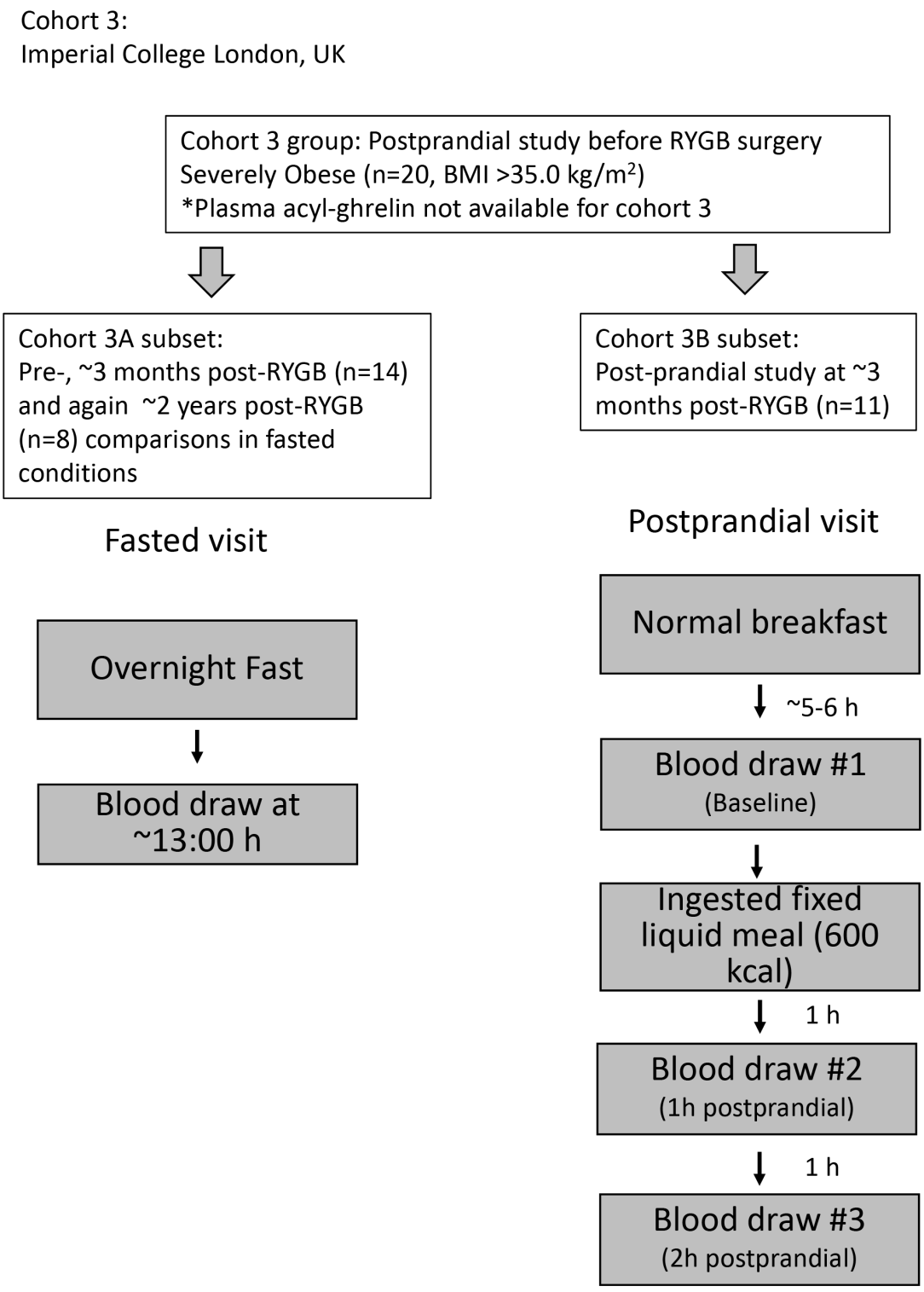
Supplemental Figure 6: Correlation of plasma LEAP2, acyl-ghrelin and LEAP2:acyl-ghrelin molar ratio with body composition, insulin resistance and serum triglycerides in fasted humans. Relationships of fasted concentrations of plasma LEAP2 (A,D,G,J,M), acyl-ghrelin (B,E,H,K,N) (UVa assay), and LEAP2:acyl-ghrelin molar ratio (C,F,I,L,O) with body mass index (BMI) (A-C), % body fat (%BF) (D-F), fasting plasma glucose (G-I), homeostasis model assessment of insulin resistance (HOMA-IR) (J-L), and fasting serum triglycerides (M-O) in Cohort 1. r_s represent Spearman correlation coefficient, n=90, except n=84 for HOMA-IR and triglycerides. Relationships of fasting plasma LEAP2 with body composition and metabolic parameters (panels D,G,J,M) are the same as those presented in Figure 4 of the main manuscript, and are again included here for comparison to the middle and right column of panels.



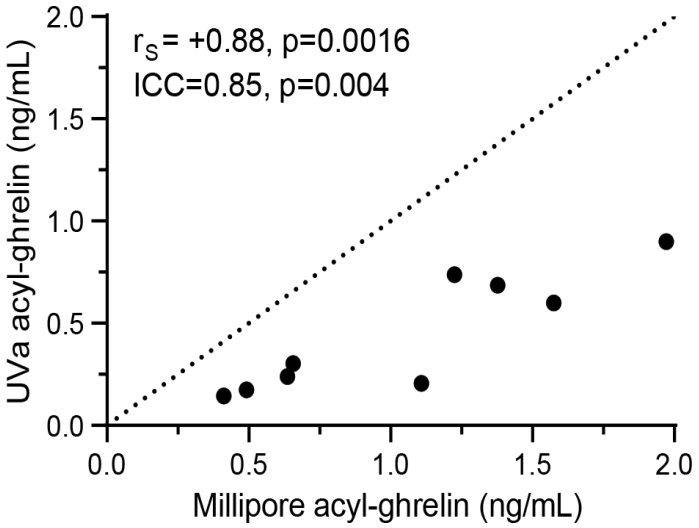
Supplemental Figure 7: Correlations of LEAP2 and acyl-ghrelin with measures of adiposity in fasted humans. Relationships of fasting concentrations of plasma LEAP2 (A,D,G,J), acyl-ghrelin (B,E,H,K) (UVa assay), and LEAP2:acyl-ghrelin molar ratio (C,F,I,L) with visceral adipose tissue (VAT) volume (A-C), subcutaneous adipose tissue (SCAT) volume (D-F), VAT / SCAT ratio (G-I), and intrahepatocellular lipid (IHCL) (J-L) in Cohort 1. Data in panels J,K and L are represented in semi-logarithmic scale (log_{10} x-axis). r_s represent Spearman correlation coefficient, n=41, except n=40 for IHCL. Relationships of fasting plasma LEAP2 with measures of adiposity (panels A,D,G,J) are the same as those presented in Figure 4 of the main manuscript, and are again included here for comparison to the middle and right column of panels.



Supplemental Figure 8: Human Cohort 2 information. Characteristics of the patients with flow chart summary of the study design.



Supplemental Figure 9: Human Cohort 3 information. Characteristics of the patients with flow chart summary of the study design.



Supplemental Figure 10: Comparison of University of Virginia acyl-ghrelin immunoassay and commercial acyl-ghrelin ELISA kit. Assay of identical human plasma samples (in duplicate; n=10) by University of Virginia (UVa) acyl-ghrelin enzyme-linked immunosorbent assay (ELISA) (2), and commercial ELISA kit used for other human samples in this study (EMD Millipore, Billerica, MD, USA; catalogue #EZGRA-88K). r_s represents Spearman correlation coefficient; ICC intra-class correlation coefficient; dotted line represents line of equality.

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